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Antioxidant strategies to mitigate oxidative stress-induced cryodamage in oocytes

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Abstract

Oocyte cryopreservation is an essential procedure in assisted reproductive technologies, aimed at preserving fertility, particularly for women undergoing IVF treatment or at risk of ovarian damage due to radiation, chemotherapy, or surgery. Despite its growing use, the survival and fertilization rates of cryopreserved oocytes remain suboptimal, largely due to cryo-induced oxidative stress. The generation of Reactive Oxygen Species (ROS) during freezing and thawing causes considerable damage to key cellular components, including proteins, lipids, DNA, and mitochondria. This oxidative stress compromises oocyte quality and reduces developmental potential. To address these challenges, the use of additives - especially antioxidants - has shown significant promise in mitigating oxidative damage. Enzymatic antioxidants such as Superoxide Dismutase (SOD) and Catalase (CAT), along with non-enzymatic antioxidants like glutathione, melatonin, and resveratrol, have demonstrated the ability to neutralize ROS and improve oocyte viability and developmental outcomes. Recent studies highlight the potential of Mitoquinone (MitoQ), a mitochondria-targeted antioxidant, to effectively counteract mitochondrial ROS and enhance cellular defense mechanisms during cryopreservation. This review explores the cellular mechanisms of cryodamage, the role of oxidative stress in oocyte cryopreservation, and the potential of various antioxidant strategies to enhance oocyte survival and function. Developing effective antioxidant supplementation approaches may significantly improve the outcomes of cryopreservation in reproductive medicine.

Keywords

antioxidants; oocyte; cryopreservation; oxidative stress

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1 Introduction

Preserving cells or gametes below zero degrees was virtually unimaginable more than three centuries ago. The discovery in 1949 that sperm could survive in glycerol at low temperatures marked the beginning of rapid development in cell cryopreservation[1]. By 1972, successful cryopreservation of mouse embryos was achieved[2]. Five years later, viable offspring were obtained following the transfer of embryos produced *via in vitro* Fertilization (IVF), representing a significant milestone in reproductive technology. During this period, the cryopreservation of mature mouse oocytes was also successfully accomplished[3]. The advent of vitrification technology in 1985 revolutionized cryopreservation by minimizing the formation of ice crystals, thereby significantly reducing the detrimental effects of low temperatures on oocytes and embryos[4]. Preserving reproductive potential has become increasingly critical for women with ovarian diseases or for those who choose to delay marriage and childbearing[5]. Oocyte cryopreservation serves as an effective strategy to store surplus oocytes and limit the generation of excess embryos beyond what is required for a single IVF cycle. Moreover, it offers hope to individuals at risk of ovarian damage due to radiation, chemotherapy, surgery, pelvic diseases, or other detrimental conditions. Despite technological advances, the developmental competence of frozen-thawed oocytes remains inferior to that of fresh oocytes. For instance, the blastocyst formation rate of vitrified-warmed bovine MII oocytes was reported to be nearly 10% lower than that of fresh oocytes[6]. Similarly, the *in vitro* maturation rate of vitrified-warmed mouse Germinal Vesicle (GV) oocytes decreased from 84% to 68%[7]. The reduced devel-

opmental potential of cryopreserved oocytes can be attributed to multiple factors, including oxidative stress from intracellular reactive species, mechanical damage due to ice crystal formation, toxicity of cryoprotective agents, and osmotic stress induced by low temperatures[3,8-11]. may result in structural injuries such as fractures in the zona pellucida, disruption of the meiotic spindle, spontaneous cortical granule exocytosis, plasma membrane damage, and impairment of cytoplasmic organelles. Consequently, these alterations can result in an increased incidence of parthenogenetic activation, lower fertilization rates, or higher rates of polyspermic fertilization[8-10]. The reduced developmental potential of oocytes can stem from several contributing factors, including mechanical damage caused by ice crystals[10], the toxic effects of cryoprotective agents, oxidative stress due to an accumulation of intracellular Reactive Oxygen Species (ROS)[11], and osmotic stress resulting from exposure to low temperatures[3]. Among these contributing factors, oxidative stress has garnered particular attention due to its profound impact on oocyte viability and function. It is primarily caused by the accumulation of intracellular ROS during the freezing and thawing process[11]. This article focuses on the role of oxidative stress in oocyte cryopreservation and explores the potential protective effects of antioxidants supplementation in mitigating such damage.

2 Cryo-damage

Numerous mechanical, thermal, and chemical stressors are exerted on cells during cryopreservation[12], potentially leading to impaired cell function or even cell death. Oocytes have generally been shown to be more vulnerable to cryo-damage than embryos at later stages. The "slow-rate" freezing technique is designed to mitigate cellular injury by controlling biophysical parameters, such as cooling and warming rates in conjunction with cryoprotectant agents. This method aims to minimize the harmful effects of elevated solute concentrations and osmotic stress, while allowing cells to be cooled to extremely low temperatures and reducing intracellular ice crystal formation[13]. During "slow-rate" freezing, extracellular ice formation causes cellular dehydration through an equilibrium-driven process. In contrast, vitrification, a rapid freezing method, employs extremely high concentrations of cryoprotectants that solidify without forming ice crystals, which are a primary cause of intracellular damage during cryopreservation.

Among the factors contributing to cellular injury, oxidative stress plays a central role during cryopreservation. One major source is the prolonged exposure of cells to ambient oxygen. Throughout the cryopreservation process, this exposure, coupled with high cryoprotectant concentrations and extreme temperature fluctuations, can generate superoxide anions and other ROS. These oxidative events contribute to osmotic stress and can adversely affect gamete physiology. For instance, increased ROS levels, including Hydrogen Peroxide (H_2O_2) , combined with a depletion of intracellular glutathione, have been associated with cytoskeletal disruption, as well as DNA and membrane damage, ultimately leading to developmental delays [10].

Additionally, cryopreservation-induced alterations in mitochondrial distribution and function can disrupt essential processes such as pH regulation and mitochondrial transport, which are critical for energy production in early embryos. Impaired energy metabolism further compromises adaptive responses such as DNA repair, thereby increasing ROS generation and exacerbating oxidative stress.

3 Reactive species and free radicals

Reactive species are oxygen (O_2) and nitrogen-containing molecules that possess one or more unpaired electrons. Among the primary types of ROS are the superoxide radical (O_2^-), H_2O_2, and the hydroxyl radical (OH•). The O_2^- has a short half-life and typically does not react with most biomolecules in the cytoplasmic aqueous environment. However, Superoxide Dismutase (SOD) catalyzes its dismutation to form H_2O_2. Compared to O_2^-, H_2O_2 has a longer half-life and selectively interacts with specific biomolecules. It can also be decomposed by antioxidant enzymes such as glutathione peroxidase and catalase, producing water and oxygen. In contrast, the highly reactive OH•, formed *via* the Fenton reaction, readily interacts with nearby molecules, oxidizing amino acids and disrupting protein structures.

A "free radical" refers to any molecule with one or more unpaired electrons that seeks electron stability by reacting, often aggressively, with surrounding molecules. Free radicals can target almost any type of biomolecule, although proteins, lipids, and nucleic acids are particularly susceptible to oxidative damage. In addition to free radicals, there are non-radical reactive species that are often less discussed. While not as reactive as free radicals, these non-radical species frequently act as intermediates in redox reactions and play key roles in the inactivation or propagation of free radical species.

Oxidative stress arises when the redox balance is disrupted and the level of free radicals exceeds the neutralizing capacity of the body's endogenous antioxidant defense system[14]. Approximately 95% of free radicals are classified as ROS[15], which include both radical forms such as O_2^-, OH•, alkoxyl (RO•), and peroxyl radicals (RO_2•), and non-radical forms like H_2O_2. Mitochondrial substrates, catalyzed by NADPH oxidase in the respiratory chain, are the primary source of intracellular ROS. While moderate levels of ROS are essential for activating intracellular signaling pathways that regulate cell growth and metabolism[16], excessive ROS accumulation can damage cellular

macromolecules and organelles. This results in oxidative stress, leading to varying degrees of damage to DNA, proteins, lipids, and carbohydrates.

ROS exert dual-edged effects on oocyte development. At physiological levels, they act as secondary messengers in cellular signaling pathways, playing vital roles in ovulation, meiosis, folliculogenesis, and embryonic development. However, excessive ROS generation induces oxidative stress and is implicated in reproductive disorders such as polycystic ovary syndrome and endometriosis[17-18].

A critical challenge is that no known enzymatic system currently exists to directly neutralize OH•, making it the most damaging among the three major ROS (Fig. 1)[19]. Although ROS can function as signaling molecules under normal physiological conditions, an imbalance that favors ROS accumulation overwhelms antioxidant defenses, resulting in redox imbalance and heightened oxidative stress. This leads to intracellular oxidative DNA damage, increased DNA strand breaks, oxidative modifications to proteins, and lipid peroxidation. Therefore, maintaining redox homeostasis and minimizing oxidative stress are essential for preserving oocyte quality[3].

The accumulation of ROS initiates Endoplasmic Reticulum (ER) stress, promotes protein misfolding, and induces calcium (Ca^{2+}) overload, which further amplifies ROS production and leads to oxidative damage to cellular lipids, proteins, and DNA originating from the ER.

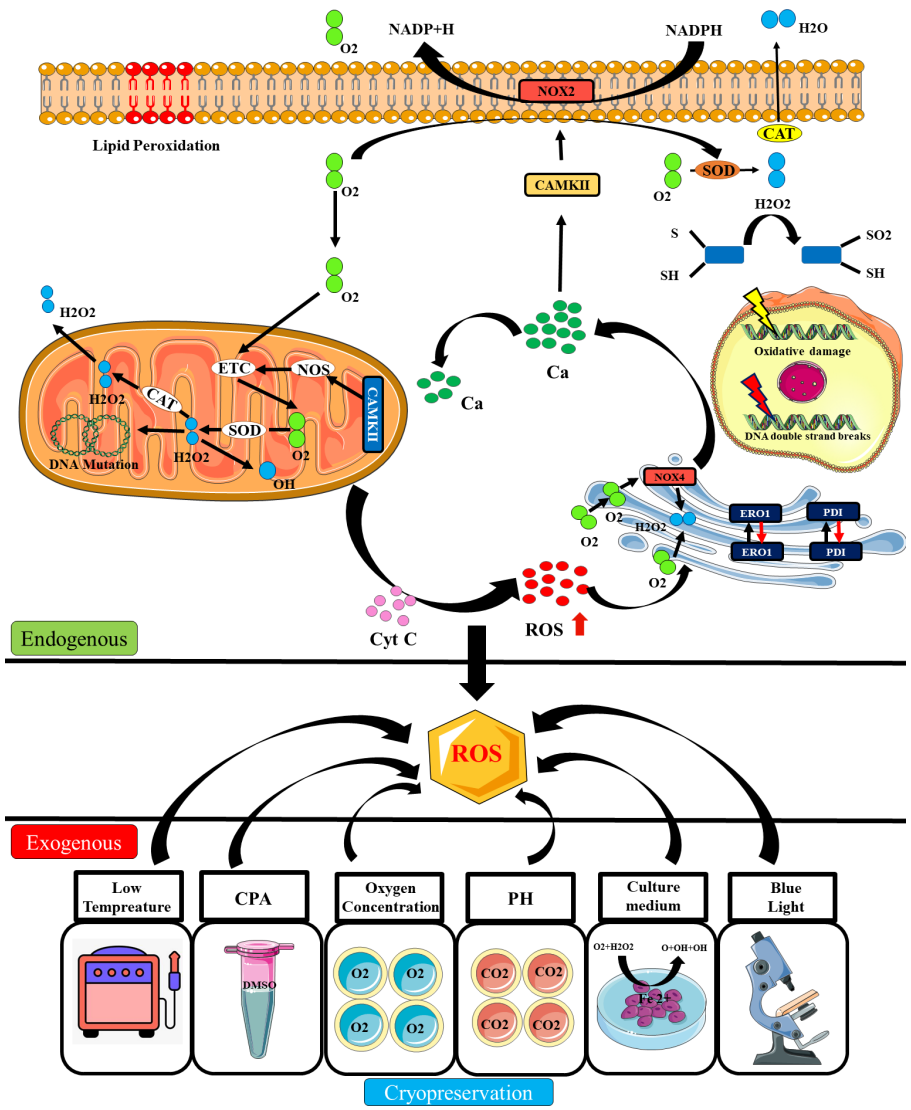

Fig. 1 Environmental factors and experimental reagents are major sources of ROS in oocyte cryopreservation. ROS, Reactive oxygen species; CPA, cryoprotective agents

4 Cellular damage during oocyte cryopreservation

4.1 Telomere shortening under oxidative stress

Oxidative stress has been shown to impair oocyte development and quality by disrupting spindle formation and chromosome assembly, or by accelerating telomere shortening, thereby interfering with meiosis[20]. Telomeres, as described by Lu *et al.*[21], are terminal DNA-protein complexes that act as protective caps at the ends of linear chromosomes. They play a crucial role in early meiosis by anchoring chromosomes, facilitating synapsis, promoting alignment, and pairing, and supporting chiasmata formation. When telomere function is compromised, chromosomal misalignment and disruption of meiotic spindles can occur, leading to meiotic failure[22-23].

While a variety of genetic and environmental factors are associated with telomere shortening, oxidative stress is the most frequently cited cause[24-25]. For example, chronic exposure to L-Buthione-Sufoximine (BSO), a compound that depletes glutathione, a major intracellular antioxidant, accelerates telomere shortening and increases the levels of oxidized proteins in CAST/Ei mice, a wild-derived strain with telomere lengths similar to those of humans[26]. Persistent inflammation has also been shown to lead to telomere disruption and premature aging in mouse models[27]. Quantitative Fluorescence in Situ Hybridization (Q-FISH) analysis of individual oocytes and embryos has demonstrated that mitochondrial dysfunction induces ROS accumulation and telomere attrition. This sequence of events ultimately results in chromosome fusions and apoptosis, further compromising oocyte viability and developmental potential[17,28].

4.2 Mitochondrial dysfunction

Mitochondria are the primary organelles responsible for energy production during aerobic respiration. They generate ATP through energy metabolism by means of redox reactions in the respiratory chain complex located on the inner mitochondrial membrane. Notably, oocytes contain the highest concentration of mitochondria compared to other cell types in the body. Mitochondria are also the principal sites of ROS generation[29-30]. Under normal physiological conditions, a small amount of ROS is produced due to electron leakage from the Electron Transport Chain (ETC)[31-32].

However, mitochondria are inherently vulnerable to ROS-induced damage. Mitochondrial genomic DNA, which encodes key components of the ETC, is particularly susceptible to oxidative stress due to its lack of protective histones, efficient repair mechanisms, and intrinsic antioxidant defenses[33]. Initial mitochondrial damage triggered by ROS leads to the accumulation of oxidative by-products. Because Mitochondrial DNA (mtDNA) is more prone to oxidative injury, this further accelerates mitochondrial dysfunction, resulting in a self-perpetuating vicious cycle. A cycle like this jeopardizes the integrity of the mitochondrial ETC, which lowers ATP synthesis, increases oxidant production, and elevates ROS levels in oocytes (Fig. 1)[3,34].

4.3 The use of antioxidants to combat oxidative damage in cryopreservation

Antioxidants are molecules capable of neutralizing or scavenging ROS before they can inflict damage on cellular components[35]. The cellular antioxidant defense system plays a critical role in mitigating oxidative damage by neutralizing harmful radicals. This system comprises both enzymatic and non-enzymatic antioxidants, which may be either endogenous or exogenous in origin[36].

Some antioxidants function synergistically to alleviate ROS-induced stress and are strategically distributed on both sides of the mitochondrial membrane. These antioxidants may be derived exogenously, for example, through dietary intake, providing essential support for cellular function under oxidative stress. Alternatively, endogenous antioxidants are synthesized within the cell and are readily available to maintain redox balance and support normal physiological processes.

4.4 Oocytes under oxidative stress from cryopreservation

Stress Granules (SGs) and Processing Bodies (PBs) are two types of ribonucleoprotein (RNP) granules in mammalian cells, composed of RNA and proteins[37-38]. SGs are dense, membraneless messenger Ribonucleoprotein (mRNP) aggregates formed in the cytoplasm under stress conditions. They contain small ribosomal subunits (40S), Poly(A)-Binding Protein (PABP), RNA- binding proteins such as TIA-1-related protein (TIA/1R), and polyadenylated mRNAs that are translationally repressed due to stress exposure[39]. In addition, SGs also harbor various non-RNA-binding proteins, including signal transduction molecules; however, they lack transcripts whose translation is activated by stress[40].

Under normal physiological conditions, PBs are rich in mRNAs, protein complexes involved in 5′-3′ mRNA degradation, such as XRN1 and DCP1, and translation repression proteins like GW182[41-42]. While PBs naturally exist and can increase in size and number under stress, the formation of SGs is specifically induced by stress conditions. The development of SGs and PBs enables cells to selectively store mRNAs, allowing for rapid restoration of translation and cellular homeostasis upon stress recovery.

Oxidative damage to RNA can lead to the formation of 8-hydrox-

yguanine (8-OHG) at guanine bases. PBs contain Y-box binding protein 1 (YB-1), which specifically recognizes and binds to 8-OHG-modified RNA[43]. Under certain stress conditions, YB-1 translocates from PBs to SGs. SGs then serve as a sorting platform for mRNAs: intact, non-oxidized mRNAs are recycled and re-enter polysomes to resume translation, while oxidized transcripts are directed to adjacent PBs for degradation. Moreover, SGs are inherently resistant to oxidative damage and contribute directly to cellular antioxidant defense. Their core components, Ubiquitin Specific Protease 10 (USP10) and Ras-GTPase-activating protein SH3 domain-binding protein 1 (G3BP1) - play crucial roles in redox regulation. Excessive G3BP1 can inhibit the antioxidant function of USP10 to maintain ROS equilibrium under normal conditions. However, in response to external oxidative stimuli, such as elevated H_2O_2 levels, this inhibition is lifted. Ataxia-Telangiectasia Mutated (ATM) protein, or its phosphorylated form, activates USP10, which subsequently reduces ROS accumulation and apoptosis[44]. Thus, SGs actively mitigate oxidative damage and support oocyte survival under cryopreservation-induced stress. The overall mechanisms involving SGs and PBs are illustrated in Fig. 2[3].

Phosphorylation of eIF2α suppresses global translation, promoting the formation of SGs where translationally stalled mRNAs are temporarily sequestered. G3BP1 interacts with and activates USP10, a key regulator of antioxidant responses, while ROS stimulate USP10 phosphorylation. Oxidatively damaged RNAs are directed to PBs for degradation, whereas intact RNAs stored in SGs can be recycled and re-engaged in translation upon stress recovery.

5 Current additives for reducing oxidative damage

Oxidative damage represents a major challenge in various biological contexts, particularly within reproductive technologies such as oocyte cryopreservation. To mitigate this damage, a range of additives are employed, among which antioxidants play a pivotal role. These antioxidants can be broadly categorized into two groups: endogenous and exogenous. Endogenous antioxidants are naturally produced by the body and can be further classified as enzymatic, such as catalase and SOD, or non-enzymatic, including glutathione and lipoic acid. These molecules function within cellular antioxidant systems to neutralize ROS and prevent oxidative stress.

In contrast, exogenous antioxidants are derived from dietary sources. These include compounds such as beta-carotene, ubiquinone (coenzyme Q10), and essential vitamins A, C, E, and K, as well as a variety of polyphenols, including phenolic acids and flavonoids. These exogenous agents complement the body's intrinsic defenses and are often incorporated into cryopreservation media to enhance oocyte viability and developmental potential following thawing, as illustrated in Fig. 3.

6 Enzymatic antioxidants

SOD, Catalase (CAT), and Glutathione Peroxidase (GPX) are the three main enzyme antioxidants found in cells[17]. SOD plays a crucial role in the initial detoxification of O_2^-, converting them into H_2O_2, which is subsequently broken down into water by CAT or GPX. Following increased expression of the SOD gene has been observed in mouse and porcine oocytes, suggesting an adaptive mechanism to scavenge ROS caused by cryopreservation. However, this upregulation is often accompanied by a decrease in Glutathione (GSH) levels, which exacerbates oxidative stress. In such cases, exogenous supplementation of SOD in the freezing solution has been shown to effectively reduce ROS levels and improve the fertilization and survival rates of mouse oocytes[45].

The upregulation of SOD gene expression in mouse and porcine oocytes following vitrification facilitates the scavenging of cryopreservation-induced ROS. However, a concurrent reduction in intracellular GSH levels exacerbates oxidative stress, necessitating the exogenous addition of SOD to the freezing medium to effectively eliminate excess ROS and enhance both fertilization and survival rates in mouse oocytes. During *in vitro* culture, CAT activity was found to be significantly elevated in vitrified-warmed sheep oocytes compared to non-vitrified controls. Likewise, the addition of catalase during the vitrification of goat ovarian tissue effectively mitigated ROS generation induced by freezing.

In addition to enzymatic antioxidants, non-enzymatic antioxidants naturally present in oocytes, such as GSH, Cysteine (CYS), and Cysteamine (CSH), also play important roles. GSH produced by oocytes helps maintain redox balance and supports cytoplasmic maturation. GSH can enhance the developmental potential of sheep embryos and protect against oxidative damage to spindle structure and function during *in vitro* maturation of bovine oocytes. Cysteine, a small thiol-containing amino acid, promotes GSH synthesis and may mitigate oxidative damage by increasing intracellular GSH levels. Cysteamine can directly scavenge OH•, helping maintain a high GSH/GSSG ratio and redox state. However, after vitrification, there is a notable decline in intracellular GSH synthesis, and the addition of CYS or CSH does not significantly improve the blastocyst formation rate of *in vitro*-warmed bovine oocytes during IVF.

7 Non-enzymatic antioxidants

Non-enzymatic antioxidants are commonly added to the vitrification, warming, or culture media to mitigate the oxidative stress associated with cryopreservation-induced oocyte

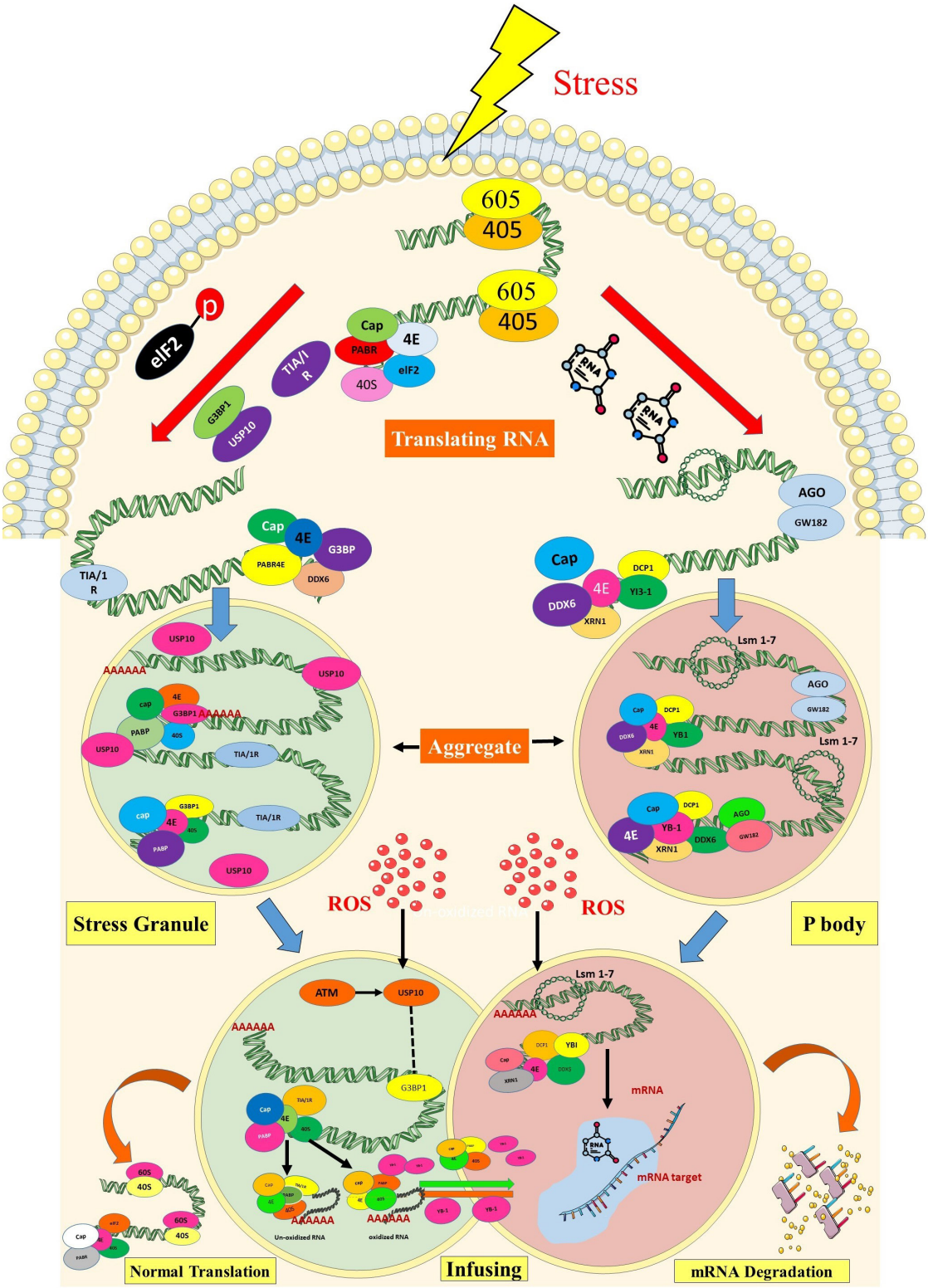

Fig. 2 During oxidative stress, Stress Granules (SGs) and Processing Bodies (PBs) play critical roles in managing oxidized RNA. ROS, Reactive oxygen species

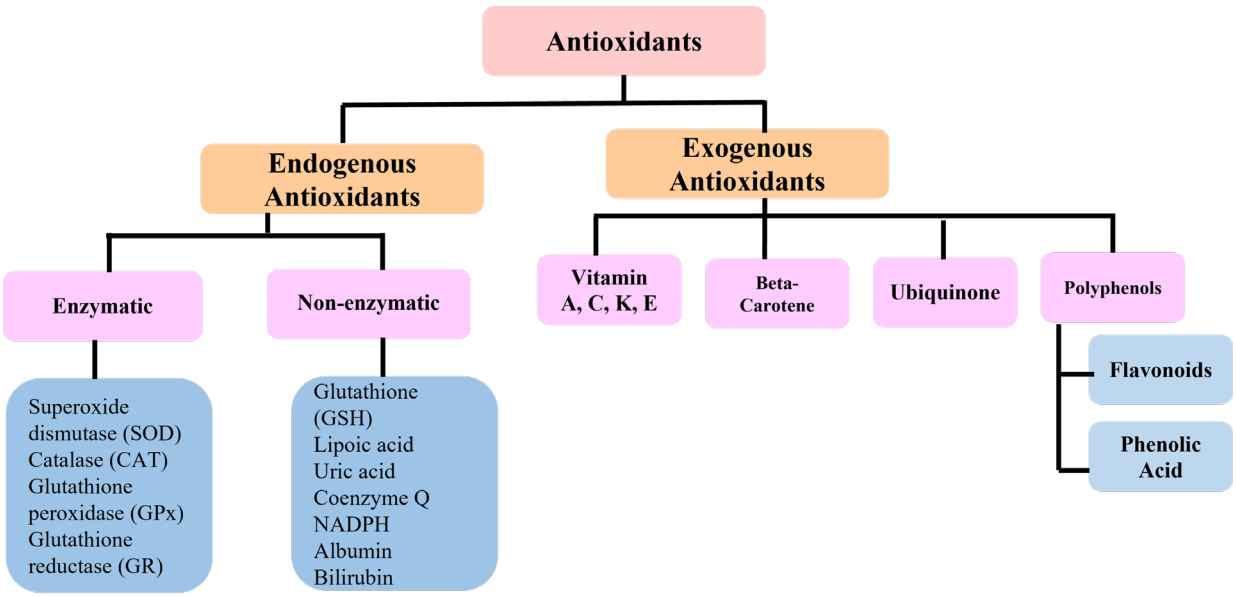

Fig. 3 Classification of antioxidants in oxidative damage

damage. Numerous antioxidants have demonstrated beneficial effects on oocyte maturation and developmental competence, including melatonin[46], resveratrol[47], L-carnitine[48], quercetin[13], vitamin E, astaxanthin, proline, and coenzyme Q10. These compounds can reduce or prevent oxidative damage in vitrified and warmed oocytes, significantly enhancing their capacity for *in vitro* development. Recent evidence suggests that mitochondria-targeted antioxidants are more effective than their non-targeted counterparts. For instance, Mitoquinone (MitoQ) has been shown to selectively scavenge excessive mitochondrial ROS and maintain mitochondrial redox homeostasis[49]. MitoQ consists of a positively charged triphenylphosphonium cation covalently linked to the benzoquinone moiety of coenzyme Q10 *via* a ten-carbon aliphatic chain. In vitrified-warmed oocytes, MitoQ has also been reported to preserve mitochondrial membrane integrity[50]. Vitamins E and C (ascorbic acid, AscAc) have also been widely studied for their antioxidant properties[51]. Ascorbic acid is considered the most important extracellular non-enzymatic antioxidant, primarily functioning to protect cells from oxidative damage induced by metal ions[51]. In contrast, vitamin Eis a lipid-soluble antioxidant embedded in cell membranes. It inhibits the formation of lipid peroxyl radicals within the plasma membrane and suppresses superoxide anion generation through NADPH oxidase activity[52]. Vitamin E has been associated with improved oocyte maturation, fertilization, and embryonic development in pig[53]. Additionally, it has been shown to enhance the reproductive potential of mouse oocytes derived from vitrified ovarian tissue by scavenging free radicals and reducing oxidative stress[54].

8 Cryopreservation benefits from the use of antioxidant supplements

To prevent cryodamage, various exogenous non-enzymatic antioxidants have been incorporated into maturation and vitrification media for pig oocytes and embryos. Among these, four antioxidants - resveratrol, GSH, Ascorbic Acid (AscAc), and β-mercaptoethanol - have yielded the most relevant and promising results. Although the exact mechanism of action is not fully understood, resveratrol has been shown to enhance the developmental competence of vitrified-warmed pig oocytes, as reflected by improved cleavage rates and the development of cleaved embryos[55]. The supplementation of both *in vitro* Maturation (IVM) and vitrification-warming solutions with resveratrol confers antioxidant and anti-apoptotic effects, preventing phosphatidylserine externalization in vitrified Metaphase II (MII) oocytes[56-57].

The addition of GSH to vitrification and warming solutions has also been reported to improve the quality and cryotolerance of IVM pig oocytes[58]. However, Somfai *et al.* found that supplementation of GSH in the *in vitro* culture medium did not enhance the cryotolerance of vitrified-warmed embryos[59].

9 Conclusion

Oocyte cryopreservation is unquestionably an indispensable tool in Assisted Reproductive Technology (ART), offering significant benefits for fertility preservation and enhancing reproductive outcomes. While the cryopreservation of oocytes at earlier meiotic stages holds promise, the use of Metaphase II (MII)

oocytes remains the most established and widely practiced approach. Thus, optimizing cryopreservation protocols at the MII stage is a logical focus for advancing the field.

Extensive research has demonstrated that cryopreservation impairs oocyte viability and developmental potential by inducing mitochondrial oxidative stress, lipid and protein peroxidation, mitochondrial DNA mutations, and reductions in ATP production and membrane potential. The release of pro-apoptotic factors further aggravates these cellular disruptions, compromising oocyte integrity. Among these effects, oxidative damage mediated by ROS during freezing and thawing is a major contributor to reduced oocyte quality.

This review highlights the critical role oof ROS in cryodamage and underscores the potential of enzymatic and non-enzymatic antioxidants in mitigating oxidative stress. Antioxidants - whether endogenous or exogenously supplied, scavenge free radicals, enhance mitochondrial function, and protect essential cellular components such as DNA, lipids, and proteins. Given the pivotal role of mitochondria in oocyte maturation, fertilization, and early embryogenesis, maintaining mitochondrial health is critical to improving outcomes.

MitoQ, a mitochondria-targeted antioxidant that selectively neutralizes mitochondrial ROS, has emerged as a particularly promising intervention. Post-cryopreservation supplementation with MitoQ and other antioxidants has shown beneficial effects, including improved oocyte survival, enhanced developmental competence, and increased fertilization efficiency. Future research should aim to optimize antioxidant formulations and explore synergistic combinations to maximize protective effects.

Enhancing cryopreservation protocols through the integration of antioxidant-based therapies holds great promise for increasing the efficacy and reliability of fertility preservation. These improvements will not only advance the effectiveness of ART but also expand its accessibility and utility, offering improved reproductive options and support for patients worldwide.

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Research ethics

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