Basic Principles of Cryopreservation of Cells and Tissues

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Contributor: Caterina Giovanna Valentini, Claudio Pellegrino, Luciana Teofili

Cryopreservation refers to the technique of storing biological materials at below-zero temperatures, slowing the rate of degradation to ensure minimal loss in function; this practice has wide-reaching applications, including basic biological research, agriculture and food industry, and medicine.

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1. Introduction

The role of hematopoietic stem cell transplantation in the treatment of hematologic and non-hematologic malignancies is rapidly expanding. Current therapeutic protocols comprise cryopreservation of hematopoietic progenitor cells (HPCs) for virtually all autologous transplants, although successful experiences with fresh grafts have also been reported [1][2]. On the other hand, grafts for allogenic use have been typically employed fresh. One notable exception is represented by umbilical cord blood (UCB) [3]: the actual transplant is harvested at the time of birth and used at a later point in time for an often-indeterminate recipient, requiring cryopreservation in a cord blood bank for an indeterminate amount of time. Notably, UCB units stored over 29 years of cryopreservation still exhibit a high quality in terms of viability of total nucleated cells and CD34+ cells [4]. In 2020, the COVID-19 pandemic has precipitously changed the practice of transplanting fresh allografts. The lockdown disrupted international and domestic travels, into the hospitals new paths dedicated to COVID-19 patients replaced pre-existent units and to access any hospital departments become much more problematic. The complex logistics of the allogenic transplant in which graft collection was accomplished to be promptly delivered on the day of planned transplantation, was seriously threatened. In addition, there was a realistic risk that the donor could became SARS-CoV-2-infected and then unable to donate after the recipient had initiated the conditioning. To circumvent the potential critical impact on allogeneic transplant candidate patients, scientific societies, donor registries, and competent authorities worldwide recommended cryopreserving and securing donations prior to initiation of recipient conditioning [5][6].

2. Basic Principles of Cryopreservation of Cells and Tissues

Cryopreservation refers to the technique of storing biological materials at below-zero temperatures, slowing the rate of degradation to ensure minimal loss in function; this practice has wide-reaching applications, including basic biological research, agriculture and food industry, and medicine. Biological materials experience a significant decrease in kinetic energy and molecular motion when exposed to ultralow temperatures below −130 °C. This slowdown causes a decline in the rates of both chemical and biological reactions. Consequently, basic cellular processes, such as metabolism, active transport, enzymatic reactions, and diffusion, also slow down. As a result, the material can remain suspended until the temperature is increased again [7].

Although ultra-low temperatures themselves do not directly cause physical damage, damage can occur during the process of freezing and subsequent thawing. When cooling aqueous solutions below the freezing point, ice crystals will form in the extracellular media. This will decrease the concentration of extracellular water in the sample, while solutes that were previously distributed in the bulk solution will become concentrated in the residual water channels between the ice crystals. Cells that are enclosed in these channels will experience an increasing solute concentration than in the sample solution without ice present. This, in turn, creates an osmotic gradient across the cell membrane, causing cellular dehydration, which ultimately leads to osmotic shock and increased toxicity.

In addition, excessive intracellular ice formation determines irreversible damage to cell membranes and intracellular organelles. In most scenarios, an intermediate cooling rate during freezing maximizes cell survival, allowing cells to gradually dehydrate and avoid excessive osmotic stress and intracellular ice formation [8]. An alternative approach to

preserve biological samples is to use extremely high cooling rates to bypass the crystalline (ice) phase and obtain an ultrahigh-viscosity amorphous glass state, which is known as vitrification. This method helps to prevent cellular damage caused by ice nucleation and intracellular ice growth, as during supercooling, ice nuclei lack sufficient time to grow due to water molecule diffusion limitations. However, vitrification requires high cryoprotectant (CPA) concentrations to increase medium viscosity resulting in osmotic stress during CPA loading and removal. Moreover, achieving homogenous rapid freezing of large sample volumes is technically challenging, limiting the application of these protocols to cellular therapies. On the other side, vitrification is the clinically preferred method to cryopreserve oocytes and embryos, with reports of better clinical outcomes compared with slow-freezing protocols [9]. Recently, Akiyama et al. demonstrated the feasibility of directly vitrifying mammalian cells in a CPA-free medium by ultrarapid cooling using inkjet cell printing, a technique named super flash freezing, thus minimizing osmotic toxicity [10].

During the thawing process, dehydrated cells can face exposure to non-physiological volumes of water or buffer solutions, which can result in swelling and cell lysis, while preformed ice crystals can undergo recrystallization, where larger crystals grow at the expense of smaller ones, adding mechanical damage. To achieve a successful rewarming, both uniform and fast heating rates are required. However, external heating, the most common applied thawing method, causes the onset of thermal gradients as the outer part of the sample melts faster than the inner part. On the contrary, nanowarming exploits the local heating effect associated with magnetic nanoparticles exposed to an alternating magnetic field, allowing homogeneous and rapid rewarming of biological samples including human induced pluripotent stem cells [11][12].

Some of these cryopreservation challenges can be partially addressed by the addition of cryoprotectants [13][14]. Dimethyl sulfoxide (DMSO) and glycerol are examples of permeating cryoprotectants that can easily enter the cells, while non-permeating extracellular cryoprotectants are made up of macromolecules such as hydroxyethyl starch (HES) and small molecules like trehalose. The specific ways in which individual cryoprotectants act, having not been fully clarified yet. It is generally believed that most cryoprotectants have multiple modes of action such as modulation of the hydrogen bonding and the properties of the cell membrane, dilute solute effects, and increase in the solution viscosity at low temperatures [14].

There has been extensive research on formulating and improving cryopreservation media to achieve the best possible cellular outcomes. Furthermore, there has been a growing interest in the characterization of innovative agents to prevent biophysical damage caused by ice growth, such as ice-binding proteins, nucleation modulators, ice recrystallization inhibitors, new macromolecular cryoprotectants, and vitrification agents [7].

In addition, the multiple stress factors of the freeze–thaw process trigger a complex molecular biological stress response, culminating in the activation of apoptotic and secondary necrotic processes, ultimately leading to cell death within hours or days after thawing, a phenomenon termed cryopreservation-induced delayed-onset cell death (CIDOCD) [15]. This discovery resulted in a paradigm shift in the cryopreservation sciences from a primarily chemo-osmometric (ice control) approach to an integrated one also combining molecular modulation of cellular pathways to minimize CIDOCD [16]. Unsurprisingly, targeting apoptotic caspase activation, oxidative stress, unfolded protein response, and free radical damage in the initial 24 h post-thaw, resulted in increased overall cell survival of human hematopoietic progenitor cells [17]. In addition to cryopreservation-related variables, the extent and timing of CIDOCD varies also according to different cell populations as biochemical pathways dysregulated after freezing and thawing may be cell specific. As an example, the post-thaw application of Rho-associated protein kinases inhibitors to T-cell cultures reduces the membrane expression of Fas death receptor, increasing cryopreserved cell yield [18].

It is important to recognize that cryopreservation can alter cell phenotype and function following thawing, including changes in surface markers levels [19] and long-term gene expression [20]. Consequently, an increasing number of assays have been developed to analyze the post-thawing cell quality, encompassing assessment of membrane integrity, molecular mechanisms, cell function, and biochemical alterations [21].

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