Cryopreservation and its clinical applications

1. Introduction

Biological and chemical reactions in living cells are dra- matically reduced at low temperature, a phenomenon that can lead to the possible long-term preservation of cells and tissues. However, freezing is fatal to most living organisms, since both intra- and extracellular ice crystals are formed and results in changes to the chemical setting of cells that lead to cellular mechanical constraints and injury.[1] The major hurdle for cells to overcome at low temperatures is the water- to-ice phase transition.[2,3] Cell injury at fast cooling rates is attributed to intracellular ice formation, whereas slow cool- ing causes osmotic changes due to the effects of exposure to highly concentrated intra- and extracellular solutions or principles of cryopreservation and its application in clinical are to mechanical interactions between cells and the extracellular ice (Fig. 1). Cryopreservation is a process that maintains biological samples in a state of suspended animation at cryo- genic temperature for any considerable period and is used to preserve the fine structure of cells.[3,4] The freezing behavior of the cells can be altered in the presence of a cryoprotec- tive agent (CPA; also called cryoprotectant), which affects the rates of water transport, nucleation, and ice crystal growth. Numerous research papers on cryopreservation have stud- ied the underlying physical and biological factors affecting the survival of cells at low temperatures during the cooling and warming processes.[5] Unlike in single cell suspensions, bulk tissues have different heat and mass transfer effects that occur during the cryopreservation of bulk tissues and thus these factors make it more difficult to achieve rapid cooling and warming rates as well as an equal distribution of CPAs.[1,6] Cryopreserved cells or tissues possess some advantages for basic research and current and future clinical applications. With the constant availability of cryopreserved cells and tis- sues, extensive quality testing can be performed to determine the suitability of the cells or tissue for transplantation without the need to obtain fresh samples.[7] The successful cryopreser- vation of cells and tissues has been gradually increasing in recent years with the use of CPAs and temperature control equipment (Table 1). In this review, we briefly summarize the

2. Cryopreservation

2.1. Cryopreservation procedure

Cryopreservation is the use of very low temperatures to pre- serve structurally intact living cells and tissues for a long period of time.[2] Depending on the cell types or given cells among different mammalian species, there is great diversity in cryobiological response and cryosurvival during the freez- ing and thawing cycle (Fig. 1 and Table 1).[5] Cryopreservation processes can

generally be grouped into the following types:

(1) slow freezing[8,9]; (2) vitrification, which involves the solid- ification of the aqueous milieu of the cell or tissue into a noncrystalline glassy phase[10]; (3) subzero nonfreezing stor- age; and (4) preservation in the dry state.[11] Generally, the storage of mammalian cells in the dry state is not readily pos- sible because of difficulties in introducing the disaccharide trehalose (disaccharide of glucose, 342 Da)[12] and amino acids (used as preservatives in plants) into the intracellular region.[13] The major steps in cryopreservation are (1): the mixing of CPAs with cells or tissues before cooling; (2) cooling of the cells or tissues to a low temperature and its storage; (3) warm- ing of the cells or tissues; and (4) removal of CPAs from the cells or tissues after thawing.[14] The appropriate use of CPAs is

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

Characteristic	Procedure	
	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, including ice crystal formation	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

[15–17]

CPA, cryoprotective agent.

therefore important to improve the viability of the sample to be cryopreserved.

2.2. Cryoinjury

The exact mechanism of cryoinjury, which is the damage of cells associated with the phase changes of water in both extra - and intracellular environments at low temperatures, has not been clearly established. [5] The cooling and thaw- ing velocities can largely affect the physicochemical and biophysical reactions, altering the survival rate. Cryoinjury mechanisms involving osmotic rupture caused by extra- or intracellularly concentrated solutes and intracellular ice for- mation have been highly suggested, both processes of which are dependent on the cooling rate (Fig. 1).[5,18] In addition, cell viability limits are defined primarily in terms of an intact plasma membrane that retains normal semipermeable prop- erties. Indeed, conditions that allow the plasma membrane to survive may not allow the survival of critical organelles within cells.[5]

2.3. CPAs

The CPA, which is usually a fluid, reduces the freezing injury from the cryopreservation process (Fig. 1). CPAs should be bio-logically acceptable, be able to penetrate the cells, and have low toxicity.[2] Various CPAs have been developed (Table 2) and are used to reduce the amount of ice formed at any given temperature, depending on the cell type, cooling rate, and warming rate.[2] In order to achieve the best survival rate of cells and tissues, the sample volume, cooling rate, warming rate, and CPA concentrations should be optimized depend- ing on the different cell types and context of tissues. [18] It should be mentioned that the macroscopic physical dimen- sion of the tissue is a major point to be defined in a cryopreservation protocol because of heat and mass trans- fer limitations in these bulk systems.[1,18] CPAs can be divided into two categories: (1) cell membrane-permeating cryopro- tectants, such as dimethyl sulfoxide (DMSO), glycerol,[19] and 1,2-propanediol; and (2) nonmembrane-permeating cryopro- tectants, such as 2-methyl-2,4-pentanediol and polymers such as polyvinyl pyrrolidone, hydroxyethyl starch, and vari- ous sugars.[1,4] Unlike synthetic chemicals, biomaterials such as alginates, polyvinyl

alcohol, and chitosan can be used to impede ice crystal growth, along with traditional small molecules. [4] The direct inhibition of ice crystal formation and application of antioxidants and other compounds have been used to attempt to reduce cell death from processes such as apoptosis during the freezing and thawing cycle. [20–23] Common CPAs are briefly addressed in the following subsections and Table 2.

2.3.1. Glycerol

Polge et al[24] discovered the cryoprotective effect of glycerol in 1949, and this polyol compound remained the most effec- tive of additives until the protective effect of DMSO was demonstrated by Lovelock and Bishop in 1959.[25] Glycerol is a nonelectrolyte and thus may act by reducing the electrolyte concentration in the residual unfrozen solution in and around a cell at any given temperature. It is widely used in the storage of bacteria and animal sperm.[26]

2.3.2. DMSO

First synthesized by the Russian scientist Alexander Zaytsev in 1866, DMSO has been commonly used for the cryopreser - vation of cultured mammalian cells because of its low cost and relatively low level of cytotoxicty.[25,27] Like glycerol, DMSO acts by

reducing the electrolyte concentration in the residual unfrozen solution in and around a cell at any given tempera - ture. However, a decline in the survival rate and the induction of cell differentiation caused by DNA methylation and histone alteration have been reported.[28,29] These negative effects of DMSO in cryopreservation create some difficulties for its use in routine clinical applications

2.3.3. Polymers

The entrapment of CPAs within a capsule during cell resus- pension in an encapsulating material is another strategy for the modulation of cell location.[4] Among encapsu- lating materials, synthetic nonpenetrating polymers can provide cryoprotection of cells within the scaffold, thereby bypassing the limitations of diffusion in higher-dimensional cryopreservation.[4] Vinyl-derived polymers, such as polyeth- ylene glycol ($C_{2n}H_{4n+2}O_{n+1}$, molecular weight: 200–9500 Da), polyvinyl alcohol [$(C_2H_4O)_n$, molecular weight: 30–70 kDa), and hydroxyethyl starch (130–200 kDa), have a capacity to decrease the size of formed ice crystals.[4,30,31]

1.1Proteins

Sericin is a water-soluble sticky protein (~30 kDa) isolated from the silkworm cocoon and has been developed as a fetal bovine serum- or DMSO-replacing CPA for human adipose tissue-derived stem or progenitor cells, or hepatocytes.[26,27] Small antifreeze proteins derived from marine teleosts or fishes have also attracted attention as CPAs. [32]

1.2 Cell BANKER

A newly developed Cell Banker series (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) allows for rapid cell cryopres-ervation at −80 ◦C, and has been shown to achieve better survival rates following freezing and thawing.[29,33] The Cell Banker series of cryopreservation media contain 10% DMSO, glucose, a prescribed high polymer, and pH adjustors. [33] Serum-containing Cell Bankers 1 and 1+ can be used for the cryopreservation of almost all mammalian cells. Indeed, con- ventional cryopreservation media include fetal bovine serum, which contains a mixture of growth factors, cytokines, and undefined substances such as bovine exosomes, rendering its use forbidden in the establishment of a standardized cryopres- ervation protocol for clinical use in humans.[34] In this aspect, the nonserum-type Cell Banker 2 is optimal for the cryopres- ervation of cells in serum-free culture conditions. Cell Banker 3 (or Stem cell Banker) is composed of 10% DMSO and other inorganic compounds (US20130198876) and satisfies the cri - terion of a chemically defined known ingredient that is xeno free and is thus suitable for the preservation of somatic stem cells and induced pluripotent stem cells.

2- Freezing method: conventional slow freezing and vitrification

Cryopreservation can be accomplished by slow freezing and vitrification methods (Table 1). The major differences between the two are the concentrations of CPAs and the cooling rates used. Theoretically, if cooling is sufficiently slow, cells could efflux intracellular water rapidly enough to eliminate super- cooling and thus prevent intracellular ice formation.[5] As a result of differences in the capacity of different cells to move water across the plasma membrane, optimal cooling rates will be dependent on cell types. Slow freezing first substitutes the water within the cytoplasm with CPAs which reduces cell damage and adjusts the cooling rate in accordance with the permeability of the cell membrane. Slow-cooling protocols involve a typical cooling rate of about 1 ◦C/min in the presence of less than 1.0M of CPA, with use of a high-cost controlled- rate freezer or a benchtop portable freezing container.[8,9] The advantages of slow freezing are that it has a low risk of contamination during the procedures and does not demand high manipulation skills. However, slow freezing

has a high risk of freeze injury due to the formation of extracellular ice (Table 1). As an alternative to the slow-freezing technique, vitrification is a process by which cell suspensions are trans- formed directly from the aqueous phase to a glass state after direct exposure to liquid nitrogen.[35] The process requires cool- ing of the cells or tissues to deep cryogenic temperatures (i.e., with liquid nitrogen) after their exposure to high con-centrations of CPA (in the ratio of 40–60%, weight/volume), with subsequent rapid cooling to avoid ice nucleation.[18] Vitri- fication is largely dependent on three factors: (1) viscosity of the sample; (2) cooling and warming rates; and (3) sam - ple volume.[18] Thus, a delicate balance must be maintained among all the relevant factors to ensure successful vitrifi- cation. There are two methods of vitrification: equilibrium and nonequilibrium. Equilibrium vitrification requires formu- lation of multimolar CPA mixtures and their injection into the cell suspensions. Nonequilibrium vitrification, which is fur- ther divided into carrier-based (including the former plastic straws, quartz microcapillaries, and cryoloops for obtaining a minimum drop volume[18]) and carrier-free systems, uses an extremely high freezing rate along with lower concentrations of the CPA mixture. A major advantage of vitrification is the low risk of freeze injury, thereby ensuring a sufficiently high cell survival rate. However, the high potential of contamina- tion with pathogenic agents is present, and the technique requires good manipulation skills.

2- Applications of cryopreservation

The applications of cryopreservation (Table 2) can be catego- rized into the following areas: (1) cryopreservation of cells or organs[5]; (2) cryosurgery; (3) biochemistry and molecular biol- ogy; (4) food sciences; (5) ecology and plant physiology; and (6) many medical applications, such as blood transfusion, bone marrow transplantation, artificial insemination, and *in vitro* fertilization (IVF) Some suggested advantages of cryopreservation include the possible banking of cells for human leukocyte antigen typing for organ transplantation, the allowance of sufficient time for transport of cells and tissues among different medical centers, and the provisionof research sources for identifying unknown transmissible diseases or pathogens.[5]

Furthermore, the long-term storage of stem cells is still the initial step toward tissue engineering, which holds promise for the regeneration of soft tissue esthetic function and for the treatment of known diseases that have currently no therapy option.[40]

1.1. Oocytes and embryos

The first case of embryo cryopreservation for fertility preser- vation took place in 1996, with the application of a natural IVF cycle prior to chemotherapy in a woman diagnosed with breast cancer. Cryopreservation of mature oocytes is a proven tech-nique for preserving the reproductive capacity. Results from a retrospective study of 11,768 cryopreserved human embryos that underwent at least one thaw cycle from 1986 to 2007 showed that there was no significant impact of the duration of storage on clinical pregnancy, miscarriage, implantation, or live birth rate, whether from IVF or oocyte donation cycles. [41] Since oocytes are highly prone to chilling injury[10]; cryopreser- vation of immature oocytes and ovarian tissue is a promising approach-with reports of live births-but the need for investi- gational improvements remain.[37,42–45]

1.2. Sperm, semen, and testicular tissue

Germ cell depletion caused by chemical or physical toxicity, disease, or genetic predisposition can occur at any age.[6] Fertil- ity preservation is of great importance to guarantee the quality of life of patients facing chemo- and radiotherapy.[46] Sperm and semen can be used almost indefinitely after proper cryo - preservation. There are new trials for cryopreserving testicular tissues in the form of cell suspensions, tubular pieces, and entire gonads,[6,47] but this technique is still premature. Overall, cryopreservation can be used as a first-line means of preser- ving fertility for men undergoing vasectomy or treatments that may compromise their fertility, such as chemotherapy, radiotherapy, or surgery.

1.3. Stem cells

Adult stem cells are capable of differentiating into multi- ple types of specific cells and can be obtained from various locations other than bone marrow, including fat tissue, the periosteum, amniotic fluid, and umbilical cord blood.[9] Stem cells can be subdivided into embryonic stem cells, mesenchy- mal stromal cells,[29,36,48] and hematopoietic stem cells, all of which are considered as goldmines for potential application in regenerative

medicine. [28,29,49,50] Clearly, the fields of tis- sue engineering, gene therapy, regenerative medicine, and cell transplantation are largely dependent on the ability to preserve, store, and transport these stem cells without modi- fication of their genetic and/or cellular contents.

1.4. Hepatocytes

Primarily isolated hepatocytes have found important applica- tions in science and medicine over the past 40 years in a wide range of areas, including physiological studies, investigations on liver metabolism, organ preservation and drug detoxifi-cation, and experimental and clinical transplantation.[7,11] In addition, there is currently increasing interest in the appli- cations of liver progenitor cells across a range of scientific areas, including both regenerative medicine and biotechnol- ogy, which raises the need for cryobanking.[11]

1.5. Others

Although primary neuronal cells and cardiomyocytes are rou- tinely used for neuroscience and cardiology research, a gold standard protocol for the preservation of these cells has not yet been developed. With the discovery of glucocorticoid-free immunosuppressive regimens,[51] pancreatic islet transplanta- tion may be considered as an alternative for the treatment of type 1 diabetes. For this reason, the development of islet cryo- preservation methods has been ongoing, but results are still suboptimal, with a survival rate of less than 50%.[51]

2- Limitations of cryopreservation

Although numerous usages of the cryopreservation technique exist, both in basic and clinical research, some limitations still exist. Cells metabolize almost nothing at low temperatures such as −196 °C (i.e., in liquid nitrogen), which has inevitable side effects, including a genetic drift toward biological vari- ations of cell-associated changes in lipids and proteins that could result in the impairment of cellular activity and struc- ture. If there were no limit to the amount of CPA that could be used, cells would be preserved perfectly.[1] In conventional settings, however, CPAs themselves can be damaging to cells, especially when used in high concentrations. For example, there is a possibility that DMSO may alter chromosome sta- bility, which can lead to a risk of tumor formation.[52,53] Apart from endogenous changes in cells, the possible infection or contamination with cells such as tumorous ones should be prevented.

2- Summary and perspectives

Improvements in the freezing and thawing rates, osmotic conditions, choice and concentration of CPAs, and equilibra- tion times in the CPAs might result in better survival and functionality of human tissue and cell samples, permitting their successful future clinical application.[6] In this review, the freezing methods (slow freezing vs. vitrification) used in cryo- preservation and several CPAs were briefly addressed. New CPAs are constantly being investigated owing to the inherent toxicity of many known agents.[4,30] A better understanding of the chemistry and biology behind freezing and thawing will be necessary for future development of this process and for find- ing the safest and most effective cryopreservation method. The successful cryopreservation of biological samples may play a pivotal role in research connected with clinical utility for all kinds of human trials. Collectively, the most prominent future goals of cryopreservation should focus on the devel- opment of procedures that minimally affect the intactness of cryopreserved cells or tissues, followed by the standardization and optimization of the technique for routine use.