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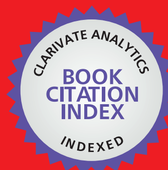

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Chapter

Cryomedia Formula: Cellular Molecular Perspective

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Abstract

The growing market of cell therapy medicinal products (CTMPs) and biopharmaceuticals demand effective cryopreservation with greater safety, of which the currently available cryoprotective agents [CPAs (e.g., dimethyl sulfoxide, glycerol, trehalose, etc.)] alone are unable to provide. This is due to the need of applying high concentration of CPAs to achieve verification that concomitant oxidative damages. Formulating cocktail of compounds with anti-freezing and antioxidants properties found to be advantageous to overcome the resultant damages. Each cocktail, however, demonstrate overlapping and/or unique protective and modulation effect patterns. The advance technology and research tools (e.g., OMICs) provide a deep insight on how the formulation of cryomedia can influence the cellular pathways and molecular interactions. In fact, this shed the light over the uniqueness of cryomedia formulation and how can they serve various application purposes.

Keywords: mammalian cells, cryoprotective agents, biological profile, toxicity, protection, formula, additive agents

1. Introduction

Cryopreservation is one of the most effective techniques that widely used for preserving living cells and organs in research and therapeutic industries [1]. The principle of cryopreservation is to protect cells from the application of super low temperature and ice crystal formation by using media that consist of antifreezing or cryoprotective (CPA) substances such as; glycerol, dimethylsulfoxide (DSMO) or trehalose. The expansion in clinical experiments for medical applications revealed the limitations of utilizing the conventional CPAs which resulting sub-optimal cell quality. This is attributed to the detrimental effects of conventional CPAs and their molecular interactions that compromise cell quality. The new research areas and advanced techniques significantly increase the demand of better cryopreservation to maintain the quality and functionality of cells and tissues.

Current trends in cryopreservation are actively focusing on identifying a safe and effective alternative CPAs to substitute or support the conventional agents. In addition, there are various cell types valuable for investigation and medical development and their different biological profile and functional mechanisms required customizing cryopreservation. However, there are limiting number of studies addressing the influence of the cryomedia formulation on the global proteomic and biomolecular profile of the cells.

2. Conventional cryomedia compositions and protection mechanisms

Cryomedia formulation usually encompass of cryoprotective agents (CPAs) and carrier media prepared at or close to cell isotonic concentrations to provide support to cells at low temperature [2]. It also may contain salts, pH buffers, osmotic agents, nutrients, antioxidants or apoptosis inhibitors [2]. There are about 56 CPAs commonly used for different cell cryopreservation [3, 4]. Glycerol and dimethylsulfoxide (DMSO) are the most common CPAs used in cryomedia formula. CPAs are classified based on the permeability through cell membrane into, permeable (pCPA), and impermeable (ipCPA).

pCPAs are generally small, non-ionic molecules that are highly soluble in water, even at low temperatures. They can pass through cellular membranes and equilibrate within the cytoplasm in exchange for intracellular water during dehydration without over dehydrating the cell [5]. They become solid at a lower temperature than water freezing point and subsequently suppress ice crystal formation [6] and mitigate cellular physical damage that could occur in cellular compartments and membranes. Moreover, pCPAs reduce salt-induced stress by dissolving solute and reducing concentrations in the remaining water fraction intracellularly until the cell is cooled to a sufficiently low temperature [6, 7]. pCPA permeability is controlled by their viscosity and the membrane properties of the cell itself [8, 9]. The latter mentioned is variable between different cell types as well as the varying ages of cells [10, 11]. Most of the pCPAs are polyols, such as glycerol and dimethylsulfoxide (DMSO), which are prominence in cryopreservation. Many successful cryopreserving protocols utilized these compounds for their high efficiency in compare to others such as methanol and ethylene glycol [6].

ipCPAs are large molecules usually comprised of long chains of polymers that are unable to permeate through cellular membranes. They are water soluble and thought to increase the osmolarity around cells, which result in cellular dehydration and reduce ice crystal formation intracellularly [12]. The combination of high concentrations of ipCPAs and fast cooling promotes vitrification and stabilizing cellular proteins and membranes [13, 14]. Their protective mechanism is based on preventing ice formation extracellularly as well as intracellular through dehydration [15]. There are several classes of ipCPAs, such as certain forms of sugars, macromolecules, and polymers. Sugars are classified based on their chemical structure into: mono-, di- and poly-saccharides (glucose, trehalose, and raffinose, respectively). A number of these sugars are permeable (e.g., glucose) and others are impermeable (e.g., trehalose). Sugars have garnered unique interest over the last decades. They have been found to protect protein activity and reduce thermal denaturing heat capacity of chemicals [16–21], which leads to protein stabilization. In particular, trehalose has been identified as a universal protein stabilizer and been involved in many freezing and desiccation studies [18–20].

Depending on the freezing mode (slow cooling or vitrification), the concentrations of CPAs are variant in the solution. For instance, slow freezing mode requires less CPA concentration than vitrification. Choosing the optimum concentration of CPA in combination with the cooling rate is crucial for successful cryopreservation [20]. For instance, when preserving human ovarian tissue following slow freezing protocol, DMSO is used with initial concentration of 7.5% and gradually increased to 12.5%. Whereas preserving the same tissue using the vitrification protocol, 20% DMSO is needed [21]. Different tissues and cells, however, demonstrate different responses to the cryopreservation approaches and CPAs. Therefore, the selection of the appropriate protocol and CPA is subject to the cryopreservation empirical success of the desired cells or tissues.

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3. Quality assessment methods of cryopreserved cells and CPA protection action

An accurate assessment of cryopreserved cells or tissues considering the viability and functionality is paramount to determine the quality and reliability of the cryopreservation protocol and solution. In the past, classical parameters, such as survival rate or motility, were the only quality measurements [22, 23]. With the evolution in technologies and developed assays, scientists can obtain more information surrounding the level of stress that heralds cellular death cascades and dynamic changes that impact cryopreserved cells' function and morphology.

Nowadays, there are a wide range of viability assays available; however, selecting the appropriate assay mainly depends on cell types to avoid inaccurate measurement. For instance, the measurement of LDH leakage in media can be used for membrane integrity assessment because of its reliability and easy performance. It is an applicable measurement in single cells as well as tissues and organs [24]. Conversely, using fluorescent probes for viability measurement is suitable for many cells excluding hepatocytes, because of their detoxification activity with respect to probes that influencing the accuracy of the measurement [25].

The emergence of developed technologies, such as genomics, transcriptomics, proteomics, and metabolomics (collectively termed OMICs), has provided a comprehensive profile of cryopreserved cells, including their stressed and compromised biological pathways, which may help designing protocols or solutions in order to modulate the damaged pathways. So far, the majority of OMICs applications in cryopreservation are limited to reproduction medicine and plants [26, 27], such as in human sperm characterization post-thaw [28]. The deep analysis OMICs stresses the importance of adopting such analytical approach in researches aiming at advancing cryopreservation and biobanking for better CTMPs outcome [25].

4. Cryoprotectant toxicity and detrimental effects

Introducing CPAs in high concentration (molars) is accompanied with non-specific adverse effects such as osmotic stress and cell dehydration [29] that also could induce the oxidative stress [30]. This can cause severe cell damage; for instance, increasing the concentration of DMSO, glycerol, and 1,2-propanediol is linked with the production of non-enzymatic formaldehyde [31], a cytotoxic compound that contributes to cell death [32]. The long exposure duration of cells to high concentration of CPA also harm cell development, as reported when exposing bovine blastocytes to a high concentration of ethylene glycol over 10 min [33]. Likewise, introducing a high concentration of propanediol to mouse zygotes was found to have a similar damaging effect on cell development to that observed in bovine blastocytes [34].

The high concentration of CPA accumulated intracellularly has a detrimental effect on cells. In cryopreserved human mesenchymal stem cells (hMSC), it has a significant effect on cellular viability, filamentous actin distribution, intracellular pH, and mitochondria aggregation [35]. It has also been found to cause abnormal spindles and morphology in human oocytes, which can potentially influence their viability post-cryopreservation [36]. Similarly, CPA causes a serious alteration in mammalian sperm viability, physiological properties, protein phosphorylation patterns [37], and can lethally damage enzymatic activity and DNA [38]. However, osmotic stress factors and associated cell shock cannot be decoupled since they interact with each other, though the resultant effects can be reversed or limited to

a certain extent by minimizing exposure time, accelerating freezing and thawing speeds, and gradually diluting CPAs in cells [39], which can increase post-thaw cell viability. These types of reported damages are considered non-specific since it is not limited to specific CPA identity. However, the molecular interaction of CPAs is more closely linked to the permeable CPAs, as they are able to interact with the cell compartments and biomolecules [30].

CPA toxicity effect can be either reversible (e.g., osmotic shock and cellular shrinkage) [40, 41] or irreversible. Notably, cryopreservation protocols involving short exposure times to CPAs can reverse the induced damages. Nevertheless, irreversible damage is common in cells lacking self-renewal or repair mechanisms, such as RBCs [42] and embryonic stem cells [43, 44].

Oxidative stress occurs during cryopreservation, mainly when adding CPAs to cells [45]. The increased oxidative stress results in more ROS production [46], which leads to a disequilibrium between the generated ROS and the cellular antioxidant capacity within the redox pathway. A decrease in cellular-reduced glutathione (GSH) content was observed during the freezing step of sperm [47], indicating that oxidative damage occurs during the initial steps of cryopreservation. Consequently, increased ROS production results in lipid peroxidation [48], DNA instability [49], protein oxidation [50], overall dysfunctional cells, and low survival rates [47, 49]. Oxidative stress has been observed when applying glycerol [51], DMSO [52], and trehalose [50] to cells.

4.1 Other biochemical effects

Cells naturally have a dynamic and complex system involving active biomolecules that respond distinctly to all forms of environmental stressors, including CPA media and temperature alterations. The cells' response to stressors involves complex biomolecular events influencing their fate. Measuring the survival rate of thawed cells is a classical parameter that is not precise when determining the efficacy of cryopreservation. This is because during the recovery period, a decrease in cellular viability occurs in different cell types [53]. This is attributed to the activation of apoptosis machinery post-thaw [54]. Xu et al. [53] reported that exposing cells to DMSO and freezing conditions activate apoptosis through extrinsic and intrinsic pathways, including caspase-8, caspase-9, and p53. Some CPAs have different mechanisms, yet they lead to the same lethal results. Propylene glycol (ProH), for instance, reduced cell viability via increasing intracellular calcium to a cytotoxic level [55].

Furthermore, the cryopreservation affects cells' biomarkers [56]. It alters the proteome profile of cells, which in some cases can bring about changes in cellular metabolism, function, and structure [57]. In previous work, there is often no clear demarcation between the effect of CPAs and the cryopreservation protocol itself. However, the exact effect of CPAs can be investigated in an experiment if cell viability and functionality are analyzed before freezing.

5. Modulating CPA damages via additive agents

Considering the aforementioned limitations in cryomedia formula, many active studies investigated the efficacy of additive agents to improve the cryomedia and modulate the resultant damages in cryopreserved cells (**Table 1**). Additive agents have variable effects on different cells. This was evidently observed in number of cases such as; quercetin, glutathione, and ascorbic acid [58]. On other hand, some other demonstrated similar efficient antioxidant protection effect on several cells

Additive agents	Example	Concentration	Cell types	Molecular and biological effects
Antioxidants	Resveratrol [61] Salidroside [62]	15 μM 200 μM	Human sperms Red blood cells	Decrease DNA fragmentation through activating AMP-activated protein kinase (AMPK) Increase glutathione reductase (GR) activity and cells stability post thawing Reduce hemolysis, lactate dehydrogenase activity and protect protein and lipid from oxidation damage
Proteins	Type III anti-freezing proteins [63] Sericin [64]	0.8 mg/ml 5%	Human carcinoma cells Human sperm	Increase cells recovery post-thawing Increase cells motility Decreased DNA fragmentation
Enzymes	Catalase [65]	40 μl/ml	Mice spermatogonia stem cells	Reduce apoptosis and ROS production Increase viability
Vitamins	Vit E [66]	100–200 μmol	Human sperm	Increase motility
Anti-apoptotic drugs	Sphingosine-1- and Z-VAD-FMK [67]	10 μM	Ovarian sheep	Preserve primordial follicular density, with normal morphology and improved proliferation

Table 1.
Cryomedia additive agents and their effects on cryopreserved cells.

(e.g., curcumin) [58]. Notably, many protective factors share their antioxidant protection effects at different concentrations (e.g., hyaluronan and glutamine [59, 60]) that commonly include reducing oxidative stress on lipid and proteins and improve viability rate.

In our published studies, the discovery of the protection potent of salidroside and nigerose was exceptional on nucleated as well as anucleated hematopoietic cells [RBCs and human leukemia cells (HL-60)] in various cryomedia formulae and freezing modes. The efficacy of these compounds was evidenced at low concentrations (200–300 μM) of salidroside and nigerose, respectively. The effect of the additive compounds was determined by analyzing both the biomolecular and proteomic profiles of the survival cells [58]. First, we examined the effect of salidroside in standard cryo-solutions (glycerol and trehalose), which are commonly used for the RBCs biopreservation, using RBCs [62]. When comparing the survival cells rate, RBCs cryopreserved in solutions contained salidroside showed higher survival rate in compare to those cryopreserved in standard cryomedia alone. On biomolecular level, salidroside improved the intracellular activity of glutathione reductase (GR), the active enzyme in the redox pathway. In addition, it reduced the level of stress resultant from freeze-thaw process, as it was measured by intracellular lactate dehydrogenase (LDH) activity [68]. Moreover, it protected RBC proteins against oxidative damages [62]. Further investigation on human leukemia cells (HL-60) using salidroside in 2% DMSO and fetal bovine serum cryosolution demonstrated similar protection effects to what have been seen in RBCs [62, 68]. Additionally, it protected lipid against oxidative stress. In the same study, we used nigerose for comparison, which showed similar protection effect on the biomolecular profile of the cells.

On top of the biological profile of cryopreserved cells, proteomic analysis revealed the specific and unique modulation effect of additive agents on compromised biological pathways [68]. Each compound was observed to have a demonstrably unique effect on the proteome pattern of cryopreserved HL-60 cells. Nigerose was strongly engaged with cell maintenance, energetic, and metabolic pathways, whereas salidroside influenced proteins associated with DNA binding and nuclear activities. Both overlapped with regards to influencing proteins associated with redox pathways. Moreover, the damaging effects of classical cryomedia were modulated by the reformulated media comprising the novel protective agents. The protective mechanisms of the compounds on the proteomic level were strongly compatible with the biochemical analysis of the cells cryosurvival rate and their resistance to stressors [68]. This has shed the light over the potency of specific effectiveness of additive agents in the cryosolution and their specific applications for preserving different cells and tissues for pharmaceutical and clinical applications.

6. Conclusion

Understanding of the protective mechanisms of cryomedia ingredients along with identifying powerful protective compounds to enhance cryomedia performance is highly demanded. Due to the wide range of preserved cells and tissues, designing the appropriate cryosolution with suitable protocol is beneficial. In fact, these are particularly important for CTMP industries and end-users at clinics, such as those with cancer and diabetes or requiring blood transfusion, organ transplantation, and infertility treatments.

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