Recent Developments in Cell Line Shipping Methods: Pivotal Gaps in Cellular Death Sciences

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January 7, 2023

Abstract

Contrary to remarkable advances within the cell therapy industry, scientists expose dissatisfied challenges associated with the preservation and post-thaw cell death globally. Post cryopreservation apoptosis is normally observed in cultures and scientists are focusing on incorporation of apoptosis inhibitors. Impressive transport of cells without affecting their survival and function is a crucial and pivotal factor in any practical cell-based therapies. Preservation of cells permits the transportation of cells between distances, as well as improvement of safety and quality control testing in clinical and research applications. The prosperity of transportation methods is evaluated through the viability and proliferation percentages of the transported cell. For many decades, the conventional methods of transferring cells globally having adverse effects and speculated to be a challenging and expensive method. The main purpose of some studies is the optimization of cell survival after cryopreservation. In the new generation of cryopreservation science, various experiments wish to discover suitable and alternative methods for cell transportation to ship viable cells at ambient temperature without dry ice or in media filled flasks. In this review we try to represent a summary of the detection of recent studies including dry preservation, hypothermic preservation, agarose-gel based method, polymer based cryogel matrix, encapsulation method, fibrin microbeads, osmolyte solution composition, collagen-based scaffold, natural zwitterionic betaine, bio-inspired cryo-ink that have been performed alternative, effective and economic methods for shipping viable cells at ambient temperature.

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Contrary to remarkable advances within the cell therapy industry, scientists expose dissatisfied challenges associated with the preservation and post-thaw cell death globally. Post cryopreservation apoptosis is normally observed in cultures and scientists are focusing on incorporation of apoptosis inhibitors. Impressive transport of cells without affecting their survival and function is a crucial and pivotal factor in any practical cell-based therapies. Preservation of cells permits the transportation of cells between distances, as well as improvement of safety and quality control testing in clinical and research applications. The prosperity of transportation methods is evaluated through the viability and proliferation percentages of the transported cell. For many decades, the conventional methods of transferring cells globally having adverse effects and speculated to be

a challenging and expensive method. The main purpose of some studies is the optimization of cell survival after cryopreservation. In the new generation of cryopreservation science, various experiments wish to discover suitable and alternative methods for cell transportation to ship viable cells at ambient temperature without dry ice or in media filled flasks. In this review we try to represent a summary of the detection of recent studies including dry preservation, hypothermic preservation, agarose-gel based method, polymer based cryogel matrix, encapsulation method, fibrin microbeads, osmolyte solution composition, collagen-based scaffold, natural zwitterionic betaine, bio-inspired cryo-ink that have been performed alternative, effective and economic methods for shipping viable cells at ambient temperature.

Keywords:

Cell shipping, cell transport, cell death, cell culture, preservation, alternative methods, ambient temperature

Running title:

Cell Line Shipping Methods

Graphical Abstract:

Facts:

- Shipping of cell lines across international borders is controversial and problematic issue.
- Contrary to remarkable advances within the cell therapy industry, scientists expose dissatisfied challenges associated with the preservation and post-thaw cell death globally.
- Representing alternative, effective and economic methods for shipping viable cells at ambient temperature is a vital requires of cell researches.

Open questions:

- Why the development of alternative procedures for the global transfer and delivery of cells is an essential issue?
- Which cell shipping methods can be alternative for conventional methods of transferring cells with low post-thaw cell death and cytotoxicity?
- When can promise to prosperity of cell transportation methods?
- What is the main problems of cell shipping process and how can overcome?

Introduction:

The influence of cell culture technology on human society is indescribable. One main aspect of cell culture is the transportation and storage of cells in which special conditions should be implemented for the maintenance of cells for this prolonged time interval. Therefore, shipping of cell lines across international borders can be controversial and problematic. Recently, evolutions and improvements in biology science, efficacy of new drugs, manufacture of vaccines and biopharmaceutics and reproductive technology has depended seriously on cell culture technology. The quality of the medium and cell culture possible problems directly influence the research results and support cell survival and proliferation and cellular functions. Cell culture possible problems including cross-contamination, bacteria, fungi and mycoplasma contamination, chemical contamination are the most complex challenges in tissue culture (Cobo et al., 2005; Nema & Khare, 2012; Yao & Asayama, 2017). Cell-based therapies involve a time lag between the preparations of tissue regeneration and implantation. Special conditions such as optimal CO2 rate and temperature should be implemented for the maintenance of cells for this prolonged time. It is obvious that while cells in suspension are being transferred in ambient conditions they may not survive for long time intervals (Reddig & Juliano, 2005). Therefore, the development of alternative procedures for the transfer and delivery of cells is important (Ehrenreich & Ruszczak, 2006; Schoenhard & Hatzopoulos, 2010). Although these procedures are complicated and expensive and involve special cell culturing conditions, but developing simple methods with minimal manipulation and maximal survival is preferable and more relevant for clinical applications. In spite of the importance of this issue, scarcely has focused on reports on the prevention of cell death in suboptimal conditions when cells are transported to the clinic (Gorodetsky et al., 2011; Suuronen et al., 2008; Theus et al., 2008).

One main aspect of cell culture is the transportation and storage of cells. Cells are often transported from one city to another and also from one country to another. Thus, convenient and low-cost methods are desirable for routine transportation of cells at long distance. There are many existing methods for cell transportation in cell culture based research. The prosperity of these methods is evaluated through the viability and proliferation percentages of the transported cell (Ebertz & McGann, 2004; Esfandiarei et al., 2007; Kumari & Kumar, 2017). Impressive transport of cells without affecting their survival and function is a major factor in any practical cell-based therapy. For many decades, the most common method of transferring cells between institutes and companies is shipping cryopreserved cells on dry ice usually treated with anti-freeze solutions; most commonly DMSO because it is less toxic than many alternatives (B. Chen et al., 2013; Hanna & Hubel, 2009). The cost of process from storage to delivery is high. In addition transit delay is repeatedly observed so by evaporation of the dry-ice, cells bathed in cryoprotectant DMSO, which is cytotoxic at ambient temperatures. Cell culture facilities will most likely continue to use this mode of shipping for the advantage of eliminating large CO2 emission associated with using dry-ice. So low-cost, simple, inexpensive and non-hazardous methods of transporting cell cultures are desirable for routine transportation of mammalian cells at long distances (Stevens et al., 2007).

The commonly used technique for cell transportation is the shipping of cryopreserved cells on dry ice. Transportation of cells in different countries by this method is limited. According to Federal Express shipping information, more than 50% of countries around the world forbid delivery with dry ice because of hazardous nature and for its possible cause of explosion and suffocation. Liquid nitrogen vapor shippers are usually designed for the safe transportation of valuable cells and biological agents because it is cost effective (Mantel et al., 1979; Junjian Wang et al., 2015). Cryopreservation is based on slowing the vital cellular functions. Formation of intra- and extracellular ice crystals can cause destruction of cell membranes and cell organelles

which leads to cell damage (D. Gao & Critser, 2000; P Mazur, 2004). On the other hand, cell dehydration due to osmotic results leads to denaturation of proteins and complete destruction of the cell (Grein et al., 2010; Karlsson & Toner, 1996). For these reasons, many researchers have focused on the optimization of cell survival after cryopreservation by investigating the influence of different cooling and thawing rates for certain cell types (Armitage, 1987; Meryman, 2007). Besides the optimization of cooling and thawing rates, studies focus on the investigation of cryoprotectants. The main properties of these chemicals for protection of cells from cellular damage during cryopreservation are the stabilization of the cell membrane (Rubinstein et al., 1995; Rudolph & Crowe, 1985) and/or the decreasing of osmotic effects (Baust et al., 2003).

Some investigators simply send cell culture flasks filled with medium, but actually agitation can cause cell monolayers to detach. Liquids pose a spill hazard, and are forbidden in the mail and air-travel (Stefansson et al., 2017; Wheatley & Wheatley, 2019). This also has limited shipping time problem that cells in media have limited viability with flask leakage. It is just suitable for transporting cells across a short distance and over a very short period of time (usually up to 24 hours). Several types of cells do not survive and grow well through transportation in cell culture flasks due to rapid exhaustion of oxygen and pH change (Junjian Wang et al., 2015). However, using liquid nitrogen for the transfer of stem cells requires a short delivery time window, is technically challenging and financially expensive (B. Chen et al., 2013). Shipping cells in liquid nitrogen is hazardous and can be prohibitively expensive. The more economical and less dangerous alternative is to ship frozen cells using dry ice (Stefansson et al., 2017). Recently, several studies intended to find suitable materials and methods for transporting cells without dry ice (Junjian Wang et al., 2015). Novel alternative procedures for transportation of viable cells at ambient temperature without dry ice or in media filled flasks have been investigated in recent experiments.

From a development perspective, a truly "ready to go" cells system for high throughput application is now possible. By these procedures scientists will have functional cells already plate to perform assays. Ambient temperature shipping is a modern method to transfer cells. Instead of sending cells in dried ice using a Styrofoam box, cells can be shipped in a standard box at room temperature. Another key aspect is the environmental and regulation aspect that must be taken into consideration. Styrofoam is non-ecofriendly and shipping in dry ice is disallowed in many locations (Stefansson et al., 2017). At atmospheric pressure, liquid nitrogen boils at -196@C, while sublimation of dry ice occurs at -78.5@C. Although cells can be kept at -78.5@C for a period, but decreasing of viability, loss of dry ice and thawing of the sample in transit will kill the cells because of DMSO toxicity (Vigneault et al., 2009). The ability of preserving cells is serious for their application in clinical and research studies. With the various diversity of cells that require to be preserved, attentive optimization of preservation protocols seems tedious and shows significant restriction (B. Chen et al., 2013; Heo et al., 2015; A. P. Li, 2007; Linnebacher et al., 2010). Preservation of cells allows the transportation between sites, completion of safety and quality control examinations. For the reason of great diversity of cells which need to be preserved, accurate optimization of preservation protocols seems tiresome and indicates remarkable limitations. In this review, we will focus on summarizing the findings of recent preservation studies that have been performed.

Search strategy:

A systematic literature search was performed using PubMed, Web of Science, Google Scholar, Scopus, and ScienceDirect as an international database from 1978 up to 2020 with English language restriction. The search key words were: Cell shipping, cell transport, cell culture, preservation. All English language and free full text paper selected.

Cryopreservation methods:

Under in vitro condition one of the vital environmental factors for cell survival is temperature. A standard condition for mammalian cells is to culture usually in the incubator which in CO2 levels are set to 5% and temperature to 37@C unless specific research purpose is required (Brown, 1990). According to previous studies low temperature can decrease cell growth rate (Azambuja et al., 1998; Kaushik & Kaur, 2005; Vergara et al., 2014); whereas mild heat stress enhances cell proliferation rate and accelerates development

(Choudhery et al., 2015; Tounkara et al., 2012). Increasingly, long-term treatment at sub-zero temperature can intensely influenced the viability rate of mammalian cells (Jin et al., 2008; Moussa et al., 2008; Juan Wang et al., 2017). Utilization of cryogenic temperatures are the most known methods for the long-term preservation of live cells. Attentive optimization of these protocols is most often inescapable for achieving high yield and survival of specific cells (Heo et al., 2015). Cryopreservation is classified into slow freezing and vitrification methods (Table 1). The main dissimilarity between these two methods are the concentrations of cryoprotective agents (CPAs) and the cooling rates used (D. Gao & Critser, 2000; Jang et al., 2017).

A common method for cell shipping is that cryopreserved cells are transported with dry ice with a foam container; which have little influence on cell features and maintains a high rate of cell viability (Prentø, 1997). However, cell shipment with dry ice is costly and forbidden by the aviation departments of many countries (Junjian Wang et al., 2015). Cryopreservation is a procedure that preserves biological samples by cooling them in very low temperatures. Cellular events such as ice crystal formation, osmotic shock and membrane damage are the main reasons that cells cannot be stored with ordinary freezing methods for a long time and it will lead to apoptosis. In recent years, the prosperous cryopreservation of cells and tissues has been gently increasing with the extension of cryoprotective agents and temperature control instruments. This review, outlines and clarifies cryopreservation processes, including slow freezing and vitrification (Jang et al., 2017).

According to the cell types, there is substantial variety in cryosurvival response through the freezing and thawing cycle. The important stages in cryopreservation are: the mixture of cryoprotectant with cells or tissues, cooling and storage, warming, removal of CPAs after thawing. The proper utilization of CPAs is therefore influential to improve the viability of the sample to be cryopreserved (H.-H. Gao et al., 2016). Cellular mechanical injury in fast freezing is associated with intracellular ice formation in water to ice phase transition. Whereas slow freezing causes osmotic rupture due to exposure to extra- or intracellular high concentrated solutions and intracellular ice formation. Freezing is lethal for most living cells however cryopreservation retains biological samples at cryogenic temperature to preserve the fine structure of cells. The freezing manner of the cells can be different in the presence of a cryoprotective agent, which influences the rates of water transport, nucleation, and ice crystal growth (Peter Mazur, 1970; Sambu, 2015).

Slow freezing

In slow cooling conditions, cells could efflux intracellular water quickly enough to stop intracellular ice formation (Schoenhard & Hatzopoulos, 2010).Optimal cooling rates are related to cell types because there are some differences in the capacity of different cells to move water across the plasma membrane. Slow freezing can lessen cell injury and maintain the permeability of the cell membrane through substitution of the cytoplasm water with CPAs (Mandawala et al., 2016; Yong et al., 2015). Freezing for long-term storage has proven to be one of the most effective methods of maintaining a stable supply of various cell types. However, cells may be damaged by environmental changes during the freezing process (Miyamoto et al., 2018). There are various factors that influence the function of cells cultured after cryopreservation and thawing, including the cryopreservation solution (Miyamoto et al., 2006; Miyamoto, Noguchi, et al., 2012; Miyamoto, Oishi, et al., 2012), biomaterials (Miyamoto et al., 2009), freezing methods (Fujioka et al., 2004), and freezing and preservation temperatures (Reubinoff et al., 2001). Among cryopreservation solutions, cryoprotective agents such as glycerol, ethylene glycol, and dimethyl sulfoxide (DMSO) are the most effective due to their high rate of penetration into cells. In addition, it has been reported that starch and oligosaccharides like trehalose and maltose are effective in suppressing damage to cells (Y. Chen et al., 1993; Crowe & Crowe, 2000; Miyamoto et al., 2018).

Cryopreservation with controlled slow cooling rate was first exposed to discussion by Polge, Smith and Parkes in 1949 using the protective properties of glycerol (GLY) (Karlsson & Toner, 1996). Dimethyl sulfoxide (DMSO) was known as a cryoprotectant in 1960 by Lovelock and Bishop which was considered to have more widespread applicability than glycerol in tissue culture (Fahy et al., 2004). The development of a cryopreservation protocol for a given cell type requires specification of: pre-freeze processing; introduction of a cryopreservation solution; freezing protocol; storage conditions; thawing conditions and post thaw assessment

(Hanna & Hubel, 2009). During cryopreservation, cells are cooled slowly in the presence of a cryoprotectant to reduce the formation of ice crystals. Because different cell types have unique biophysical and biological characteristics (Stacey & Masters, 2008). During cryopreservation, slower than optimal freezing rates can cause cell damage by the formation of intracellular ice and have deleterious effects on cell viability, while concentrations of cryoprotectant that are too high could be toxic to the cells. To prevent this, an ideal cooling rate and cryoprotective agent (CPA) should be chosen (Heo et al., 2015).

CPAs are classified into intracellular and extracellular agents. The intracellular one penetrates inside the cells and prohibits ice crystal buildup and membrane rupture. The extracellular compounds do not penetrate the cell membrane and decrease the hyperosmotic effect through the freezing duration. Among CPAs, DMSO, propylene glycol (PG), ethylene glycol (EG) and glycerol (GLY) are intracellular compounds. Extracellular CPAs are known as trehalose, sucrose, dextrose, and polyvinylpyrrolidone. DMSO is the frequently utilized CPA. It provides a high rate of cell survival during the freezing time but causes chemical cytotoxicity at room temperature which can lead to cell injury osmotically. This cytotoxicity obligates the researchers to perform the experiment quickly. Moreover, different experimenters need diverse amounts of time to thaw their cells completely and to wash them free of CPA. Consequently, less cytotoxic CPAs are required for the standardization of this course of actions (Janz et al., 2012; Syme et al., 2004; von Bomhard et al., 2016). An alternative method extensively used by local companies or laboratories is directly to ship the cultured cells in the flask fully filled with cell culture medium; but the disutility of this method is to not be appropriate for long-distance shipment (Juan Wang et al., 2017).

Vitrification

It is now believed that with current methods of cryopreservation, unrestricted ice formation in cells and tissues at subzero temperatures is the most vital factor that is seriously important in survival procedures during freezing and thawing. Recently, this important problem has been efficaciously circumvented in some tissues by using ice-free cryopreservation techniques based on vitrification which is a fast freezing method that has become increasingly popular (Taylor et al., 2019). This hopeful and simple process keeps away cells from injuries caused by their dehydration during slow freezing. To prevent intracellular ice buildup, a higher concentration of CPA is necessary, but its cytotoxic effect can be reduced by immediate freezing in liquid nitrogen (LN2). The study of evaluation of comparison between vitrification and controlled slow freezing methods under standardized conditions has been performed recently (Table 1.). According to scientific reports, the cell toxicity depends on the chemical properties of CPAs and cell types. The investigation around the effect of various CPAs and their combinations on cell types cannot be predicted well, and hence it dependes on more experimental approaches (Fan et al., 2009).

Vitrification known as ice-free cryopreservation is an alternative preservation for slow-freezing technique which uses very high concentration of cryoprotectant cocktails to prevent ice formation by directly transforming from the aqueous phase to a glass state after direct exposure to liquid nitrogen (Fuller et al., 2016). Cryoprotectant concentrations as high as 4-8 M are typically required. Because such concentrations are very toxic for most cell types, individual protocols are optimized for each cell type depending on its sensitivity to the cryoprotectant (Heo et al., 2015). Vitrification significantly relies on three main factors including: viscosity of the sample; cooling and warming rates; and sample volume. A fine harmony must be maintained among all the related factors to certify a successful vitrification. There are two methods of vitrification that are classified into individual two methods: equilibrium and none quilibrium. Equilibrium vitrification needs formulation of multimolar CPA mixtures and their injection into the cell suspensions. Nonequilibrium vitrification is divided into carrier-based systems and carrier-free systems. A major advantage of vitrification is the low risk of freeze injury, therefore warrant a sufficiently high cell survival rate. It has reported that the probability of contamination with pathogenic agents is high so the technique requires good manipulation proficiency (Yavin & Arav, 2007).

Alternative procedures for vitrification protocol optimization have centralized on enhancing the cooling rate, which could permit lower concentrations of cryoprotectant for glassy-state achievement. Technologies to allow faster cooling rates have been described in which cells are placed inside thin open straws, cryotops,

electron microscopy grids, or cryoloops. More recently, scientists validated the use of quartz micro-capillaries for vitrification, which enabled ultrahigh cooling rates and required significantly lower concentrations of cryoprotectants than standard vitrification protocols (2.0 M) (Criado et al., 2011; He et al., 2008; Kuwayama et al., 2005; H.-J. Lee et al., 2009; Vajta et al., 1998). To dominate the restriction of current cryopreservation and vitrification protocols, a vitrification protocol has developed that can be easily standardized and performed without changes for a wide variety of cell types. To improve previous quartz capillary technique, fused silica microcapillaries have been employed, which are industrially manufactured to have well controlled dimensions and physical properties. Compared with most current cell vitrification technologies, this method contains fused silica microcapillaries performed like a low-thermal-mass, high-heat conductivity cell container to raise the cooling rate by an order of magnitude. The vitrification process is simple and can be done in less than 15 minutes, significantly faster than conventional vitrification techniques (Heo et al., 2015).

Vitrification is a high warming and cooling rate method. Cryopreservation strategies focus on the use of cryoprotectants (CPAs) as supplements prior to cooling and the rates of cooling/ warming of the temperature of the sample. Vitrification can eliminate intracellular ice crystals, but high concentrations of CPAs are required to achieve the vitreous state (Vajta & Nagy, 2006; Zhou et al., 2010). CPAs function in a dual capacity by decreasing freezing temperature and increasing viscosity so that instead of crystallizing the syrupy solution becomes an amorphous ice; it 'vitrifes'. Intracellular ice crystals which organize among cooling and warming duration are the main reason for cell damage (K.-H. Lee et al., 2013). During cooling time, microscopic ice crystals appeared that may cause recrystallization in the warming process by transfer of water molecules from smaller ice crystals to larger ones, which can reach sizes that seem to be lethal for the surrounding cells. According to Hopkins et al. differences in warming rates are correlated directly with prior cooling rate because of the accumulation of tiny ice fragments in vitrified cells (Hopkins et al., 2012). A faster cooling rate is essential for a corresponding warming rate to prevent recrystallization. Coupled with moderate cooling rates, high warming rates of 117,500 C/min can protect cells from damage due to recrystallization (Peter Mazur & Seki, 2011).

It is evident that vitrification has more benefits to offer as a cryopreservation procedure than do slow cooling methods. First, vitrification or ultra-rapid techniques prevent cryoinjuries to viable cells from ice crystal formation during the cooling process. This is due to the use of highly concentrated CPAs and very high cooling and warming rates. Second, the exposure time of the vitrification process to room temperature is shorter than for traditional freezing methods. Living cells and tissues should not remain long in such non-physiological conditions before their storage at low temperatures. Third, vitrification is cheaper than stepwise methods. Fourth, vitrification is not requiring sophisticated machinery, and it is easy for technicians to learn vitrification protocols through a short practical training course. More over the survival rates of vitrified embryos are often reported higher than those of frozen embryos (Campos-Chillon et al., 2006; Do et al., 2014).

Although vitrification has several advantages over other cryopreservation procedures it nevertheless indicates drawbacks and disadvantages. While vitrification minimizes the time exposure of ova and embryos at room temperature (Campos-Chillon et al., 2006), this technique may lead to the contamination of embryos during the vitrifying process as a result of direct exposure to liquid nitrogen (Vajta et al., 1998). Environmental and water-borne bacteria and fungi have been identified in liquid nitrogen. It is clear that vitrification is often an open system; therefore, there is a high level of microbial contagion when vitrified samples are banked in liquid nitrogen (Morris, 2005). Hence, it is necessary to measure the contamination risks in cell and tissue banking. However, it seems that the contamination risks for cryopreserved samples are forsaken (Do et al., 2014). Vitrification methods of cryopreservation may bring new chances in research protocols. It is yet an experimental procedure. There are two major concerns about vitrification toxicity of high concentration of cryoprotectants used and microbial contamination of liquid nitrogen. The problem is to discover a protocol to successfully vitrify cells (Barun, 2015). Modifications to the vitrification process have focused on the type and concentrations of CPAs and on cooling and freezing rates which improves the vitrification process. Table 2 summarizes the advantages and disadvantages of slow freezing and vitrification techniques (Sharma, 2005).

Limitations of cryopreservation

The metabolism of cells almost is lessen at low temperatures such as -196 @C which has inescapable side effects, such as genetic drift due to biological variations in lipids and proteins that could lead to impairmentation in cellular function and structure. If there were no restriction to the amount of utilized CPA, cells would be preserved excellently (Jang et al., 2017). In regular and standard settings, however, CPAs themselves can be deleterious for cells, particularly when used in high concentrations. For example, DMSO may alter chromosome stability which can led to a risk of tumor formation. Apart from endogenous changes in cells, the possible infection or contamination with cells should be prohibited (Jenkins et al., 2012; Yong et al., 2017). Although cryo-preservation is still undoubtedly the best present method for long-term preservation of stem cells, its disadvantages have driven research into alternatives. Cryo-preservation has long been associated with cell membrane damage and apoptosis (Trusal et al., 1984; Yong et al., 2017). Furthermore, cryo-preservation is labor intensive and operator dependent and typically uses equipment that is not suitable for cell therapy such as liquid nitrogen, which is not GMP compliant (B. Chen et al., 2013).

Cell death induced by cryopreservation

Accumulating evidence indicate that apoptotic cell death and necrosis are initiated by a variety of microenvironmental perturbations including metabolic uncoupling/coupling, ionic imbalances, energy deprivation, caspase activation, membrane phase transitions and alterations, free radical production, cellular acidosis, cytoskeleton disassembly, protein denaturation, water solidification, cell volume excursions, hyperosmolarity, and protein denaturation (Galluzzi et al., 2018; Messmer et al., 2019; Nazio et al., 2019). It has been reported fallowing cryopreservation, post-thaw apoptosis in a wide variety of cell types is a significant drawback faced by cryobiologists. Cryopreservation-Induced, Delayed-Onset Cell Death (CIDOCD) is the timing of cell death following cryopreservation which has been targeted with scientists to improve cryopreservation. It is the prime goal of researchers to generate cryopreservation solutions inhibiting signaling molecules associated with apoptosis and necrosis down the cell death cascades (Bissoyi et al., 2014). Cryoinjury-induced cell death is initiated by the intrinsic or extrinsic apoptotic pathways. According to recent studies activation of caspases 3, 8, and 9 are happened after thawing process. Various caspase inhibitors provide protection against apoptosis (Chatterjee & Gagnon, 2001; Fowke et al., 2000). Although further study is required to provide a detailed role of caspases in the cryopreservation-induced apoptosis pathways because some studies reported caspase-8 and caspase-9 activity inhibition without enhancing post-thaw cell viability (Heng et al., 2007; Xu et al., 2010).

Under stressful conditions like cryopreservation, cellular oxidative stress is occurred. various reactive oxygen species (ROS) activates the multiprotein complex, including Apaf-1, cytochrome c, and caspase 9, and thus eventually leads to apoptosis.50 activation and accumulation of p53 in the cytosol through the intrinsic pathway can induces the expression of prooxidant genes at high concentrations of ROS caused by the procedure of freezing in cryopreservation (Barbaric et al., 2011). Therefore by suppressing ROS and using strong antioxidants can achieve cell recovery after cryopreservation. Also it has been reported that in the presence of the p53 inhibitor, there is a reduction in caspase 9, but not in caspase 8 activity (Melino, 2011; Sablina et al., 2005). The p38 MAPK is activated during freezing-thawing and cryopreservation processes which can induces apoptosis. P38 MAPK inhibitors can reduce apoptosis and thus led to improvement in viability of cryopreserved cells (Omori et al., 2007). During cryopreservation, alteration of actin cytoskeleton may occur and induce cell apoptosis (Bishop & Hall, 2000). Rho-associated kinase I (ROCK-I) protein which is implicated in the regulation of apoptosis can triggers a signaling pathway that leads to actin–myosin coupling to the plasma membrane. A potent ROCK inhibitor may blocks apoptosis and improves survival of many cryopreserved cells. Figure 1. shows the mediators of cell death during cryopreservation and the effective apoptosis inhibitors (Bissoyi et al., 2014; Heng et al., 2006).

Alternatives to Dimethylsulfoxide

The standard and commonly used cryoprotective agent is dimethyl sulfoxide (DMSO), which is the cause of cell toxicity and represents some side effects. DMSO has also been recognized to show toxic effects, reduce

the expression of key factors related to stemness, and induce epigenetic changes. Hence, there is an elevated request from health care authorities to discover non-toxic cryopreservation alternatives of DMSO (Svalgaard et al., 2018). The functions of dimethyl sulfoxide (Me_2SO) as a supplement in the cryopreservation medium are to protect the cells from excessive dehydration during the freezing process and to inhibit intracellular ice formation. Due to the fact that Me_2SO is toxic and associated with clinically significant side-effects as well as the fact that serum could possibly transfers pathogens, both are unsuitable for the storage of hMSC for clinical applications. Moreover, Me_2SO can lead to uncontrolled differentiation of stem cells. Thus Me_2SO must either be replaced for cryopreservation or it has to be removed prior to transplantation, for instance by diafiltration. Despite the possibility of removal, it is more beneficial to replace Me_2SO by non-toxic agents. Non-toxic alternatives to Me_2SO include, for example, glycerol or compatible solutes such as Proline and ectoin (Hegner et al., 2005; Ji et al., 2004; Woods et al., 2007).

The glycerol protection of cells from osmotic damage is represented by reducing the intracellular water loss and indeed reducing intra and extracellular ice formation (Morris et al., 2006). For cryopreservation of blood cells and adult stem cells, glycerol has already been used successfully in concentrations between 5% and 20% (v/v) (De Vries et al., 2004; Scott et al., 2005). Ectoin was detected first in extreme halophilic bacteria (*Ectothiorhodospira halocholris*) to protect these bacteria against high salt concentrations and high temperatures in their environment. Today, ectoin is also used for the stabilization of proteins and cells during the freezing process in concentrations between 0.1% and 1% (w/v). This effect is based on the stabilization of the hydrate envelope of enzymes and the regulation of the osmotic balance in cells. Proline is a non-essential amino acid and a special cryoprotectant, because it has low- and macromolecular behavior. Proline can diffuse into the cells and forms hydrogen bonds typical for low-molecular cryoprotection. Moreover, depending on the concentration, proline forms aggregates which act like macromolecular cryoprotectants (Grein et al., 2010; Troitzsch et al., 2008). Proline can preserve enzyme activity and membrane stability after freeze-thaw cycles (Rudolph & Crowe, 1985) and increases the post-thaw vitality of spermatozoa in concentrations between 5 mM and 20 mM (Y. Li et al., 2003). In a study which ectoin was the cryoprotectant, the results indicated high post-thaw cell survival of up to 72% whereas after cryopreservation with glycerol and proline, the hMSC cells were completely dead or had only poor cell survival. Indeed, following the cryopreservation with proline, the altered morphology of cells was seen. These results show that glycerol and proline are not appropriate for cryopreservation of hMSC. In contrast, ectoin has the qualification for being the alternative of Dimethylsulfoxide in a serum-free cryomedium (Grein et al., 2010). Pentaisomaltose as an extracellular cryoprotectant is agreed for clinical utilization. It is less likely than DMSO to interact with the intracellular molecules. Cryopreservation in DMSO or pentaisomaltose have been compared in cell survival in vitro, also in long-term cryopreservation in the in vivo models (Svalgaard et al., 2018).

Alternatives to Cryopreservation:

Dry preservation

One of the alternatives for cryopreservation is elusion of low temperature storage and transport by desiccating cells which could be kept at room temperature. If possible this would be economically and practically good alternatives for long-term storage of cells (Coopman, 2013). Freeze drying (lyophilisation) is useful for preservation of bacterial cells and fungi. Briefly, the process comprises three stages: freezing in order to partly crystallise the solvent so that it can be separated from the solutes, sublimation of the resultant ice (primary drying phase) and finally, a secondary drying phase where the majority of the remaining moisture is removed by desorption (Sundaramurthi & Suryanarayanan, 2012; Wolkers et al., 2002). Just as cryoprotectants are used in cryopreservation, cells can be pre-treated and indeed lyophilised in the presence of lyoprotectants, to minimise cell damage during the process. One compound that has been particularly researched is trehalose, a hydrophilic disaccharide which is known to accumulate in desiccation tolerant organisms (Crowe et al., 2005).

According to Watanabe K. et al. studies; Pv11 was the first dry-preservable animal cell line which showed improved survival and achieved proliferation after dry storage. The method is mentioned in figure.2 including preincubation, desiccation and rehydration processes. As a result, they managed to obtain successful

drying and storage of Pv11 cells at room temperature by preincubation in trehalose solution for 48 h before dehydration to achieve successful dry preservation of Pv11 cells. They extended this dry storage up to 251 days and the cells still kept nearly 7% viability (Watanabe et al., 2016).

Hypothermic Preservation

Hypothermic preservation is pausing of cells at hypothermic temperatures (1°C-35°C) (J. Yang et al., 2017). It is another potential alternative to cryopreservation for mammalian cells where cells are stored at low but not freezing temperatures, effectively pausing the cells by slowing down metabolism, but without the dangers of ice formation. At mild hypothermia (25-35°C) cells proliferation is still seen and recombinant protein expression may be improved by culturing cells at reduced temperatures (Miyamoto et al., 2009). More severe hypothermia and refrigerated storage (0-10°C) is needed to arrest growth and pause the cells but this would nonetheless be preferable to storing cells in liquid nitrogen and the use of dry shippers. This would be a short term preservation method and indeed, it is already used in the pharmaceutical industry as it can uncouple cell culture from cell-based assays. It allows researchers to store cells for several days if a piece of equipment is double-booked or breaks down, rather than having to dispose of plates of cells, saving time and money (Coopman, 2013; Wise et al., 2009).

Agarose-Gel Based Method

In the state of cell transportation, agarose was extensively utilized in viral plaque assays at concentration of 0.8%. Rigidity might be a main factor for supporting adherent cells and prevention of cells. For this reason the agarose-medium gel should have enough rigidity. One of the important conditions for the utilization of agarose-medium gel method is the temperature adjustment. It should be between 1 to 37 ^{o}C since greater than 37 ^{o}C or lower than 0 ^{o}C can be damageable for cells. On the other hand agarose concentration should be adjusted between 0.8 to 1.2%. Because the agarose layer at concentration less than 0.8% is not hard enough and at the concentration above 1.2%, it becomes dry and easy to crack. The higher concentrations of agarose (higher than 1.2%) might be the reason for low cell viability. Thus, agarose concentration at 1 % is an optimal condition for this cell transportation method because it has the highest cell recovery viability and is firm enough to form an appropriate layer of gel to support the cell during the transportation.

According to Yang L. and et al study on the agarose gel based method, the numbers of cell colonies were detected for testing the viability and function of cells after transportation. Cell morphology which were being kept in medium containing agarose gel at room temperature were similar to those in regular cell culture condition. The results of colony formation detection indicated that the morphology and size of cell colonies for three cell lines were not changed after recovering from the transportation by this method (L. Yang et al., 2009).

Wheatley S. P. et al. represented a gel based method which provided transit of up to 2 weeks without compromising cell recovery. Its application will help scientists and permit them to send cultured cells across the world without requiring dry-ice and at ambient temperatures. In this research privileged recovery is observed when cells are transported at 5×10^{6} cells/ml at the temperature of 4–27°C for 1 week. They counseled to ship cells in small aliquots of Transporter (100 µl) in microfuge tubes and diffuse the gel pellet prior seeding (Wheatley & Wheatley, 2019).

6.3.1. HemSol gel

From a development viewpoint, a truly "ready to go" cell system, shipping of both suspension cell and adherent cell cultures at ambient temperature is now possible. Cells can be prepared in a multi-well plated, packaged with HemSol gel and sent to end users. According to Stefansson S. and et al studies, HemSol gel can protect platelet and red blood cells function in cold storage up to 6 days. They also analyzed live cells' function during transportation with HemSol gel method. End users will receive functional cells and already plate to perform assays. Thus, HemSol gel is a noteworthy alternative to standard dry ice shipment and could develop the utilization of cells in pharmaceutical and biotechnology studies. The cells which shipped in HemSol gel represented more than 95% viability and restored biological functions in 2 hours, whereas,

cells that shipped in dry ice needed more than 24 hours to recover and indeed they required media change for removal of the DMSO (Stefansson et al., 2017).

Polymer Based Cryogel Matrix

Recently, matrices synthesized at subzero temperature using cryogelation technology known as cryogel have been reported for cryopreservation (Katsen-Globa et al., 2014; Vrana et al., 2012). These polymeric cryogels possess three-dimensional (3D) structure and have already been used as a scaffold for tissue engineering applications (Hanna & Hubel, 2009). Cell-scaffold construct for regenerative medicine was cultured*in vitro* which was then transplanted in *in vivo* (Sarkar & Kumar, 2016; Umemura et al., 2011). Pausing or storing of the developed tissue engineered construct is prohibited during this entire process. Therefore, cryopreservation of cell-scaffold construct having viable and functional cells is a preferable approach to meet the demand. Due to their ready-to-use nature, they could be immediately utilized. These cryogel-based methods for cryopreservation have advantages over the gel-based method because in the former cell encapsulation and gel formation do not occur simultaneously (Vrana et al., 2012).

Cryogels were preferable over traditional gel carriers in the field of biochromatography and related biomedical studies. These matrices approximately simulate the three-dimensional structure of native tissue extracellular matrix. Indeed, mechanical, osmotic and chemical stability of cryogels make them wonderful polymeric materials for the construction of scaffolds in tissue engineering investigations and in vitro cell culture, immobilization of biomolecules, capturing of target molecules, and controlled drug delivery. Cryogels are known as gel matrices which are produced by polymerization at low temperatures (below zero). While the polymerization process happens in the unfrozen part, ice crystals perform as pore forming agents. Figure 3 represents the Schematic view of cryogel formation and Figure 4 shows the physical characterization, digital image and SEM image of HEMA agarose (HA) and gelatin cryogels (Bakhshpour et al., 2019).

Bioreactors are specified as devices which protect biological reactions. They have a significant role in the cell cultivation providing control and monitoring of pH, temperature, oxygen and nutrient decomposition in microbiology. When tissue engineering is taken into consideration, bioreactors can be applied to develop new functional tissue in vitro with the production of regulatory signals in an appropriate physical environment. In several research publications, it has been mentioned that the cryogels have vital function in bioreactor platforms. A research about the storage and transportation ability of the cells have reported the utilization of both HEMA-agarose (HA) and gelatin cryogels for this aim. These materials also can be applied in cell cultures for both in vitro and in vivo studies. In these conditions, they influence the improvement of cell migration, proliferation and differentiation as well as enhanced mechanical stability (Bakhshpour et al., 2019). Scanning electron microscope (SEM) analysis of both the cryogels were performed for surface morphological analysis. The homogenous pore distribution was observed under SEM. Presence of pores associated in easy recovery of cells. Hence, pore walls provide adherent surface to the cells and help for cell proliferation (Kumari & Kumar, 2017).

Kumari J. and et al have tested the possibility of using cryogels for cell transportation. According to this study cryogels could be integrated with the methodology of using cryogel for cryopreservation. Cells received from the cryogel were functional and viable following the simulated transportation using 2-hydroxyethyl methacrylate (HEMA)-agarose (HA) cryogel. Further, we have also shown that the cells stored in the gelatin cryogel remain fit for cryopreservation even after the simulated transportation for up to five days. The results show that cryogels not only overcome the above-mentioned problems of transportation of cells but also provide a ready-to-use scaffold for further engineering of adherent cells, including cryopreservation. Application of cryogels for transport and storage also shows another advantage such as well cell proliferation during transportation. Thus cryogels could be a preferable solution for tissue engineering including cell-transportation and cell-cryopreservation (Kumari & Kumar, 2017).

In summary, HA and gelatin cryogels have the potential of transporting and storing adherent-cells. As both the cryogel have different surface properties, they can be used for various applications. Kumari J. and et al had produced HA cryogel for the purpose of cell transportation with the chance of cells that can be eluted

following the transportation. On the other hand, they suggested gelatin cryogel that can be utilized for both cell transportation and cryopreservation. In both, cells were alive and had the capability of proliferation after simulated transportation. The adherent-cells kept in HA cryogels and C2C12 cells remain up to 25% more viable in comparison with the adherent-cells kept in cryovial as a suspension culture under the same simulation conditions for transportation. It is demonstrated that the adherent-cells can be stored in gelatin cryogels and C2C12 cells, up to five days at room temperature and representation viability up to 80%. It has further shown that these adherent-cells indeed remain fit for cryopreservation even after the simulated transportation in cryogels for up to five days. This suggests that cryogels can minimize the effect of shear force, which is the main cause for the reduction of viability in transporting cells in suspension. Cryogels act as an efficient, low-cost storing and transporting matrix at room temperature and at cryo-conditions, and hence it may show serious outcome for ready-to-use transplantation, in vitro drug testing, and regenerative medicines. These pave the way for establishing cryogels as a superlative solution for tissue engineering, including cell-storage, cell-transportation, and cell cryopreservation (Kumari & Kumar, 2017).

According to some investigations, transportation of mammalian cells for long distance at ambient temperature can be performed through agarose gel-or matrigel-based methods. Also it is shown that these procedures retain a high rate of cell recovery after transportation for a few days. These procedures are complex and labor-consuming. Whether mammalian cells to be shipped in a simple mode at ambient temperature remains obscure (Junjian Wang et al., 2015; L. Yang et al., 2009). Novel polymer based cryogel matrix method is simple, practical and affordable because specific equipment and commercial medium aren't needed. In this method cells were directly suspended in the culture medium. It can be used for cell shipping at short and long distances. However, it should be performed carefully and cautiously for the shipment on clinical purpose where specific equipment and techniques are highly required (Wille et al., 2014). It not only expands the comprehension of the effect of hypothermia on cell survival, but also provides an alternative approach for cell shipment. According to this method, the approach for cell shipment would be useful to biological and biochemical laboratories (Juan Wang et al., 2017).

Encapsulation Method

Cell microencapsulation technology is an important technique for prevailing over the bioprocess challenges. In this process cells are microencapsulated in alginate to progress a unified bioprocess for cryopreservation of pluripotent hESCs. The existence of components of the extracellular matrix on microcarrier cultures (e.g. collagen, laminin) may have participation with cell survival enhancement during freezing and thawing (Ji et al., 2004; Kim et al., 2004), by reducing post-thaw apoptosis. The alginate microcapsule permits extra increase of post-thaw cell viability up to 3 times more compared to non-encapsulated cultures. Although the underlying mechanisms are still obscure, several experiments demonstrate that maintaining cell-cell/matrix contacts improves hESC recovery following cryopreservation (Hunt, 2007; Karlsson & Toner, 1996). Cell entrapment within alginate microcapsules may help assist hESCs in the presence of harmful effects of cryopreservation, by preventing the disruption of cell-cell and cell-matrix contacts, decreasing exposure to cryoprotectants and preventing the damage caused by intracellular ice formation (Malpique et al., 2010; Zimmermann et al., 2005).

Hydrogels such as alginate, poly (lactic-co-glycolic acid)/ poly (l-lactic acid) scaffolds, agarose, chitosan and hyaluronic acid have been used in hESC culture. Alginate is the most commonly used encapsulation material owing to its biocompatibility, biosafety and permeability. Although Nie et al reported a novel technique for the cryopreservation of hESCs adherent on microcarriers, this protocol required more optimization in order to omit animal feeder cells and enhance cell attachment/survival after thawing (Serra et al., 2011).

Recently the frequent application of hydrogels as cell carriers for delivery of stem cells are now known to be challenging conventional cryo-preservation methods (Gorodetsky et al., 2011). The cell samples used for study of alginate hydrogels as cell carriers were human mesenchymal stem cells (hMSC) and mouse embryonic stem cells (mESC). These cells were successfully stored inside alginate hydrogels for 5 days under ambient conditions in sealed cryo-vials. After extraction of cells from alginate gel, cell viability results show 74% survival rates for mESC and 80% for hMSC, which were compared with survival rate results gained following

cryo-preservation. Indeed, the proliferation rate and detection of mRNA and protein levels in hMSC and mESC extracted from alginate hydrogels were also comparable to cryo-preservation (B. Chen et al., 2013).

Stem cells in suspension do not survive for extended time intervals during transportation under ambient conditions (Reddig & Juliano, 2005) within appropriate delivery times and at reasonable financial cost. Hence, cryo-preservation of stem cells was previously known invalid owing to the adverse effects of DMSO (Chakrabarti & Schutt, 2001). Thus, appropriate conditions for the preservation of transported stem cells are demanded. A hydrogel that is chemically inert, structurally uniform and biocompatible, may be useful for a simple and economical storage method which subjects stem cells to minimal manipulation and preserves their viability and phenotype (B. Chen et al., 2013).

The major properties of alginate based cell encapsulation such as biocompatibility and rapid gelation provide the success of cell encapsulation. The mechanical properties of alginate hydrogels are mainly related to the ratio of components used for gelation including polysaccharides and the cationic cross-linker (Martinsen et al., 1989). Although calcium is the conventional crosslinking ion for alginate gels, other cations such as strontium and barium are appropriate (Mørch et al., 2006; Place et al., 2011). Strontium-alginate microbeads were known to be more stable than calcium alginate beads (K. L. Chen et al., 2007). The mechanical properties of alginate gels in the presence of live cells also can be controlled by modification of the internal porosity of these gels. The addition of hydroxyethyl cellulose to alginate, led to gels with controllable pore size, which relate to the improved viability of cells immobilized within this scaffold (Wright et al., 2012).

Having found that calcium alginate gels were too fragile, and agitation would release the encapsulated cells resulting in their death. We surmised that the transport of cells would cause significant agitation to the gels resulting in their disintegration. Therefore to avoid possible alginate hydrogel fragmentation during long distance transportation, strontium was chosen to cross-link with alginate gels in this study. Previously strontium was found to have a greater affinity to alginate than calcium, resulting in alginate hydrogels with improved stability. Strontium alginate hydrogels have successfully been used in bone tissue engineering. We further improved the gels overall mechanical strength by encapsulating within it an inert nylon mesh. Together the composite material, comprised of strontium alginate gel and nylon mesh, produced an extremely robust and effective carrier for hMSC and mESC. Chen B. et al. results clearly show that both mESC and hMSC stem cell markers were positive following extraction from the alginate gels remarkably even after 5 days at ambient (room temperature) in a sealed cryo-vial (B. Chen et al., 2013).

According to Swioklo S. et al. investigation alginate-encapsulation method was applied to improve hypothermic preservation of human adipose-derived stem cells. They evaluated the essential conditions required for hASCs cell survival and function over short-term periods in hypothermic storage. Due to inert hydrogel characterization of alginate, cells are separated from each other resulting in little or no physical cell-cell interaction that may offer protection at higher temperatures. After 24 hours encapsulation in culture, it was not detected any change in metabolic activity per cell. The cytoprotection was maintained in cell densities of up to 2×10^6 cells/mL, resulting in 70% viability. Assessment of Morphology and proliferative potential of hASCs following storage showed that Growth kinetics had not changed in all samples up to day 6. In this research it was demonstrated that cell density could have an effect on viable cell recovery (Stacey et al., 2017; Swioklo et al., 2016).

Fibrin Microbeads

Fibrin microbeads (FMB) are human fibrin-based, dense, nonimmunogenic, and slowly biodegradable particles with prolonged shelf life that were developed as cell carriers. FMB is a simple and highly qualified tool to isolate mesenchymal stem cells (MSCs) from different mixed sources and to support the expansion of many matrix-dependent cells in a three-dimensional (3D) culture with potential application in cell-based regenerative medicine. Cells which were loaded on FMB and cultured in slowly rotating 3D conditions in suspension can attain a high density (Ben-Ari et al., 2009; Gorodetsky et al., 1999; Shimony et al., 2006).

A number of other biodegradable microspheres and microcarriers were suggested for cell culturing in 3D (Chung & Park, 2009; Kang et al., 2008; Park et al., 2011), but they do not represent multifunctional features

and do not provide stem cell separation, expansion, and support them while differentiating at the same time. They are acting as the carrier for cell implantation. According to one study, FMB-based technique was used for delivery of mesenchymal cells and other matrix-dependent cells in sealed vials at room temperature for long time intervals of > 10 days. They reported high survival rate, without any need for further supportive infrastructure. The role of a possible protective mechanism of MSC attached to FMB by hypoxia induced factor (HIF)-1 is discussed (Gorodetsky et al., 2011). They tested the rate of proliferation for the cells after room temperature storage. They reported that after recovery time, cells keep their properties which was performed before and after 6 days in room temperature storage. The results displayed that the cells not only survived well but also have the same proliferation rate. Figure 5 represents the setup of cells cultured on fibrin microbeads (Gorodetsky et al., 2011).

Osmolyte Solution Composition

Natural osmolytes like sugars, sugar alcohols and amino acids work by stabilization of biological systems exposed to environmental extremes. Raman images shows the influence of osmolytes on ice crystal shape, which reflects the interactions between osmolytes and water. C.-H. Pi et al. optimized DMSO-free cryoprotectants for Jurkat cells as a model CD3+ T cell. They applied mixtures of osmolytes formulations of SGI (sucrose-glycerol-isoleucine), TGI (trehalose-glycerol-isoleucine) and MGI (maltose-glycerol-isoleucine). They designed a comparative study to evaluate the effects of three DMSO-free osmolyte-based formulations to provide better awareness to the potential of these cryoprotectants in terms of preserving a heterogeneous population. DMSO-free cryoprotectants indicated competitive post-thaw recovery of PBMCs (CD45+) and higher preservation capability for primary T cells and helper T cells. In their previous work they represented the effect of combining osmolytes on preservation quality and characterized the cell and osmolytes interactions on Jurkat cells. They detected that multicomponent osmolyte solutions including sugars, sugar alcohols and amino acids were effective in cryopreserving cells (C.-H. Pi et al., 2020; C. Pi et al., 2019). In the other study they characterized the "sweet spot" for the preservation of a T-cell line using osmolytes (C.-H. Pi et al., 2018).

Traditional knowledge designates that appropriate cryopreservation includes the control of ice formation in the freezing process. The thermophysical properties of each cryoprotectant solution were evaluated and discovered that the formulation of trehalose-glycerol-isoleucine had the lowest enthalpy of melting, indicating less ice formation. Although this formulation was not the optimal cryoprotectant for every cell type, it proved that the mass of ice formation in the freezing process is not the unique factor for cell recovery. Biology is a contributing factor of the cryopreservation process. The results indicate that the biological interactions between cryoprotectants and water and also between cryoprotectants and cells can associate with improvement of the recovery of cell types which are mainly sensitive to cryopreservation. These cell types have various responses to cryoprotectants obviously, and there may not be a global cryoprotectant that can optimize all branches at the same time. Finally, understanding the knowledge of freezing responses of different subsets of PBMCs will permit for more potent and successful cryopreservation of PBMCs (C.-H. Pi et al., 2020).

Trehalose-Contained Solution Formula

Trehalose is disaccharide of glucose which has the ability of stabilizing and preserving cells or tissues through the freeze and thaw procedures (Erdag et al., 2002). Albeit the mechanisms were unknown, it could be used alone as an excellent alternative CPA in the cryopreservation of red blood cells and germ cells (Eroglu et al., 2000; Satpathy et al., 2004). A few studies have investigated trehalose ability to keep the cells safe from apoptosis in relatively high temperature (i.e., 48C). In a study, the effect of trehalose was evaluated on improvement of the survival of MSCs at 48C. Di G. et al developed a solution formula that could effectively preserve MSCs at 48C for up to 2–3 weeks (Di et al., 2012).

Trehalose is known as a nontoxic compound which has been commonly used as natural CPA for cryopreservation of cells and hypothermic storage of human tissues (Erdag et al., 2002; Pu et al., 2005). It was demonstrated that the addition of trehalose to the cryopreservation medium remarkably increased the sur-

vival of human hepatocytes and amniotic fluid-derived stem cells (Katenz et al., 2007; Seo et al., 2011). Also, the low concentrations of intracellular trehalose could increase the survival rate of human cells during cryopreservation (Eroglu et al., 2000; Satpathy et al., 2004). However, the exact mechanism of efficient preservation of biological systems by trehalose during freezing and drying is obscure. It is believed that trehalose has the ability to stabilize phospholipids and proteins through its direct interaction with polar groups of these biomacromolecules during freeze-thawing process (T. Chen et al., 2001; Crowe et al., 2003).

Cryoprotectants are classified into two types of Penetrating and nonpenetrating (McGann, 1978). The penetrating types comprise glycerol, 1,2-propanediol and dimethyl sulfoxide. The nonpenetrating cryoprotectants involves polyvinylpyrrolidone, trehalose, fructose, sucrose and glucose. Trehalose is found in numerous organisms, such as nematodes and yeasts, which are capable of surviving during freezing and drying. Previous studies have used trehalose for the purpose of human cell cryopreservation such as platelets, red blood cells, sperm, oocytes, pancreatic islets and fetal skin. Hence, the administration of an alternative protocol with trehalose for cryopreservation of stem cells obtained from umbilical cord blood and bone marrow has previously been reported. Also in a study by Scheinkonig et al. cryopreservation of stem cells obtained from mobilized peripheral blood stem cells has been reported. Conversely, in another study, trehalose was used to cryopreserve pure Hematopoietic stem cells and progenitor cell (Beattie et al., 1997; Erdag et al., 2002; Eroglu et al., 2002; Martinetti et al., 2017; Motta et al., 2014; Scheinkönig et al., 2004; Wolkers et al., 2001; Zhang et al., 2003).

The application of the anhydrobiotic compound trehalose (a,a-trehalose, a-D-glucopyranosyl-a-D-glucopyranoside) has been known as a cryo/lyoprotective agent with or without other CPAs for a wide variety of therapeutic applications ranging from blood banks to reproductive technology (Eroglu et al., 2000; Y.-A. Lee et al., 2013) Trehalose forms hydrogen bonds with biomolecules and preserves their functional conformations during water deficiency (Lawson et al., 2011). Indeed trehalose acts as a nonpermeating molecule to form a stable glassy matrix with low molecular mobility that suspends any intracellular degradative biochemical processes in response to water loss by prohibiting the resultant lipid phase transition (Hara et al., 2017).

Adding relatively low concentrations of dimethyl sulfoxide (DMSO) and trehalose as cryoprotective agents (CPAs) for the evaluation of their synergistic effect on long term cryopreservation process was subjected by some studies (Beattie et al., 1997; Choi & Chang, 2003; Yokomise et al., 1995). Lee L. Q. Pu et al. suggested a cryopreservation method containing trehalose as a cryoprotective agents. They developed a practical technique by adding trehalose alone as CPAs. It considered to represent better long-term preservation method for adipose tissues because the viability rate of cells was significantly increased. Further studies are needed to refine their method for cryopreservation with trehalose as a CPA. Since non-toxic characteristics of trehalose, it can be used alone, as a single CPA, in long-term preservation of adipose tissues and the removing stage does not important (Pu et al., 2005).

Sucrose-Based Media

Non-DMSO cryoprotectants such as sugars have been commonly utilized to be an alternative of DMSO in the cryopreservation of various mammalian cell types. According to research results sugars can be effective in combination with sugar alcohols and amino acids in the cell preservation process. Numerous studies have been performed to figure out the mechanisms of protective effects of sucrose, most of which aimed at the interactions between sucrose and water, protein and membranes. Yu G. et al. explored the interactions between sucrose and water in a frozen sucrose solution. They visualized the interactions between sucrose and cell membrane at a low temperature directly and the mechanisms of its protective effects on cells. They detected the strengthened hydrogen bond network in concentrated sucrose solution. They suggested that protective properties of sucrose might originate from its direct interaction with cells. Recently, the high spatial resolution of Raman spectroscopy allowed us to visualize the distribution of extracellular and intracellular ice, cryoprotectants and the size of ice crystals formed in different combinations of cryoprotectants during freezing time. They quantified the variation in the sucrose concentration in a gap between a cell and the ice phase by using Raman spectroscopy images. The sucrose concentration at the interface between cell and

extracellular ice was lower than that in bulk nonfrozen solution (Yu et al., 2018). Shanina I. V. et al. had a study on comparison of a sucrose-based solution with other preservation media in hepatocyte cells. According to their results, sucrose-based solution could be a less complex alternative solution for cold storage of isolated hepatocytes. The stability of liver cells at low temperature were conducted by exclusion of trypan blue dye and morphological appearance. Dye exclusion in sucrose-based solution was higher than in other mediums (Shanina et al., 2000).

Collagen-Based Scaffold

Cell interaction with scaffolds occurs via chemical groups or ligands on the material surface. Natural extracellular materials (e.g., collagen) are the Precursor of scaffolds. The ligands of Arg-Gly-Asp have naturally found in the material surface. The pore size of a scaffold shows an essential component for cell attachment, survival, and function (Daei-farshbaf et al., 2014; Ranucci et al., 2000). Porosity and the water-uptake capacity of the scaffold are also vital parameters in cell-scaffold interaction. Custom-made scaffolds frequently lack reproducibility. Ruo M. et al after characterization of the scaffold, shipped cells on scaffold over 10 days. Optimaix-3D was the name of a scaffold which was reproducibly manufactured in a controlled freezing process from a defined collagen dispersion (Schoof et al., 2001). Primary human hepatocytes need the interaction with Extracellular matrix (ECM) components, such as collagen, to maintain their specific functions. The ECM protein collagen presents a significant function in the maintenance of organs and tissue (Baiocchini et al., 2016). So it is known as a biomaterial utilized in medical and bioengineering sciences. It is biodegradable and in contrast to albumin or gelatin only weakly antigenic (C. H. Lee et al., 2001). Collagen is also one of the vital ECM proteins of the healthy liver. Therefore, the Optimaix-3D scaffold could be an appropriate carrier for primary human hepatocytes for transportation and/or metabolic studies (Khan & Khan, 2013).

According to Ruo M. et al studies, the viability of the cells can be maintained significantly better by transport on the Optimaix-3D Scaffold from Matricel compared to the conventional shipment as a cooled suspension. Additionally, the utilization of Optimaix-3D Scaffold led to maintenance of the important hepatic functions including drug metabolism, urea production, and albumin synthesis over the period of 10 days. Hence, the scaffold has nearly the same stiffness as a healthy liver. With its high porosity and permeability, it is not only ideal for supplying the cells with nutrients, but also for use within a bioreactor. Due to the good biocompatibility of the collagen used for the scaffold, it might be also possible to use a scaffold seeded with primary human hepatocytes in regenerative medicine (Ruoß et al., 2018).

Natural Zwitterionic Betaine

Betaine can perform as a highly promising CPA. Betaine is a zwitterionic and hydrophilic molecule rich in abundant microorganisms, plants, and animals. Betaine has efficient effects on ice formation and can regulate osmotic stress. Betaine is a well-known osmoprotectant capable of protecting cells against osmotic stress (Courtenay et al., 2000). Also zwitterionic betaine could produce a monolayer of water around the proteins to maintain their stability and functions. Moreover betaine is related with the freezing tolerance of various plants. According to Kishitani et al. studies the winter-type barley by accumulation of betaine at high levels in their leaves during cold acclimation can protect them from freezing injury (Nomura et al., 1995). Yang J. et al. represented cell cryopreservation using betaine as a nontoxic alternative to DMSO. In order to their findings ultra-rapid freezing was more straightforward than the conventional controlled-rate freezing protocol due to diverse cell types cryopreservation, better post-thaw survival efficiency and low cytotoxicity (J. Yang et al., 2016).

The interaction of water molecules had shown an exact relation with ice formation. For this reason for minimizing the ice formation, CPAs are commonly hydrophilic molecules indicating strongest inhibition to water crystallization and excellent capability of decreasing the water chemical potential. Zwitterionic betaine is well known for its highly hydrophilic property; it can strongly bind water molecules via ionic solvation effects due to its charged groups (Shao & Jiang, 2015). As well as ice injury, solute injury occurred by osmotic stress is also responsible for cell death during the freezing and thawing process. Following cells were exposed in hypertonic betaine medium, they could still assume their spindle shape was similar to the control

cells. Interestingly, adding betaine into the hypertonic NaCl medium was able to significantly improve the viability of cells. These events indicated that by accumulating this natural osmoprotectant named betaine, cells would adapt to external osmotic stress. Therefore, betaine highlighted the uniqueness for protecting cells from solute injury. This natural zwitterionic molecule is a nontoxic CPA and enables cells to survive ultrarapid cryopreservation. Cellular uptake of betaine was ultra-rapid for intracellular protection during the freezing process. Due to betaine privileged cell cryopreservation ability, it has the possibility to be an alternative CPA for the conventional toxic ones (J. Yang et al., 2016). Figure 6 proposes the mechanism of cell cryopreservation using betaine with ultrarapid freezing.

Bio-Inspired Cryo-Ink

One of the important challenges in conventional vitrification is ice crystallization, which happens during rapid rewarming. It occurs as a result of the latent heat of fusion, causing hemolysis of the cells. It has reported a new technology-enabled capability of printing viable and functional cells. To avoid such crystallization, Assal R. E., et al applied a novel and innovative cryo-ink unified with a cryo-printer that can transform a bulk volume of human blood into nanoliter droplets on a cryo-paper. In this method, the cryo-ink solution includes ectoine, trehalose, and polyethylene glycol. The cryo-ink as a cryoprotective agent can assist the cells overcome the shock until the cooling and rewarming processes of cryopreservation. The cryo-ink solution used in this study posed complementary enhancing effects. Ectoine is present in the extremophilic bacteria which has the ability of adapting to extreme thermal and osmotic stress conditions. Indeed PEG suppresses the freezing point of the solution. Ultra-rapid rewarmation through immersing the printed nanoliter droplets on cryo-paper in warm media ([?]37 degC) can avoid crystallization (Figure 7). Furthermore, utilizing bio-inspired CPAs; such as ectoine and trehalose, in low concentrations eliminate extensive washing steps. Assessment of RBC morphology following ectoine-based vitrification and rewarming indicated that approximately 88% of RBCs during the ectoine-based vitrification maintained their original form with low irreversible morphological alterations compared to low-glycerol slow freezing and low-glycerol vitrification methods. Furthermore they measured band-3 phosphorylation, CD35 expression, intracellular nitric oxide (NO), and intracellular reactive oxygen species (ROS) for evaluating the functional properties of EV recovered RBCs (El Assal et al., 2014).

As a result of using DMSO, a usual toxic cryoprotectant, current cryopreservation protocols intail deterioration in NK cell viability and functionality which associated with side-effects in human. For this reason in another recent research which performed by Assal et al., the human natural killer cells were cryopreserved by slow freezing method using a cocktail of biocompatible bioinspired cryoprotectants including dextran and carboxylated ε-poly-L-lysine. They assessed morphology of RBC following ectoine-based vitrification and rewarming (Figure 8). It is important to notice that the anti-tumor functional activity and viability of recovered NK cells assessed and demonstrated higher cytotoxic potency against leukemia cells compared to cells cryopreserved with DMSO based solutions (El Assal et al., 2019). Dextran acts as a cryoprotective for various cell types. Carboxylated PLL similar to antifreeze proteins (AFPs) inhibits ice crystal growth and recrystallization during cryopreservation. By combining both dextran and carboxylated PLL, they showed synergic cryoprotective effect. These molecules indicate high affinity to water and thus could also they have the ability to remove intracellular water during freezing. The combination of dextran and CPLL could also interact with concentrated salts controlling the degree of dehydration to a level sufficient to avoid intracellular ice formation during freezing (Jain et al., 2014).

Discussion:

It is inevitable that for research studies, the mammalian cells have to be shipped globally from one laboratory center to another (Juan Wang et al., 2017). According to some ancient cell shipping procedures they need a special container and dry ice or need to fill flasks of live cells with medium. In comparison, a agarose-medium gel based method for transporting live cells directly in a plate (e.g. 6-well plate) has advantages of simple, low priced, no requirements for special container and dry ice, and no liquid in the cell plate. Cells shipped in live condition have a fast recovery process following the receiving time compared with the traditional methods. Therefore, agarose-medium gel based method is a helpful and convenient technique for cell transportation

between laboratories around cities or countries. In this method the appropriate time for cells transporting should be in 3 days (no more than 5 days) following the coverage of the cells with the agarose-medium gel (L. Yang et al., 2009).

A novel and easy utilization of alginate hydrogel encapsulation, may offer an inexpensive and powerful alternative to cryo-preservation for the transport and storage of stem cells for clinical and research studies. The appropriate storage duration for stem cells encapsulated within alginate gels is about five days under ambient conditions in an air-tight environment. This method also affords to allow the easy recovery of cells, and retain their viability and phenotype. It is a practical technique for the transport of stem cells, which will be beneficial for clinical and research studies (B. Chen et al., 2013).

Cells sealed on Fibrin microbeads at room temperature were protected, but did not represent any elevation of their hypoxia-induced factor-1a expression. Although the density of cells sealed on Fibrin microbeads incubated to reach confluence, the density of cells sealed on Fibrin microbeads at room temperature did not alter even after 10 days at room temperature. After 6 day storage of cells on Fibrin microbeads at room temperature, no alteration of pH and gas level were detected in the medium. Fibrin microbeads are types of cell carriers which could be useful for transferring progenitor cells at room temperature for long term intervals between different research centers (Gorodetsky et al., 2011).

Development of non-toxic and animal serum-free preservation medium is an important challenge for storage and distribution of mesenchymal stem cells. Therefore, it is inevitable to develop a formula that could preserve MSCs at 48C for up to 3 weeks. There is a formula which says trehalose is the important ingredient for maintaining viability of MSCs. Cells preserved in the formula contain trehalose still representing 70% viability for 3 weeks and they are similar to those of freshly harvested mesenchymal stem cells in terms of growth kinetics, expression of cell surface antigens, and differentiation potential. In summary, storage of MSCs in the medium makes it far easier for transporting the cells from processing units to clinical sites (Di et al., 2012). In particular, the efficacy of trehalose has been reported for clinical purposes in blood stem cells. The aim of the current study was to establish an efficient method for biological research based on the use of trehalose, to cryopreserve pure peripheral blood stem cells. The efficacy of trehalose was assessed in vitro and the cell viability was evaluated. The results showed that trehalose could improve cell viability after thawing compared with the standard freezing procedure. These data could confirm the potential of trehalose application for cell cryopreservation studies in future (Martinetti et al., 2017). Indeed the effect of combining osmolytes on preservation quality indicated that multicomponent osmolyte solutions including sugars, sugar alcohols and amino acids were effective in cryopreserving cells (C.-H. Pi et al., 2020; C. Pi et al., 2019). Numerous studies have been performed to figure out the mechanisms of protective effects of sucrose at low temperature of preservation. Recently, the high spatial resolution of Raman spectroscopy allowed us to visualize the distribution of extracellular and intracellular ice, cryoprotectants and the size of ice crystals formed in different combinations of cryoprotectants during freezing time. They quantified the variation in the sucrose concentration in a gap between a cell and the ice phase by using Raman spectroscopy images. The sucrose concentration at the interface between cell and extracellular ice was lower than that in bulk nonfrozen solution. It is suggested that protective properties of sucrose might originate from its direct interaction with cells (Yu et al., 2018).

The Optimaix-3D Scaffold is appropriate for Primary human hepatocytes long-term culture and transport. The novel collagen-based scaffold is characterized by its pore size, porosity, water-uptake capacity, permeability and stiffness. It was further tested whether the 3D cultivation permits the cells to be sent more convenient and to be cultivated over 10 days without loss of viability. Hence, the effect of the scaffold cultivation on important metabolic functions of Primary human hepatocytes was tested for up to 10 days and compared to conventional 2D cultures. The basal urea production, the one of important metabolic functions of Primary human hepatocytes that cultured on optimaix-3D scaffolds was about 50% higher than Primary human hepatocytes cultured in 2D. Hence, basic urea production of Primary human hepatocytes in 2D cultures decreased quickly within 10 days. In contrast, the basic urea production in 3D cultures remained stable over 10 days. According to the results obtained from albumin synthesis tests, albumin synthesis in

optimaix-3D cultures enhanced over the time compared to 2D cultures (Ruoß et al., 2018).

It is evident that vitrification has advantages over traditional slow freezing methods as a means of cryopreservation of mammalian ova and embryos. The most remarkable characteristic of vitrification is the prevention of ice crystal formation during the cooling process (Campos-Chillòn et al., 2006; Taylor-Robinson et al., 2014). Also, this procedure is less time-consuming and relatively inexpensive compared to conventional freezing protocols (Vajta et al., 1998). Although significant efforts have been made to appoint a novel vitrification procedure, large-scale application of IVF bovine embryos remains a relatively unexplored area of research. Evidently, vitrification offers significant potential applications for IVF and cloned bovine embryos in the field (Do et al., 2014).

Conclusion:

According to study of all of these kinds of cell shipping methods, we reach some conclusion about them. Based on the evaluation of appropriate time for cells transporting in these methods it is apparent that Cells preserved in the formula containing trehalose represented 70% viability for 3 weeks. After that Optimaix-3D Scaffold permits the cells to be sent more conveniently and to be cultivated over 10 days without loss of viability. Following that the density of cells sealed on Fibrin microbeads at room temperature did not alter even after 10 days at room temperature. After 6 day storage of cells on Fibrin microbeads at room temperature, no alteration of pH and gas level were detected in the medium. The appropriate storage duration for stem cells encapsulated within alginate gels is about five days under ambient conditions in an air-tight environment. The appropriate time for cells transporting by agarose-medium gel based method was 3 days (no more than 5 days). After cells were exposed in hypertonic betaine medium, it was able to significantly improve the viability of cells for 3 days and they could still attach to cell culture substrates. Altogether, we concluded that the preservation of cells by formula containing trehalose is much more convenient with the higher appropriate storage duration compared to other methods which were mentioned in this review. Although it should mentioned that the appropriate method for cell shipping is cell line dependent and it must be examined which method is the best one for different investigations.

Abbreviations

DMSO: dimethyl sulfoxide; CPAs: cryoprotective agents; PG: propylene glycol; EG: ethylene glycol; GLY: glycerol; GMP: good manufacturing practice; LN2: liquid nitrogen; Me2SO: dimethyl sulfoxide; hMSC: human mesenchymal stem cells; SEM: scanning electron microscope; HA: HEMA agarose; hESCs: human embryonic stem cells; hMSC: human mesenchymal stem cell; mESC: mouse embryonic stem cells; FMB: fibrin microbeads ; HIF-1: hypoxia induced factor-1; ECM: extracellular matrix; IVF: In vitro fertilization

Declarations:

Acknowledgements

Not applicable.

Authors' contributions

SHH and MH designed and drafted the manuscript, collected the references and carried out the primary literature search. SB and SKH modify the manuscript and participated in discussions. All authors read and approved the final manuscript.

Funding

Not applicable.

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

This is not applicable for this review.

Consent for publication

This is not applicable for this review.

Competing interests

The authors declare that they have no competing interests.

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Table 1: Comparison between the slow-freezing and vitrification methods

Table 2. Advantages and disadvantages of commonly used cryopreservation techniques

Figure 1. Mediators of cell death during cryopreservation and the effective apoptosis inhibitors during cryopreservation.

Figure 2. Schematic protocol for dry-preservation of Pv11 cells. Preincubation step: Pv11 cells were collected by centrifugation and resuspended into a 600 mM preincubation trehalose mixture. The cell suspension was then incubated in a culture flask for 48 h at 25 C. Pictures show the aspect of Pv11 cells before and after preincubation.

Figure 3. Schematic view of cryogel formation

Figure 4. (A) For the formation of cryogels, (1) a hydrogel precursor solution is frozen (T < 0C). This process leads to a phase separation of the solvent (e.g., water) into a frozen phase (ice crystals) and a nonfrozen phase around ice crystals where the gel precursors (monomers, polymer, crosslinker, and initiator) are expelled. (2) Next, concentrated gel precursors are crosslinked around ice crystals (porogens). (3) Following cryogelation, thawed ice crystals give rise to a macroporous sponge-like hydrogel, known as a cryogel. (B) Digital image of (a) HA cryogels and (b) gelatin cryogels. (C) Scanning Electron Microscopy image of (a) HA cryogels and (b) gelatin cryogels.

Figure 5. The setup of cells cultured on fibrin microbeads (FMB) in regular tubes placed in a slow rotator in a CO2 incubator (A). The cells grown in special tubes with a covered perforated cover for air exchange with preservation of sterility, as shown in (B). Attached cells on the surface of the FMB are shown in (B) by electron scanning microscopy (C).

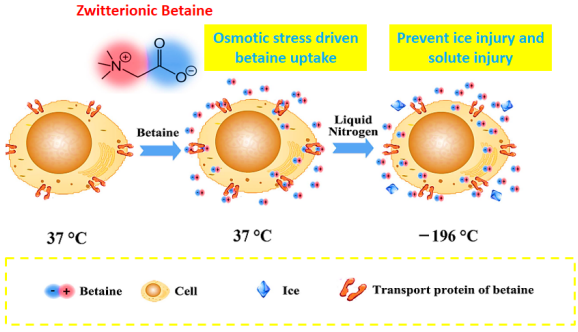
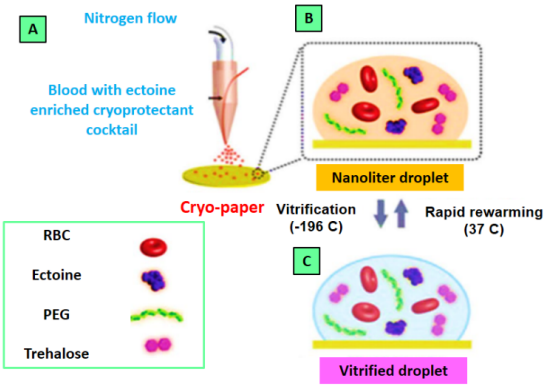


Figure 6. A proposed mechanism of cell cryopreservation using betaine with ultrarapid freezing. During the

freezing process, the uptake of betaine by cells via transport proteins is induced by osmotic stress (middle) and prevents the intracellular and extracellular ice injuries as well as solute injury.



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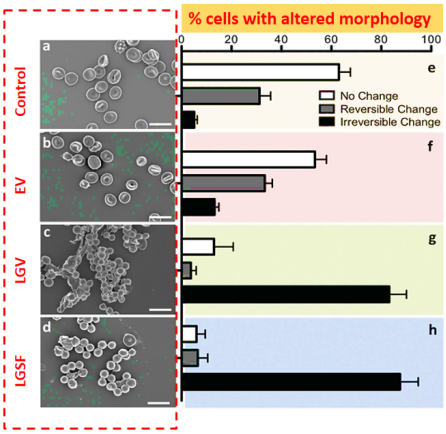

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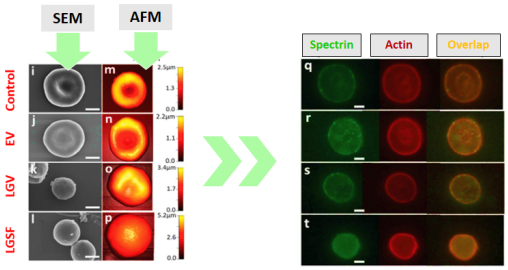
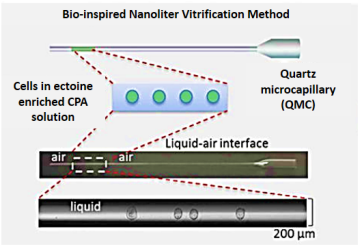
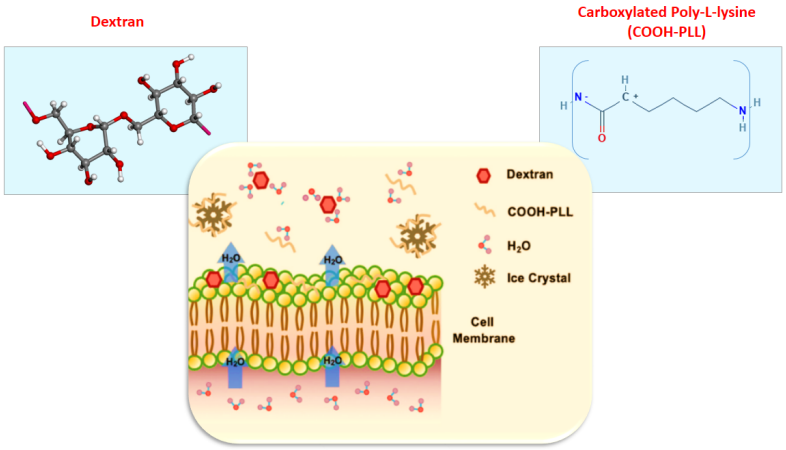


Figure 7. *Droplet-based vitrification of red blood cells. A) The essentials of the experimental setup for the droplet formation. B) Schematic magnified view of a single droplet on the cryo-paper including RBCs, ectoine, trehalose, and PEG. C) Schematic view of the vitrified droplet in (B). **Assessment of RBC morphology following ectoine-based vitrifi cation and rewarming. a–d) Scanning electron micrographs of RBCs: a) selected directly from blood, and recovered after: b) ectoine-based vitrifi cation (EV), c) low-glycerol vitrifi cation (LGV), and, d) low-glycerol slow freezing (LGSF). e–h) The percentage (%) of RBCs within a population representing: e) untreated fresh blood, and blood recovered from: f) EV, g) LGV, and, h) LGSF: biconcave shape, and reversible and irreversible shape changes (n, number of cells = 114–309, and N, number of donors = 3–9). Error bars in fi gures represent the standard error of the mean. i–l) Higher magnifi cation (30 000×) of SEMs of: i) fresh RBCs, and RBCs recovered after: j) EV, k) LGV, and, l) LGSF. m–p) Atomic force micrographs of: m) fresh RBCs, and RBCs recovered after: n) EV, o) LGV, and, p) LGSF. q–t) Fluorescence micrographs showing distribution of the spectrin-actin network in RBCs. q) RBCs in fresh blood, and RBCs recovered from: r) EV, s) LGV, and t) LGSF. Scale bar represents 10 μm in a–d and 2 μm in i–l and q–t.



A)



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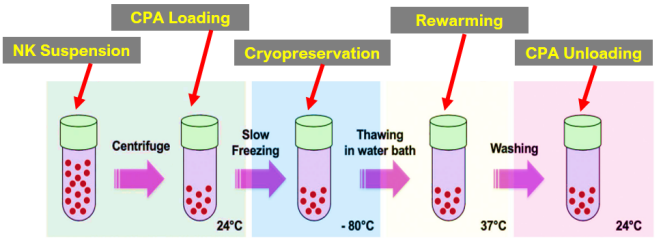
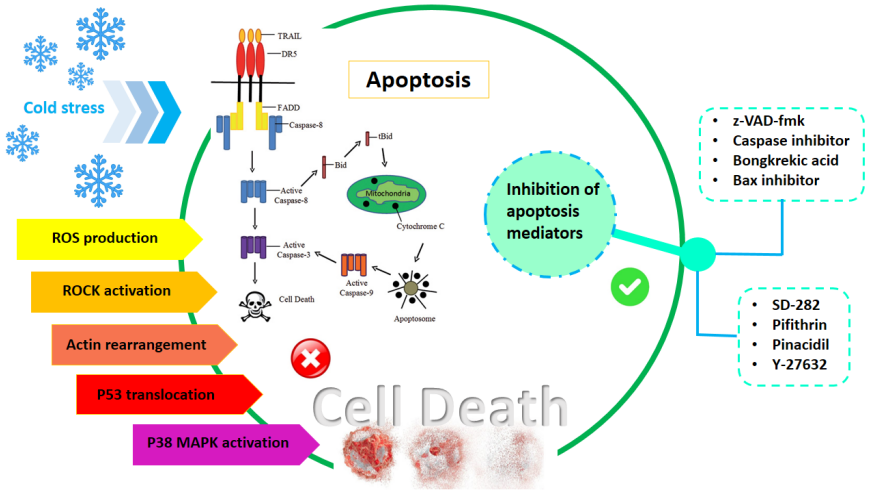
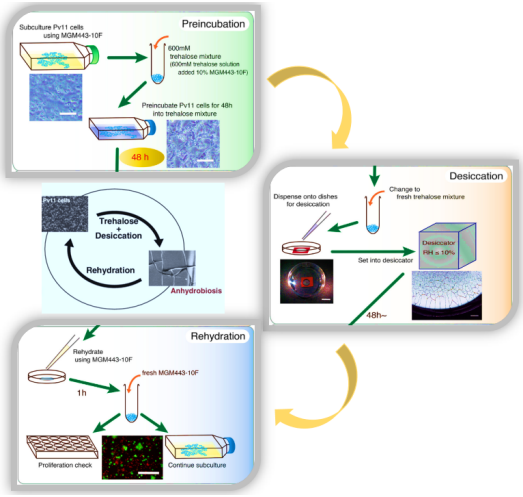


D)

Figure 8. A) Schematic view of bio-inspired nanoliter vitrification method. B) Schematic showing the chemical structures of dextran and carboxylated poly-L-lysine (CPLL). C) Schematic demonstrating the potential mechanism of action of dextran and CPLL during cryopreservation of natural killer (NK) cells. The synergic effect of CPAs is related to their high affinity to cell membrane, water molecules, and solutes. This characteristic might provide cell protection while removing intracellular water, restricting solute diffusion, and controlling the degree of dehydration to a level sufficient to minimize intracellular ice formation during cooling. Carboxylated PLL also might limit cryoinjury to cells by binding to ice crystals and inhibiting their growth and recrystallization during rewarming. D) Schematic showing the cryopreservation protocol used for preservation of natural killer (NK)-92 cells.





Characteristic	Slow freezing	Vitrification
Working time	More than 3 h	Fast, less than 10 min
Cost	Expensive, freezing machine needed	Inexpensive, no special machine needed
Sample volume (μL)	100-250	1-2
Concentration of CPA	Low	High
Risk of freeze injury	High	Low
Post-thaw viability	High	High
Risk of toxicity of CPA	Low	High
Status of system	Closed system only	Opened or closed system
Potential contamination with pathogenic agents	Low	High
Manipulation skill	Easy	Difficult

	Slow freezing	Vitrification
Advantages	• Stability from relatively non-toxic cryoprotectants	• No special equipment needed, fast procedure, fast recovery
Disadvantages	• Requires expensive equipment, slow recovery, low applicability to tropical species	• Vitrification solutions are toxic to many plants, cracking is possible, requires careful timing of solution changes