

Cryopreservation: A Review Article

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Abstract

Organelles, cells, tissues, or any other biological construction can be preserved using a method called cryopreservation, in which samples are cooled to extremely low temperatures. The reaction of the living cell to the formation of ice is both theoretically intriguing and practically useful. Since osmotic shock, membrane damage, and ice crystal formation during freezing and thawing will result in cell death, other viable tissues and stem cells, which are of much importance for uses in basic research and medical applications, may not be preserved by simple cooling or freezing for large periods. With the aid of cryoprotective agents (CPAs) and temperature control technology, the successful cryopreservation of cells and tissues have been rising in recent time. Sometimes excessive use of cryoprotective agents may damage the original structure of preserved tissue. Therefore, cryoprotective agents should be used appropriately, and their quantities should be regulated. Excessive cooling may damage the membrane structure of the cell, so cooling should be done appropriately. Slow freezing and vitrification method are the two procedures that may be used for cryopreservation. Vitrification's main benefit is that it significantly reduces the likelihood of freeze damage, making it possible to maintain a high enough cell survival rate. Good manipulation skills are also required, and there is a considerable risk of infection with pathogenic pathogens. [As1] Fruitful cryopreservation of cells or tissues and their therapeutic use will require ongoing knowledge of the physical and chemical features which take place in the freezing and thawing cycle. We briefly discuss representative cryopreservation techniques and their clinical uses in this study.

Categories: Transplantation, Healthcare Technology, Anatomy

Keywords: advances, clinical applications, cpas, cryoinjuries, cryopreservation

Introduction And Background

Cryopreservation is an important method in tissue engineering, and it is much helpful in the storage and preservation of tissue for the long term. The primary factor influencing the preservation of cells or tissues in the long term is a decrease in the biological and chemical reactions of living cells at low temperatures. However, freezing kills the majority of live tissue because it causes the formation of extracellular and intracellular ice crystals, which changes the chemical makeup of the cell and causes harm [1]. Excessive cooling of tissue may damage its original structure and function of the tissue. The transition from the liquid to the solid phase is the main challenge for cells to overcome at low temperatures. Cryoprotectants are substances that are used in the preservation of tissues or cells. These substances reduce the rate of formation of ice crystals in the cytoplasm of cells at a given temperature, but the condition is that these cryoprotective agents should penetrate the cytoplasm of cells and should have low toxicity. It should be noted that intracellular freezing is toxic, and extracellular freezing is harmless. Densely packed cells may also be damaged by the stress created during the process of preservation. Vitrification is an important method in preventing the formation of ice in the intracellular space. The process of osmosis plays an important role in the action of cryoprotectants [2]. Temperature history and cooling rate play an important role in cryobiology. Therefore, the measurement of temperature and heat transfer are important aspects of cryobiology. The thermal properties of cryoprotective agents play an important role in their activity. Therefore before using any cryoprotective agent, its physical and chemical properties should be taken into consideration [3]. Cryopreservation aids in keeping biological material in a stable state at cryogenic temperature for any significant amount of period and helps preserve the original cell structures [4]. Biological processes in the living cells are temporarily paused at low temperatures. This allows us to preserve the cells or tissues for the long term for many medical applications and research purposes. However, there is a contradiction that living cells may get damaged by cryopreservation itself. Because it is difficult for the cell to adapt at such low temperatures while the temperature changes take place during the cooling and warming process. Therefore, the physical and biological factors should be taken into consideration [5]. Cryoprotective agents (CPAs), which regulate the rate of ice crystal formation, water transport, and nucleation, can change a cell's freezing behavior. These cryoprotective agents play an important role in the preservation of sperm cells, oocytes, hepatocytes, embryos, etc. [6]. In the area of fundamental research and its therapeutic application, cryopreserved cells and tissues are very significant. Without obtaining a fresh sample, quality testing can be done to assess whether a cell or tissue is suitable. The method of cryopreservation is important in treating various disorders related to organ failure and metabolic disorders. Cryopreservation serves as an important alternative for the complex and costly treatments of various disorders [7]. We succinctly summarize the cryopreservation principle and its clinical use in this review.

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Review

Cryopreservation

Cryopreservation is the process of preserving living cells and tissues at very low temperatures for an extended duration of time [8]. Types of cryopreservation processes can typically be categorized: Slow freezing [9] vitrification (the aqueous environment of the cell or tissue solidifies into a non-crystalline transparent phase) [10], non-freezing subzero preservation, and dry state preservation [11]. Due to the challenges of introducing the disaccharide trehalose, it is difficult to store mammalian cells in a dry state [12]. The mixing of CPAs with the cell or tissue before chilling is one of the key processes in cryopreservation, low-temperature cooling of cells or tissues and their storage, cells and tissue warming, and removal of CPAs from tissues or cells during thawing [13]. Therefore, it's crucial to employ CPAs properly to increase the sample's resistance to cryoinjury.

Cryoinjuries

It is unclear how exactly cells are damaged by cryoinjury, which occurs when water undergoes a phase transition in both extracellular and intracellular settings at low temperatures [14]. According to McGann, Yang, and Walterson's 1988 study on the process of cryoinjury, intracellular ice production and solute concentrations outside or inside cells can trigger osmotic rupture [14,15]. The cooling rate influences both processes [16]. The boundaries of a cell's viability are determined by an intact plasma membrane with its natural semipermeable features. The circumstances that permit the survival of the plasma membrane may preclude the endurance of vital structures within the cells [17].

CPAs

CPAs are substances that are utilized to lessen the freezing damage caused by the cryopreservation process. CPAs should have low toxicity, can enter cells, and be biologically acceptable [18]. To achieve the highest possible endurance rate, the warming rate, cooling rate, sample volume, and CPA concentrations should be optimized for the various cells and tissue contexts [16]. Because bulk systems have constraints on heat and mass transmission, it must be noted that the large size of the tissue is an important aspect to be included in strategies of cryopreservation [16]. CPAs can be categorized into two types.

- 1) Cryoprotectants that permeate cell membranes include Dimethyl sulfoxide (DMSO), glycerol [19], and 1,2-propanediol.
- 2) Membrane-impermeable cryoprotectants include polymers such as polyvinyl pyrrolidone, hydroxyethyl starch, 2-methyl 2,4-pentanediol, and other sugars [20]. Liquid nitrogen is used as a cryoprotectant [12].

Glycerol

Cryoprotective effects of glycerol were first found in 1949 by Polge et al. [21]. These polyol compounds remained the most efficient additives until the protective action of DMSO was proven by Lovelock and Bishop in 1959 [22]. Nonelectrolyte glycerol has the potential to function by diluting the electrolyte concentrations of the unfrozen fluid remaining in and around the cell at any temperature. It finds widespread use in the preservation of microorganisms and sperm [23].

DMSO

Because of its inexpensive price and relatively lower degree of cytotoxicity, DMSO has been broadly employed for the cryopreservation of cultured mammalian cells since it was earlier produced by a Russian scientist in 1866 [22]. DMSO is one of the most used cryoprotectants [24]. In the same way, as glycerol does, DMSO lowers the electrolyte content in the unfrozen fluid around a cell at any temperature. DNA methylation and histone modification, on the other side, have been connected to decreased cell survival and the activation of cell differentiation [4]. Because of these concerns, the widespread clinical use of DMSO for cryopreservation presents certain challenges.

Polymers

To further modify cell location, CPAs may be entrapped within a capsule upon resuspension of cells in an encapsulating substance [4]. Cryoprotection of cells inside the scaffold may be achieved using synthetic nonpenetrating polymers, allowing for higher-dimensional cryopreservation without the diffusion constraints that plague other enclosing materials [4]. Some vinyl-derived polymers may reduce the size of newly formed ice crystals. These include polyethylene glycol (C₂nH₄n+2O_n+1, molecular weight: 200-9500 Da), polyvinyl alcohol [(C₂H₄O)_n, molecular weight: 30-70 kDa], and hydroxyethyl starch (130-200 kDa) [25]. Polymers are also important cryoprotectants [26].

Proteins

Sericin is a water-soluble sticky protein (50 kDa) obtained from silkworm cocoons that act as CPAs for hepatocytes, human adipose tissue-derived stem cells, or progenitor cells [23]. Additionally, small antifreeze

proteins isolated from marine teleosts or fishes have garnered interest as potential CPA [27].

The CELLBANKER saga

More cells survive the freezing and thawing processes when using the recently created CELLBANKER series (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) [28]. Cryopreservation media from the CELLBANKER series include 10% DMSO in addition to glucose and the high polymer that has been recommended and pH buffers [29]. CELLBANKERS 1 and 1+, which include serum, are suitable for the cryopreservation of most mammalian cell types. Traditional cryopreservation media includes fetal bovine serum, which is not allowed in the development of a standardized cryopreservation procedure for clinical application in humans since it includes a variety of growth hormones, cytokines, and unknown compounds such as bovine exosomes [30]. When it comes to cryopreserving cells under serum-free culture conditions, the nonserum type CELLBANKER 2 is ideal. With a composition of 10% DMSO and other inorganic compounds (US20130198876), as CELLBANKER 3 (or stem CELLBANKER) is Xeno free, it meets the requirement of a chemically defined known component; therefore, it is appropriate for the preservation of somatic stem cells and induced pluripotent stem cells.

Traditional slow freezing and vitrification are the two types of freezing techniques

Slow freezing and vitrification are two procedures that may be used for cryopreservation. The concentration of CPAs and the cooling speed employed are the main distinguishing features. A modest enough cooling rate would theoretically allow intracellular water to be effluxed quickly enough to remove supercooling and avoid ice formation inside the cell [5]. It is important to note that ideal cooling speeds will vary between cell types due to variances in their capacities to transport water across the plasma membrane. By slow freezing, water in the cytoplasm is first replaced with CPAs, decreasing cell damage, and the cooling rate is adjusted according to the permeability of the cell membrane. When using controlled rate freezers or portable freezing containers on a benchtop, the slow cooling procedure calls for a cooling rate of roughly 1 C/min in the presence of less than 1.0M of CPA [8]. Slow freezing's benefits include a reduced contamination risk and little manipulation expertise required. Slow freezing, on the other hand, poses serious threats of freeze damage because of the accumulation of ice outside of cells. Rather than using a lengthy freezing method, vitrification involves exposing aqueous cell suspensions to liquid nitrogen, which causes them to immediately transition into a glass state [31]. After exposing cells or tissues to a high concentration of CPA (in the ratio of 40%-60%, weight/volume), they must be rapidly cooled to minimize ice nucleation before being frozen at deep cryogenic temperatures (i.e., using liquid nitrogen) [18]. The speeds of chilling and warming, the volume of the sample, and the sample's viscosity are the most important variables in the vitrification process [18]. So, it's important to maintain a careful equilibrium between all the important components for good vitrification. Both equilibrium and nonequilibrium processes may be used for vitrification. To achieve equilibrium vitrification, multimolar CPA mixes must be formulated, and injected into cell suspensions. Extremely high freezing rates and lower concentrations of the CPA mixture are utilized in nonequilibrium vitrification, which is further subdivided into carrier-based (including the former plastic straws, cryoloops, and quartz microcapillaries for obtaining a minimum drop volume [18]) and carrier-free system. Vitrification's main benefit is that it significantly reduces the likelihood of freeze damage, making it possible to maintain a high enough cell survival rate. Good manipulation skills are also required, and there is a considerable risk of infection with pathogenic pathogens (Table 1).

Characteristics	Slow freezing	Vitrification
Sample volume (uL)	100-250	1-2
Working time	More than 3 hours	Fast less than 10 min.
Cost	Expensive	Comparatively cheaper
Concentration of CPAs	Low	High
Risk of toxicity of CPAs	Low	High
Manipulation skills	Easy	Difficult
Past thaw viability	High	High
Status of system	Closed system only	Opened or closed system

TABLE 1: Comparison of slow freezing and vitrification method

CPA: cryoprotective agents

Examples of cryopreservation in use

The following types of cryopreservation applications can be made. 1) Cryopreserved tissues and cells [5]. 2) Molecular biology and biochemistry. 3) Cryosurgery. 4) Numerous medical procedures, including in vitro fertilization (IVF), artificial insemination, and bone marrow transplantation [11]. Some benefits of cryopreservation include the preservation of cells for human WBC antigen typing for organ transplantations and providing an adequate period for cell and tissue transportation between various medical locations [5]. Preservation of stem cells for a long period is the primary step in tissue engineering, which helps in the reformation of soft tissue's appreciative function and treatment for known diseases for which there is currently no available therapy [32].

Embryos and oocytes

The initial instances of embryo cryopreservation for the preservation of fertility occurred in 1996 with the use of natural in-vitro fertilization. Before starting chemotherapy, it happened to a woman who had been diagnosed with breast cancer. The tried-and-true method of cryopreserving mature oocytes can be used to maintain reproductive capability. The duration of preservation had no discernible effect on miscarriage, live birth rate or implantation, or clinical pregnancy, whether from an oocyte donation cycle or IVF, according to the results of a previous study of 11,768 cryopreserved human embryos that undergoes at least one thaw cycle between 1986 and 2007. The main issue with oocyte cryopreservation is cooling damage. As a result, there is always a need for improvement in research methods [32]. These methods can also cause injury to cells [33].

Tissue from the testicles, sperm, and semen

Any age can experience the loss of germ cells brought on by disease, physical or chemical toxicity, or genetic predisposition. Fertility preservation is crucial for a patient receiving chemotherapy and radiation therapy to ensure their quality of life [34]. After adequate cryopreservation, sperm and semen can be used for nearly endless periods. There are currently new studies being conducted to preserve testicular tissue as tubular portions, cell suspensions, and whole gonads; however, this technology is still in its infancy [34]. For males undergoing vasectomy or other medical procedures that could harm their fertility, such as radiotherapy, chemotherapy, or surgery, cryopreservation can be used as a primary method of saving fertility.

Stem cells

Adult stem cells have the capacity to differentiate into a variety of distinct cell types. Other than bone marrow, they can be obtained from a variety of sources, such as adipose, the periosteum, the adipose tissue, amniotic fluid, and umbilical cord blood [9]. Mesenchymal stromal cells, embryonic stem cells, hematopoietic stem cells [28], and other subtypes of stem cells are all considered to be a gold mine for positive use in regenerative medicine [35]. The skill to retain, preserve, and pass these stem cells without modifying their hereditary and biological contents is crucial for the field of regenerative medicine, gene therapy, and cell transplantation.

Hepatocytes

Over the past 40 years, physiological studies, research on hepatic metabolism, drug detoxification, organ transplantation, and experimental and clinical transplantation are just a few of the areas where previously isolated liver cells have been found of significant importance in science and medicine [11]. Additionally, there is a growing demand for cryobanking due to the use of liver progenitor cells in various scientific fields, including regenerative medicine and biotechnology [11].

Skin

Genetically modified pig xenografts might take the place of human cadaveric allografts (HCA) in the treatment of badly burnt patients. However, if long-term storage and preservation were achieved without cellular viability loss, their therapeutic usefulness would be significantly boosted. A frozen human allogeneic skin graft is considered a suitable substitute when freshly obtained allogeneic skin grafts are not available. However, little is known about the functional and histological effects of cryopreservation on allogeneic skin transplants, especially those that overcome hurdles caused by mismatched histocompatibility. Therefore, cryopreservation of the skin is important in skin grafting [36].

Others

Although primary neuronal cells and heart muscle cells are frequently employed in cardiology and neuroscience research, no established technique for the preservation of these cells exists. Pancreatic islet transplantation may be considered an option for glucocorticoid-free immunosuppressive regimens for the treatment of Type 1 Diabetes [37]. That is why islets cryopreservation technology is currently being developed, but the results are still not ideal, with a success rate of less than 50% [37].

Requirements for cryopreservation

Even though the cryopreservation technology is very useful in scientific and clinical research, there are still certain restrictions. As a result of a genetic shift toward biological variation cell-associated alterations in proteins and lipids that could damage cellular activity and structure, the metabolic rate of the cell is nearly nil at low temperatures like -196°C (i.e., in liquid nitrogen). CPAs themselves can harm cells if used excessively [1]. DMSO has the capacity to change chromosome stability, which increases the likelihood of tumor formation [38]. The potential for contamination or infection with cells like tumors ones should be considered in addition to endogenous changes in cells.

Advances of cryopreservation

When all other therapeutic options have failed, organ transplantation may be the best option. Currently, the donor organ utilized for clinical transplantation is kept at above-zero temperatures. Although these techniques for preservation are tried and true, they are also quite simple; therefore, they only provide a storage window of 4-12 hours at most. Some pieces of research sought to lengthen the organ storage duration by strengthening protectant and HLA matching to boost the utilization of preserved organs and prolong the long-term life of organs. Even with these increases, the supply of organs for transplantation is still insufficient to meet the current clinical demand. In addition, numerous organs were lost owing to insufficient storage time, HLA mismatch, unsuitable recipients, or long travel distances. Therefore, it has become a common objective among academics throughout the world to find ways to preserve organs for extended periods (weeks to months). Cryoprotecting chemicals, freezing and thawing techniques and other concerns associated with organ cryopreservation are discussed in this article [39].

Conclusions

From this review article, we can conclude that cryopreservation is a principal technique for the long-term preservation of cells and tissues. Long-term storage of cells or tissues at low temperatures can alter the functioning of cells or tissues. Excessive use of CPAs can also have effects on the cells or tissues. Cryopreservation also has great clinical significance in clinical applications. Understanding the principle behind the chemistry and biology of the freezing and thawing process could allow the development of more efficient procedures for cryopreservation and further expand their clinical applications.

Additional Information

Disclosures

Conflicts of interest: In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

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