frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Opinion
PUBLISHED 15 June 2023
DOI 10.3389/fbioe.2023.1215591

OPEN ACCESS

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RECEIVED 02 May 2023
ACCEPTED 08 June 2023
PUBLISHED 15 June 2023

CITATION
Zhang W, Liu X, Hu Y and Tan S (2023), Incorporate delivery, warming and washing methods into efficient cryopreservation.
Front. Bioeng. Biotechnol. 11:1215591.
doi: 10.3389/fbioe.2023.1215591

Incorporate delivery, warming and washing methods into efficient cryopreservation

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KEYWORDS

cryopreservation, DMSO, cryoprotectant delivery, novel warming, automatic washing

1 Introduction

Cryopreservation is a popular and crucial method for long-term storage of cells, tissues, and other biological samples at low temperatures. During this process, the cells are in a state of "suspended animation" to inhibit biological and chemical reactions (Pegg, 2015; Jang et al., 2017; Chang and Zhao, 2021). Recently, there are two main strategies of cryopreservation: slow freezing and vitrification (Kometas et al., 2021). Slow freezing refers to the freezing of biological samples at a rate of 1°C/min. This can be achieved through laboratory freezing tubes and programmed cooling boxes (Garcia-Flores et al., 2023). Vitrification means that when a small biological sample cools at a very fast rate, the internal water will be transformed into a glassy state (Schulz et al., 2020). The devices for vitrification are various, such as cyrotop (for oocytes) (Miao et al., 2022), plastic straw (for spermatids) (Patra et al., 2021), and cryomesh (for islets) (Zhan, Rao, et al., 2022). However, low temperature can cause a range of damage to biological samples, including protein denaturation (Chen et al., 2022), membrane damage (Lee et al., 2023), oxidative stress (Gualtieri et al., 2021). Since DMSO was first used in bull sperm cryopreservation in 1959, it has been found that the addition of a certain concentration of DMSO could resist these cryodamages (Lovelock and Bishop, 1959; Stubbs et al., 2020). Unfortunately, DMSO can lead to various problems such as differentiation of human stem cells (Davidson et al., 2015), hemolysis (Yi et al., 2017), and alterations in DNA methylation (Verheijen et al., 2019) at body temperature (37°C).Therefore, a series of novel CPAs, such as AFP, proline, etc., have been developed for DMSO-free cryopreservation (Li et al., 2020; Weng and Beauchesne, 2020), and these CPAs can be classified as permeable or impermeable according to whether they can enter cells (Weng et al., 2019; Yong et al., 2020). But none of them can replace DMSO totally. Currently, the most common cryopreservation process involves three steps: 1) mixing DMSO with biological samples and storing them at low temperature; 2) thawing by convection rewarming; and 3) removing DMSO by centrifugation and washing (Jang et al., 2017; Whaley et al., 2021). Although this protocol is widely used in clinics and laboratories, there are still some challenges.

Besides the toxicity of DMSO, commonly used convective rewarming can lead to ice recrystallization and devitrification because of its slow rewarming rates (Marquez-Curtis et al., 2015; Wang et al., 2016). Also, uneven temperature distribution and thermal gradients can induce thermal stress and destroy the biological samples, especially for larger volumes (Taylor et al., 2019). Finally, the manual centrifugation and washing to remove CPA is not only demanding for operators, but may also lead to complex procedures and unintended cell loss (Shu et al., 2014; Hornberger et al., 2019). In general, these procedures may cause damage instead of thoroughly cleaning (Fois et al., 2007).

To solve the problems discussed above, advanced cryopreservation technologies must be employed. Initially, impermeable CPA is widely used in cryopreserving biological samples

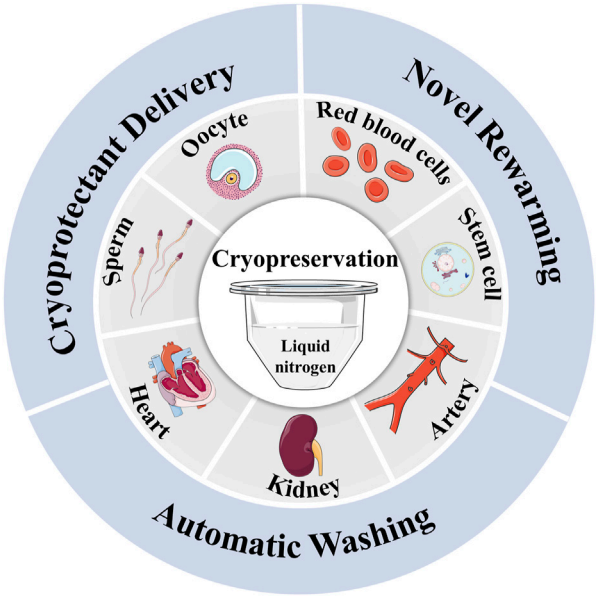

FIGURE 1
The novel delivery, warming and washing methods for cryopreservation of biological samples.

such as oocytes and red blood cells due to its non-toxicity, high efficiency, and stability (Stoll et al., 2012; Zhang et al., 2016; Huang et al., 2017). However, its impermeability hinders its application in preventing intracellular damage. The use of delivery methods like nanoparticles (Rao et al., 2015) and membrane perturbation (Janis et al., 2021) are required to ensure its presence inside or outside the cells. In addition, novel warming methods such as nanowarming offers a faster and more even heating option compared to convective rewarming. It is especially important in cryopreserving large volume biological samples (Manuchehrabadi et al., 2017). Furthermore, high-quality washing methods have become effective way of convenient removal of CPA (Lusianti and Higgins, 2014; Zhao and Fu, 2017). Therefore, the adoption of these advanced cryopreservation technologies provides an opportunity to achieve efficient and high-quality cryopreservation (Figure 1).

2 CPA delivery methods

On the one hand, trehalose has the ability to form hydrogen bonds with biomacromolecules and promote hydration, enabling cell components to maintain functional conformations. It can also slow metabolic activity by forming glassy substrates with extremely low molecular mobility (He, 2011; Ntai et al., 2018; Hu et al., 2023). On the other hand, AFP can enhance the resistance of cells to cryoinjury by inhibiting ice crystal growth and interaction with membranes (Kim et al., 2017; Baskaran et al., 2021). Due to these properties, trehalose and AFP have gained attention as non-toxic and reliable CPAs (Lee et al., 2013; Huang et al., 2017). However, unlike DMSO, trehalose and AFP cannot penetrate the cell membrane, which limits their use in protecting cells from intracellular ice crystal damage (Chang and Zhao, 2021; Hu et al., 2023). Therefore, effective methods for delivering trehalose and AFP are essential for successful cryopreservation. These methods include nanoparticles carriers and membrane perturbation delivery, depending on cellular structure and function. It must be noted that trehalose cannot be metabolized in cells and the safety of intracellular AFP is unclear, which may hinder their translation to clinic (Campbell and Brockbank, 2012; Dovgan et al., 2017). Detailed information has been summarized in Table 1.

2.1 Nanoparticles carriers

Endocytosis is one of the mechanisms by which nanoparticles (NPs) can deliver trehalose into cells (Stewart and He, 2019). For instance, cold-responsive nanocapsules (CR-NCs) encapsulated trehalose by microfluidics have successfully maintained the glucose-regulating function of pancreatic β cells after cryopreservation (Cheng et al., 2019). Some pH-responsive delivery systems, such as genipin-cross-linked Pluronic F127-chitosan nanoparticles (GNPs) (Rao et al., 2015) and chitosan-tripolyphosphate (CS-TPP) nanoparticles (Yao et al., 2020) have also shown efficient intracellular delivery of trehalose. Remarkably, Poly (l-alanine-co-l-lysine)-graft-trehalose (PAK_T) was synthesized as a natural antifreezing glycopolypeptide (AFGP). It can be used as a carrier for trehalose delivery while also mimicking a CPA to inhibit ice recrystallization and protecting cells (Piao et al., 2022).

2.2 Membrane perturbation delivery

Membrane perturbation is another method to deliver impermeable CPAs into cells. The effectiveness of this approach has been demonstrated by the delivery of AFPIII via the location of cell-penetrating peptide pep-1 (Tomás et al., 2019). In non-endocytic human red blood cells, trehalose can be delivered by altering membrane permeability, which depends on the interaction between the polymer attached to the hydrophobic side group and the membrane lipid bilayer (Liu et al., 2022). Phenethylamine-grafted PGA (PGA-g-PEA) synthesized from hydrophobic PEA-modified PGA enhances trehalose loading capacity and reduce hemolysis of red blood cells by self-forming nanoparticles in a phosphate buffer solution (Zhang et al., 2020). Besides nanoparticles, ultrasound and microbubbles can also induce transient perforations to achieve trehalose loading into human red blood cells (Janis et al., 2021).

3 Warming methods

Convective rewarming, which means immersing biological samples in a water bath heated to 37°C, is still considered the gold standard for rewarming (Wolkers and Oldenhof, 2015). However, the slow heating rate resulting from convective rewarming can lead to ice recrystallization and devitrification (Wang et al., 2016). Additionally, convective rewarming may not provide even heating, particularly as the volume of biological samples increases. The resulting thermal gradients can cause biological samples to crack (Mfarrej et al., 2017; Sharma et al.,

TABLE 1 Examples of CPA delivery methods and warming methods.

Methods			Cell types	Positive effects	References
CPA delivery methods	pH-sensitive systems delivery	GNPs[a]	hADSCs[b]	Cell viability↑	Rao et al. (2015)
				Functional survival↑	
				Attachment efficiency↑	
				Proliferative potential↑	
		CS[c]–TPP[d] nanoparticles	NK cells[e]	Cell viability↑	Yao et al. (2020)
				Survival rate↑	
				Cytotoxicity↑	
				Proliferative potential↑	
	Cold-responsive systems delivery	PNP[f]	MDA-MB-231 cancer cells[g] and hADSCs	Cell viability↑	Zhang et al. (2019)
				Attachment efficiency↑	
				Survival rate↑	
				Proliferative potential↑	
				Functional survival↑	
	Membrane perturbation delivery	Apatite nanoparticles	sRBCs[h]	Survival rate↑	Stefanic et al. (2017)
				Cell viability↑	
		ε-PL[i] and PVP[j]	hRBCs[k]	Trehalose permeability↑	Liu et al. (2022)
				Survival rate↑	
				The amount of PS[l] exposure↓	
		Sonoporation	hRBCs	Cell recovery rate↑	Janis et al. (2021)
				Trehalose permeability↑	
		Ultrasound-integrated PDMS[m]-based microfluidic	hRBCs	Trehalose permeability↑	Centner et al. (2020)
				Cell viability↑	
		Glycopeptide of saccharide-grafted ε-poly (L-lysine)	hRBCs	Survival rate↑ hemolysis↓	Gao et al. (2022)
	Other delivery methods	PAK_T[n]	MSCs[o]	Cell viability↑	Piao et al. (2022)
				Proliferative potential↑	
				Functional survival↑	
				Ice Recrystallization↓	
		EGFP-ApAFP752[p]	HEK 293T[q]	Survival rate↑	Sreter et al. (2022)
				Intracellular AFP concentration↑	
Warming methods	Magnetoresponsive induction heating	msIONPs[r]	HDFs[s], porcine carotid arteries, porcine heart valve leaflet, porcine femoral arteries	Cell viability↑	Manuchehrabadi et al. (2017)
				Survival rate↑	
				Damaged cells↓	
		sIONPs[t]	Rabbit kidney	Tissue integrity↑	Sharma et al. (2021)
				Tissue viability↑	
	Photoresponsive induction heating	Gold nanorods and pulsed lasers	Zebrafish embryos	Functional integrity↑	Khosla et al. (2020)
				Survival rate↑	
		2D-GO-MoS_2 NSs[u]	HUVECs[v]	Warming rate↑	Panhwar et al. (2018)

(Continued on following page)

TABLE 1 (*Continued*) Examples of CPA delivery methods and warming methods.

Methods			Cell types	Positive effects	References
				Cell viability↑	
				Survival rate↑	
				Functional integrity↑	
	Photo- and magnetoresponsive induction heating	Graphene Oxide-Fe_3O_4 Nanocomposite	MSCs	Proliferative potential↑	Cao et al. (2019)
				Cell viability↑	
				Functional integrity↑	
	Rapid joule heating	Electrical conductor and voltage pulse generator	Adherent fibroblast cells, *Drosophila* embryos and rat kidney slices	Survival rate↑	Zhan, Han, et al. (2022)
				The structural integrity of the kidney slices↑	
	Radiofrequency Heated Metal Forms	Al foil metal	2 mm thick porcine aortas	Tissue viability↑	Han et al. (2020)
	Infrared radiation heating	Focused halogen IR lamp	Heterogeneous human epithelial Caco2[w] and RPE[x] cell lines	Cell viability↑	Bissoyi and Braslavsky (2021)
				Cell adhesion ↑	

[a]GNPs: genipin-cross-linked Pluronic F127-chitosan nanoparticles.
[b]hADSCs: human adipose-derived stem cells.
[c]CS: chitosan.
[d]TPP: tripolyphosphate.
[e]NK cells: natural killer cells.
[f]PNP: PLGA (poly (lactic-co-glycolic acid))—pNIPAM-B (poly (N-isopropylacrylamide-co-butyl acrylate)) —PF127(Pluronic F127).
[g]MDA-MB-231 cancer cells: human breast cancer cells.
[h]sRBCs: sheep red blood cells.
[i]ε-PL: ε-poly (L-lysine).
[j]PVP: poly (vinyl pyrrolidone).
[k]hRBCs: human red blood cells.
[l]PS: protoplasmic surface.
[m]PDMS: polydimethylsiloxane[n]PAKT:poly (l-alanine-co-l-lysine)-graft-trehalose.
[o]MSCs: mesenchymal stem cells[p]EGFP-ApAFP752: enhanced green fluorescent protein (EGFP) -tagged antifreeze protein.
[q]HEK 293T: human embryonic kidney cell line.
[r]msIONPs: mesoporous silica coated iron oxide nanoparticles [s]HDFs: human dermal fibroblasts.
[t]sIONPs: silica-coated iron oxide nanoparticles.
[u]2D-GO-MoS2 NSs: Two-dimensional graphene oxide molybdenum disulfide nanosheets.
[v]HUVECs: human umbilical vein endothelial cells.
[w]Caco2: colorectal adenocarcinoma.
[x]RPE: retinal pigment epithelium.

2021; Sharma et al., 2023). To overcome these limitations, a sequence of methods for rapid and even rewarming were developed, such as nanowarming, rapid joule heating (Zhan, Han, et al., 2022), infrared radiation heating (Bissoyi and Braslavsky, 2021). These methods have been summarized in Table 1.

3.1 Magnetoresponsive induction heating

Néel and Brownian relaxations caused by magnetic moment oscillation can induce the thermal effect of magnetic nanoparticle under an alternating magnetic field (AMF) (Syme et al., 2004; Cazares-Cortes et al., 2017). Therefore, the addition of magnetic nanoparticles to the cryoprotectant solution under an AMF can improve the thermal conductivity of biological samples, resulting in relatively even and rapid heating. This method minimizes damage to biological samples caused by slow and uneven rewarming (Etheridge et al., 2014; Liu et al., 2018). The vitrified organs, including rat hearts (Joshi et al., 2022) and rabbit kidneys (Sharma et al., 2021), have been successfully rewarmed utilizing magnetic iron oxide nanoparticles (IONPs), and the integrity of their structure and function is maintained. But the potential cytotoxicity of nanoparticles must be considered. However, due to the limitation of warming rate, the application of magnetically responsive nanoheating requires high molarity CPA, which brings potential toxicity to biological samples.

3.2 Photoresponsive induction heating

Gold nanorods and carbon black micron-sized particles have also been utilized in rewarming methods to achieve photoresponsive inducing heating (Khosla et al., 2020). Laser vibration in the gold nanoparticles induces heat dissipation. This enables ultra-rapid rewarming of cryopreserved zebrafish embryos and improves embryo survival. However, physical damage from injection site increased the probability of ice formation during rapid freezing (Khosla et al., 2019; Khosla et al., 2020). Carbon black micron-sized particles can suddenly heat up and emit heat after absorbing laser infrared energy. This hate will be transferred to the biological sample through the solution to achieve rapid and even heating (Panhwar et al., 2018).Nonetheless,

photoresponsive nanoheating is difficult to apply to large-scale biological samples (Zhan, Han, et al., 2022).

4 Washing methods

Currently, the removal of DMSO from biological samples still relies on manual centrifugation, which requires skilled operators to remove the supernatant and replace it with a washing solution (Shu et al., 2014). However, cell loss is unavoidable during centrifugation, and residual DMSO can be highly toxic (Syme et al., 2004). Fortunately, several techniques for DMSO removal have been developed to address these challenges.

The hollow fiber module with semi-permeable membrane uses the pressure and concentration difference between the cell membrane and the fiber membrane to remove CPA from cells. This technique can also be scaled up for large cryopreserved cell suspensions (Ding et al., 2010). Dual-flow microfluidic devices have been specifically designed to remove intracellular DMSO in a limited time, which is essential for clinical applications (Fleming Glass et al., 2008). Dilution filtration system has been demonstrated to be more efficient and cost-effective than conventional multistep centrifugation or automated centrifugation (Zhou et al., 2011). Sepax-2 and Lovo devices have also been proven effective in removing DMSO from thawed hematopoietic progenitor cells (HPC), while maintaining the viability of CD34 cells before clinical infusion. However, the washing scheme must be flexible, convenient and low-cost for more common applications (Abonnenc et al., 2017; Mfarrej et al., 2017).

5 Conclusion

With the advancement of modern biotechnology, conventional cryopreservation obviously failed to keep pace with current needs. This review generalized the recent advances of delivery, warming and washing methods used in cryopreservation. Delivery methods helped to overcome the major limitation of the ultra-low permeability of impermeable CPAs, enabling their intracellular and extracellular cryopreservation. The use of novel warming methods can achieve rapid and even rewarming while avoiding the adverse effects of devitrification on biological samples. The emergence of various washing methods created a novel platform for convenient and efficient CPA removal. It must be noted that all the novel methods for cryopreservation have not been widely used neither in laboratory nor in clinic due to the high cost and complex operation protocol. Future studies need to focus on making new methods less difficult to perform without reducing their effectiveness, so that they can be applied by more researchers.

Author contributions

WZ: conceptualization and writing-original draft. XL: conceptualization and supervision. YH: investigation and software. ST: writing-review and editing, and supervision. All authors contributed to the article and approved the submitted version.

Acknowledgments

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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