

# New strategies for germ cell cryopreservation: Cryoinjury modulation

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Cryopreservation is an option for the preservation of pre- or post-pubertal female or male fertility. This technique not only is beneficial for human clinical applications, but also plays a crucial role in the breeding of livestock and endangered species. Unfortunately, frozen germ cells, including oocytes, sperm, embryos, and spermatogonial stem cells, are subject to cryoinjury. As a result, various cryoprotective agents and freezing techniques have been developed to mitigate this damage. Despite extensive research aimed at reducing apoptotic cell death during freezing, a low survival rate and impaired cell function are still observed after freeze-thawing. In recent decades, several cell death pathways other than apoptosis have been identified. However, the relationship between these pathways and cryoinjury is not yet fully understood, although necroptosis and autophagy appear to be linked to cryoinjury. Therefore, gaining a deeper understanding of the molecular mechanisms of cryoinjury could aid in the development of new strategies to enhance the effectiveness of the freezing of reproductive tissues. In this review, we focus on the pathways through which cryoinjury leads to cell death and propose novel approaches to enhance freezing efficacy based on signaling molecules.

**Keywords:** Apoptosis; Autophagy; Cryoinjury; Cryopreservation; Fertility preservation; Necroptosis

## Introduction

Fertility preservation refers to the techniques used to preserve germ cells or reproductive tissue, thereby enabling the future production of biological offspring. Cryopreservation is an option for preserving the fertility of both pre- and post-pubertal male and female patients. This technique relies on a variety of cryoprotective agents (CPAs), which can be either non-permeable or permeable, along with methods such as slow freezing, vitrification, and freeze-drying

[1,2]. These freezing techniques not only are beneficial for human clinical applications, but also play a crucial role in the breeding of livestock and endangered species.

Unfortunately, frozen germ cells are subject to cryoinjury, which can result from the excessive generation of reactive oxygen species (ROS), apoptosis, and cold shock during freezing. As a result, various CPAs and freezing methods have emerged to minimize the extent of cryoinjury [3-6]. In the process of developing these solutions, most research has been dedicated to preventing cell death induced by cryoinjury, which is primarily caused by apoptosis. Despite considerable research effort aimed at minimizing apoptotic cell death during freezing, a low survival rate or compromised function of cells after freeze-thawing is often still observed. This suggests that additional cell death pathways may be involved in cryoinjury.

Unsurprisingly, various cell death pathways (including necroptosis, autophagy-dependent cell death [ADCD], NETosis, ferroptosis, parthanatos, and the mitochondrial permeability transition pore) have been discovered in recent decades in addition to apoptosis, which is the most widely recognized cell death pathway [7]. While necroptosis and autophagy have been associated with cryoinjury, our under-

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standing of the contributions of these pathways is still incomplete [8-11]. Consequently, gaining insight into the molecular mechanisms of cryoinjury could offer new strategies to enhance freezing efficacy.

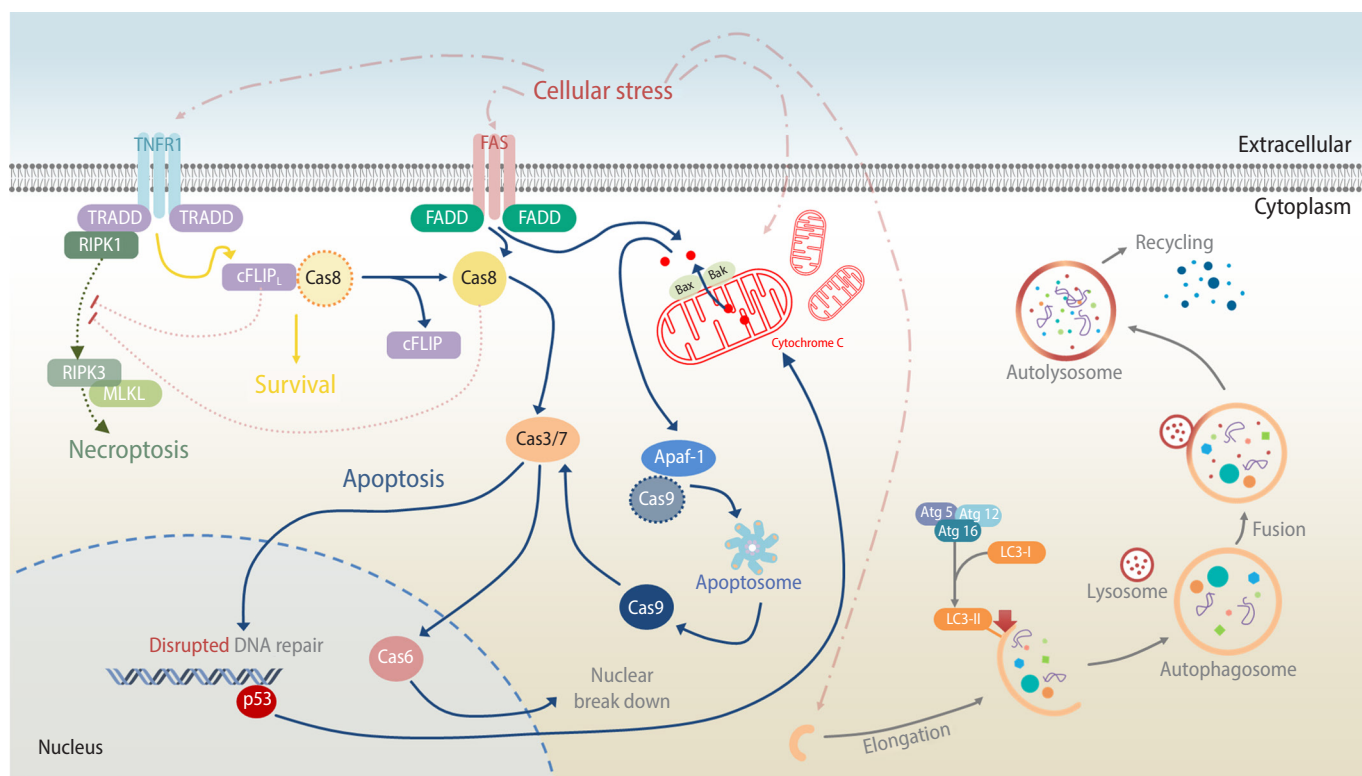
The compounds identified through the study of cryoinjury pathways may serve not only as cryoprotectants but also as supplements to culture media following thawing. Cryoinjury is primarily understood to occur during the freezing process; however, research indicates that damage continues to occur after thawing, particularly within the first 24 hours of post-thaw culture. This suggests the necessity for careful rescue of frozen cells and tissues, even post-thawing [12]. To meet this need, researchers have sought to improve cell survival and restore cell function using agents such as apoptosis inhibitors and antioxidants before freezing or after thawing to prevent cryoinjury, yielding positive results for freeze-thawed cells [13-16]. As previously suggested, the clear identification of cryoinjury pathways will facilitate the development of new strategies to be used along-

side existing techniques.

Understanding the fundamental pathways of cryoinjury is crucial for manipulating cryoinjury at a molecular level and enhancing fertility preservation strategies through improved freezing efficacy. In this review, we focus on the pathways through which cryoinjury induces cell death and propose novel approaches to enhance the freezing efficacy of reproductive tissues, based on signaling molecules.

### Cell death pathways

In recent decades, our understanding of various cell death pathways has substantially improved. These pathways include the well-known apoptosis, necroptosis, and necrosis, as well as other pathways such as ADCD, NETosis, ferroptosis, parthanatos, and the mitochondrial permeability transition pore [7]. This review primarily focuses on apoptosis, necroptosis, and ADCD. These pathways have



**Figure 1.** Apoptosis, necroptosis, and autophagy. In extrinsic apoptosis, activated Fas-associated protein with death domain (FADD) interacts with procaspase-8, resulting in the formation of cleaved caspase. Intrinsic apoptosis involves the release of cytochrome C from the mitochondria, which forms apoptosomes and allows procaspase-9 to be cleaved. These cleaved caspases eventually follow a common apoptosis execution pathway. Necroptosis is triggered when the activation of caspase-8 is compromised. The maturation of the autophagosome involves the conversion of microtubule-associated protein 1A/1B-light chain 3 (LC3)-I to LC3-II through the conjugation of autophagies (ATGs). These then fuse with the lysosome to degrade intracellular content and recycle the components. TNFR1, tumor necrosis factor receptor 1; TRADD, TNFR1-associated death domain protein; RIPK, receptor-interacting protein kinase; cFLIP<sub>L</sub>, cellular FLICE (FADD [Fas-associated death domain]-like IL-1β-converting enzyme)-like inhibitory protein long form; Cas, caspase; MLKL, mixed lineage kinase domain-like protein; Apaf-1, apoptotic protease activating factor-1.

been studied in relation to their roles in germ cell cryopreservation (Figure 1).

### 1. Apoptosis

Apoptosis, or programmed cell death, is a crucial process in maintaining tissue homeostasis. It contributes to cell turnover, embryonic development, and the functioning of the reproductive system [17,18]. During apoptosis, cells undergo a series of morphological changes, such as cell shrinkage and the fragmentation of cells, organelles, and DNA into apoptotic bodies [19,20]. This process can be initiated by two primary pathways. The extrinsic pathway is activated by cell-surface death receptors, such as tumor necrosis factor (TNF) receptor 1, Fas, and death receptors, which interact with their respective ligands, including TNF- $\alpha$ , Fas ligand, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). In contrast, intrinsic apoptosis is initiated when pro-apoptotic proteins from the B-cell lymphoma 2 (BCL-2) family permeabilize the outer mitochondrial membrane within cells [21]. Both apoptotic pathways lead to the activation of a family of proteases and caspases. In the extrinsic pathway, the activation of cell-surface death receptors results in the recruitment of the adaptor protein Fas-associated protein with death domain (FADD). The activated FADD then interacts with procaspases 8 and 10 to form cleaved caspase, marking the onset of apoptosis. In the intrinsic pathway, the activation of BCL-2 proteins leads to the permeabilization of the outer mitochondrial membrane, a step often referred to as the point of no return. This allows for the release of cytochrome C into the cytoplasm. Once in the cytoplasm, cytochrome C forms apoptosomes with apoptotic protease activating factor 1. These apoptosomes enable the cleavage of procaspase-9. The cleaved caspases 8, 10, and/or 9 then proceed along a common apoptosis execution path, which involves the activation of caspase-3 and/or caspase-7 [21].

### 2. Necroptosis

Necroptosis is a regulated, caspase-independent form of cell death that serves as an alternative pathway to bypass resistance to apoptosis. Cells undergoing necroptosis exhibit necrotic characteristics, such as swelling and rupture of the cellular membrane. This pathway can be initiated by death receptors, toll-like receptors, TNF, Z-DNA binding protein 1, or certain viral infections, particularly when the activation of caspase-8 is compromised [22-24]. While necroptotic cell death can be triggered by pro-inflammation or as an alternative to apoptosis, our focus is on the former, given that necroptosis induced by cryoinjury is seldom associated with viral infection or inflammation. Furthermore, apoptosis and necroptosis are closely regulated by each other, with caspase-8 playing a pivotal role as a mediator of both apoptotic and necroptotic pathways. This is because caspase-8

not only regulates apoptosis but also serves as a key component of the ripoptosome [25]. In the absence of caspase-8 activity, receptor-interacting protein kinase (RIPK) 1 autophosphorylates RIPK3, leading to the formation of the ripoptosome. The RIPK1/RIPK3 complex then phosphorylates mixed lineage kinase domain-like protein (MLKL), resulting in the creation of the necrosome. This structure triggers cell death by destabilizing mitochondria, perturbing the membrane, and causing cell lysis [26-28].

### 3. Autophagy: a double-edged sword

Autophagy is a process in which cellular components such as mitochondria, organelles, and endoplasmic reticula are degraded by lysosomes, allowing them to be recycled and used in cellular function during periods of stress or starvation. This makes autophagy a crucial player in maintaining cellular homeostasis. The autophagy process can be broken down into five stages: (1) initiation; (2) nucleation and phagophore formation; (3) phagophore expansion; (4) fusion with the lysosome; and (5) degradation [29]. The primary regulators of autophagy are the mammalian target of rapamycin, which functions as an inhibitor, and adenosine monophosphate-activated kinase, which acts as an activator. The initiation of autophagy triggers the regulation of nucleation by various protein complexes, including autophagy-related proteins. Subsequently, phagophores marked with microtubule-associated protein 1A/1B-light chain 3 (LC3) mature into autophagosomes. These autophagosomes then fuse with lysosomes to create autolysosomes, where the cargo is degraded [30].

While autophagy is typically viewed as a survival mechanism, its deregulation has also been linked to a non-apoptotic form of cell death known as either ADCD or autophagic cell death [7,31-33]. Morphologically, ADCD is characterized by the extensive presence of autophagic components in the cytoplasm, devoid of other forms of programmed cell death. This process can be halted either pharmacologically or genetically [31,33]. ADCD can occur via an autophagic flux-dependent or flux-independent pathway. The former, known as autosis, is a non-apoptotic form of cell death that is autophagy-dependent and results from excessive or uncontrolled autophagy levels [34]. The latter is triggered by glycosylceramidase beta 1 and leads to autophagic cell death [35].

## Cryoinjury-induced cell death in germ cell freezing

Cryopreservation is a method used to preserve the fertility of both male and female patients, whether pre- or post-pubertal. However, the process of freezing and thawing often exposes cells or tissues to various forms of stress, including extensive ROS generation, oxidative stress, apoptosis, and cold shock. These factors can reduce the efficacy of freezing [3-6]. Additionally, CPAs, which are necessary for bio-

logical freezing, can have adverse effects on frozen cells or tissues, such as osmotic stress or toxicity [4,36]. In this context, it is crucial to understand the molecular mechanisms of cryoinjury that occur during freezing in order to overcome these limitations and improve the efficacy of freezing. Therefore, this review is focused on the mechanisms of cell death induced by cryoinjury, including apoptosis, necroptosis, and ADCD, in the context of germ cell cryopreservation (Table 1).

Cryoinjury-induced apoptosis has been observed in a broad range of cell types, including oocytes, ovarian tissue, spermatozoa, testicular tissue, and spermatogonial stem cells (SSCs). This phenomenon has been reported across various species, such as mice, cattle, pigs, and humans, and through different methods, namely slow freezing and vitrification. In the vitrification of bovine oocytes, it was discovered that the oocytes degenerated during subsequent culture, as evidenced by signs of apoptosis like DNA fragmentation and caspase-3 activation [37]. Similarly, an increase in apoptotic levels was noted in murine metaphase II (MII) oocytes and porcine oocytes post-vitrifica-

tion [38,39]. More specifically, in 2015, Dai et al. [40] demonstrated that porcine MII oocytes experienced apoptosis due to the generation of ROS, the upregulation of intrinsic apoptotic pathways, and impaired mitochondrial function following vitrification. Numerous studies have also shown that cryopreservation is linked to apoptosis in sperm freezing, with a higher activation of pan-caspases in frozen spermatozoa compared to fresh ones [41-45]. One study revealed that vitrified prepubertal mouse testicular tissue exhibited a high degree of apoptosis [46], and slow-frozen porcine testicular tissue was separately associated with elevated levels of apoptosis [47]. Furthermore, several studies have determined that the freezing efficiency of SSCs, regardless of the species, was reduced due to apoptosis when slow freezing was used [48-50].

As previously noted, apoptosis is not the sole process accountable for cryoinjury that results in cell death during freezing, just as additional cell death pathways exist beyond apoptosis [51,52]. Unfortunately, unlike apoptosis, which is recognized as a cause of cell death during freezing, the mechanisms of cryoinjury-induced necroptosis

**Table 1.** Cryoinjury-induced cell death mechanisms in germ cell freezing

Pathway	Cell type	Freezing method	Cryoinjury	Reference	
Apoptosis	Bovine oocyte	Vitrification	(↑) DNA fragmentation (↑) Caspase-3 activation	[37]	
	Murine MII oocytes	Vitrification	(↑) Apoptotic level	[38]	
	Porcine oocytes	Vitrification	(↑) Apoptotic level	[39]	
	Porcine MII oocytes	Vitrification	(↑) ROS generation (↑) Intrinsic apoptotic pathways (↑) Impaired mitochondria	[40]	
	Bull sperm	LN <sub>2</sub> vapor procedure	(↑) Pan-caspase activity	[41]	
	Human sperm	LN <sub>2</sub> vapor procedure	(↑) Pan-caspase activity	[42]	
	Human sperm	LN <sub>2</sub> vapor procedure	(↑) DNA fragmentation (↑) Caspase-3 activation	[44]	
	Murine prepubertal testicular tissue	Vitrification	(↑) Apoptotic level	[46]	
	Porcine prepubertal testicular tissue	Slow freezing	(↑) Early apoptosis	[47]	
	Murine SSCs	Slow freezing	(↑) Early apoptosis	[48]	
	Bovine prepubertal SSCs	Slow freezing	(↑) Cytochrome C release	[49]	
	Necroptosis	Murine SSCs	Slow freezing	(↑) RIP1 expression	[10]
		Ovarian tissue	Vitrification	Higher intact follicle ratio in the necrostatin-1-treated groups	[11]
		Murine oocytes	Vitrification	Non-significant difference of necroptosis-associated genes due to unique localization patterns of pMLKL and pRIPK1 in oocyte	[54]
Autophagy	Stallion sperm	Stored at 5 °C	(↑) LC3B expression	[8]	
	Murine immature oocytes	Vitrification	(↑) Beclin-1 expression (↑) Number of GFP-LC3 puncta	[55]	
	Murine oocytes	Vitrification	(↑) Beclin-1 expression	[56]	
	Murine SSCs	Slow freezing	(↑) Excessive autophagy (↑) Beclin-1, ATG7, p53, LC3II/I, and Lamp2 expression	[9]	

MI, metaphase II; ROS, reactive oxygen species; LN<sub>2</sub>, liquid nitrogen; SSC, spermatogonial stem cell; RIP1, receptor-interacting protein 1; pMLKL, phosphate mixed lineage kinase domain-like protein; pRIPK1, phosphate receptor-interacting protein kinase 1; LC3, microtubule-associated protein 1A/1B-light chain 3; GFP, green fluorescent protein; ATG7, autophagy-related 7.

or ADCD remain largely unknown. Recent research has indicated that the inhibition of necroptosis in SSCs is crucial for maintaining male fertility during gonadotoxic treatment [53]. In this context, necroptosis could be a contributing factor to cryoinjury resulting in cell death. This hypothesis is backed by studies that have reported a cryoprotective effect when necrostatin-1 (a receptor-interacting protein [RIP] 1-targeted inhibitor of necroptosis) was employed as a CPA in SSC or ovarian tissue freezing [10,11]. However, another study indicated that the inclusion of necrostatin-1 decreased the survival rate of vitrified oocytes, suggesting that the positioning of pMLKL and pRIPK is unique in mouse oocytes and that necroptosis plays a role in preserving oocyte integrity post-vitrification [54].

The role of autophagy, a crucial process for cellular homeostasis, in determining cell survival or death following freeze-thawing remains a topic of debate. Gallardo Bolanos et al. [8] posited that autophagy aids in the survival of spermatozoa during cooled storage. In a similar vein, Gao et al. [55] demonstrated that inhibiting autophagy triggered intrinsic apoptosis in immature oocytes during the vitrification-warming process, while Bang et al. [56] depicted autophagic activation as a natural adaptive response to cold stress in vitrified-warmed oocytes. Conversely, Jung et al. [9] suggested that autophagy could contribute to cryoinjury during SSC cryopreservation, indicating that excessive autophagy could result in cell death after freeze-thawing.

## New strategies for germ cell freezing

While numerous modifications to freezing protocols (such as alterations to CPAs, freezing rate, cell concentration, and storage temperature) have been explored to preserve pre- or post-pubertal female or male fertility, the molecular mechanisms underlying cryoinjury in germ cell freezing remain unclear. As previously noted, the majority of research has focused on apoptosis, with few studies investigating necroptosis or autophagy. This lack of understanding will impede the development of innovative techniques to enhance cryoprotective efficacy until we fully comprehend the pathways involved in cryoinjury. Consequently, emerging strategies for germ cell freezing are intended to modulate target signaling molecules based on a more comprehensive understanding of cryoinjury pathways; this moves beyond current freezing strategies that focus solely on apoptosis, as well as the development of new CPAs. Furthermore, the primary objective is to target and inhibit various signaling molecules in the cell death cascades that trigger apoptosis, necroptosis, and autophagy, not only during freezing but also before and after thawing. In Table 2, we provide a summary of the targeted CPAs used in germ cell freezing.

### 1. Targeting CPAs

Apoptosis can be initiated by two distinct pathways: the extrinsic and intrinsic pathways. These pathways can be activated during low-temperature freezing, as evidenced by the protective role of specific cysteinase inhibitors (for example, Z-Val-Ala-Asp-(OMe)-fluoromethylketone [Z-VAD-FMK], a pan-caspase inhibitor; Z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone [Z-IETD-FMK], a caspase-8 inhibitor; and Z-Leu-Glu(O-Me)-His-Asp(O-Me)-fluoromethylketone [Z-LEHD-FMK], a caspase-9 inhibitor) when used as CPAs in germ cell freezing. Ha et al. [57] revealed that the proliferation capacity of SSCs was increased in frozen SSCs treated with a mixture of 200 mM trehalose and 15  $\mu$ M Z-VAD-FMK. This treatment also reduced early apoptosis after thawing through pan-caspase inhibition. In a similar vein, Niu et al. [58] reported a decrease in caspase activity and early apoptosis in vitrified porcine oocytes treated with Z-IETD-FMK and Z-LEHD-FMK. In 2020, Colombo et al. [59] also discovered that Z-VAD-FMK reduced DNA damage and caspase activity in vitrified

**Table 2.** Functions of cryoprotective agents

Target	Component	Function
Apoptosis inhibitor	Z-VAD-FMK	Pan-caspase inhibitor
	Z-IETD-FMK	Caspase-8 inhibitor
	Z-LEHD-FMK	Caspase-9 inhibitor
	Bongkreikic acid	mPTP opening inhibitor
Antioxidant	Coenzyme Q10	Increases the production of key antioxidants
	Melatonin	Mitochondrial-targeted antioxidant
	Vitamin C	Scavenger of free radicals
	Vitamin E	Lipophilic chain-breaking antioxidant
	MnTBAP	Cell-permeable superoxide dismutase mimetic
	Pentoxifylline	Phosphodiesterase inhibitor
	NAC	Reduces various radicals by donating 1 electron
	Leptin	Supports antioxidant enzyme activity
	Selenium	Antioxidant to break down peroxides
	Y-27643	ROCK inhibitor
Necroptosis inhibitor	Necrostatin-1	RIPK inhibitor
Autophagy inhibitor	3-methyladenine	PI3K inhibitor
	Melatonin	Inhibits autophagosome formation

Z-VAD-FMK, Z-Val-Ala-Asp-(OMe)-fluoromethylketone; Z-IETD-FMK, Z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone; Z-LEHD-FMK, Z-Leu-Glu(O-Me)-His-Asp(O-Me)-fluoromethylketone; mPTP, mitochondrial permeability transition pore; MnTBAP, manganese (III) tetrakis (4–69 benzoic acid) porphyrin; NAC, N-acetyl-L-cysteine; ROCK, Rho-associated coiled-coil containing kinases; RIPK, receptor-interacting protein kinase; PI3K, type III phosphatidylinositol 3-kinase.



oocytes. These findings suggest that inhibiting apoptotic proteolytic activation allows frozen germ cells to minimize cell death induced by cryoinjury. This conclusion is consistent with the findings of Jung et al. [60], who showed that extrinsic apoptosis, which involves the proteolytic activation of caspase-8, is increased in frozen SSCs. Therefore, Z-IETD-FMK can significantly enhance the cryoprotective efficacy of SSCs by suppressing caspase-8 activity during freezing. Furthermore, bongkreikic acid, an inhibitor of mitochondrial permeability transition pore opening, has been shown to improve the freezing efficacy of stallion sperm. This is achieved by reducing caspase activity and increasing the mitochondrial membrane potential [61].

Antioxidants are often used as CPAs to reduce apoptosis, as excessive ROS generation during freezing can cause cell death or damage upon thawing. For instance, vitrification of bovine oocytes with 50  $\mu\text{M}$  of coenzyme Q10, an antioxidant, significantly improved oocyte survival and mitigated premature cortical granule (CG) exocytosis, thereby preserving the CG migration pattern [62]. Similarly, freezing SSCs with 100  $\mu\text{M}$  of melatonin, a mitochondria-targeted antioxidant, greatly enhanced survival, reduced ROS production, and decreased intrinsic apoptosis [63]. Vitamin C, known for its potent antioxidant properties due to its strong reducing power, has been found useful in preserving DNA integrity, sperm motility, acrosome membranes, and plasma after freezing bovine sperm with vitamin C [64]. Vitamin E, a lipophilic chain-breaking antioxidant, has also been effectively used as a CPA for the freezing of ram sperm [65-67]. Moreover, resveratrol, a powerful non-flavonoid antioxidant, has shown beneficial effects on post-thaw sperm survival, fertility, and quality by inhibiting ROS production [68-70]. In addition, previous research has suggested that an increase in intracellular TNF- $\alpha$  after freezing is implicated in TNF- $\alpha$ -mediated necroptotic cell death [53]. The activation of the RIP complex also induces ROS production in the mitochondria and mediates plasma membrane rupture [71]. As such, the necroptosis inhibitor necrostatin-1 has shown beneficial effects on the proliferation rate while reducing necroptosis and apoptosis after thawing in SSC cryopreservation; it exerts these impacts by reducing ROS generation, apoptosis, and RIP1 activity [10]. The addition of pifithrin  $\mu$  (a p53 inhibitor) or 3-methyladenine, which blocks autophagosome formation by inhibiting type III phosphatidylinositol 3-kinase, has been found to enhance cryoprotective effects and help maintain normal stem cell characteristics after thawing during SSC freezing [9]. Mitophagy, a selective autophagy that removes aged and damaged mitochondria, is also activated in frozen oocytes to improve survival by eliminating damaged mitochondria [55]. In 2020, Feng et al. [72] demonstrated that melatonin inhibits autophagosome formation and regulates the levels of autophagy-related proteins, thereby reducing cryoinjury-induced excessive autophagy in goat SSC freezing.

Interestingly, autophagy does not consistently safeguard frozen germ cells; in fact, excessive autophagy can lead to cryoinjury-induced cell death [73]. Furthermore, while each pathway regulator may have cryoprotective effects resembling those of CPAs, the intricate nature of these pathways prevents complete rescue from cryoinjury-induced cell death. This is due to our limited understanding of the complex interplay among these pathways. This underscores the need for further molecular mechanistic studies to enhance our comprehensive understanding of cryoinjury-induced cell death.

## 2. Pre-freeze or post-thaw care

Efforts have been made to develop effective CPAs to prevent cryoinjury during the freezing process. However, these CPAs only partially mitigate cryoinjury, as damage continues to occur even after thawing. This highlights the necessity for careful handling and recovery of frozen cells and tissues post-thaw [12]. In this context, Hwang and Hochi [14] discovered that a brief post-thaw culture of oocytes with Y-27643, a Rho-associated protein kinase inhibitor, prevented the formation of multiple asters. This formation is a key factor limiting the embryonic potential of vitrified-warmed bovine oocytes [14]. Additionally, Girka et al. [13] found that treatment with 5 and 10  $\mu\text{M}$  Y-27632 effectively improved spindle rescue following vitrification-warming. In 2018, Pero et al. [74] proposed that the use of 20 mM Z-VAD-FMK during *in vitro* culture post-thawing could partially reduce cryoinjury-induced apoptosis by suppressing active caspase-3. Pagano et al. [16] demonstrated that treating with 100  $\mu\text{M}$  Z-VAD-FMK enhanced the mass motility of bovine sperm prior to freezing and maintained sperm membrane integrity post-thawing. Finally, Jung et al. [15] reported that supplementing post-thaw media with 200  $\mu\text{M}$  Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone (Z-DEVD-FMK) enhanced the proliferation potential of frozen SSCs, safeguarding them against ROS generation and apoptosis following cryo-thawing.

Numerous studies have demonstrated that incorporating antioxidants into media during *in vitro* maturation prior to freezing can enhance embryo development in various species, including mice [75,76], sheep [77], and cattle [62,78]. Furthermore, a range of antioxidants, such as selenium, manganese (III) tetrakis (4-69 benzoic acid) porphyrin (MnTBAP), pentoxifylline, N-acetyl-L-cysteine (NAC), and leptin, have been found to mitigate oxidative stress related to cryoinjury-induced damage when added to the *in vitro* culture media used in sperm freezing. Gavella and Lipovac [79] found that pre-incubating human sperm with 3.7 or 10 mM pentoxifylline, a phosphodiesterase inhibitor that also protects against lipid peroxidation by hydrogen peroxide, for 30 minutes at 37 °C reduced oxidative stress and enhanced fertility potential post-thawing. Oeda et al. [80] reported that incubating human semen with 1 mg/mL NAC for

20 minutes at room temperature significantly lowered ROS levels and improved sperm motility. Boroujeni et al. [81] found that rinsing with 5 µg/mL selenium after thawing can enhance human sperm parameters and survival. Fontoura et al. [82] found that adding leptin, a hormone that regulates food intake behavior, to human sperm prior to freezing supports antioxidant enzyme activity and provides additional antioxidant protection. Additionally, Treulen et al. [83] demonstrated that pre-incubating with MnTBAP, a cell-permeable superoxide dismutase mimetic, for 4 hours at 38 °C improved stallion sperm motility and reduced oxidative stress, while maintaining normal fertilization potential post-thawing.

While the addition of antioxidants or apoptosis inhibitors to *in vitro* culture media may help reduce oxidative stress, the use of these compounds in freezing is not always effective. Prolonged exposure or high concentrations can disrupt the appropriate physiological balance, as apoptosis is instrumental in removing damaged cells in adults and regulating embryonic development [84]. Similarly, excessive antioxidant supplementation may disrupt the oxidation-reduction balance, resulting in reductive stress [85]. However, the apoptosis or oxidative stress induced by cryoinjury often results in considerably more additional apoptotic/oxidative stress, which affects cell survival [86]. Therefore, the inclusion of antioxidants or apoptosis inhibitors could be beneficial in alleviating cell death caused by cryoinjury, provided optimization and functional tests for fertility are conducted.

## Conclusion

Cryopreservation of germ cells, reproductive tissue, and SSCs is an essential technique for preserving the fertility of patients with infertility or adolescents diagnosed with cancer. The primary objective of this technique is to ensure high viability and normal function post-freezing. Despite concerted efforts, current cryopreservation methods for reproductive tissues lack a comprehensive understanding of the underlying mechanisms of cryoinjury. Cryoinjury appears to be implicated in various cell death pathways, not just apoptosis, which has been the primary focus of research to date. However, the pathways involved in cryoinjury are not yet fully understood. As such, it is imperative to conduct in-depth investigations into the potential processes and pathways that lead to cryoinjury during freezing. We propose that future research should expand to include various cell death mechanisms associated with cryoinjury. Furthermore, we suggest that this research should incorporate both foundational and recent findings to enhance freezing efficacy by modulating signaling molecules and to devise new solutions that can be applied before freezing and after thawing.

## Conflict of interest

No potential conflict of interest relevant to this article was reported.

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Conceptualization: SEJ, BYR. Data curation: SEJ. Funding acquisition: BYR. Project administration: BYR. Visualization: SEJ. Writing-original draft: SEJ. Writing-review & editing: SEJ, BYR.

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