

Review Article

Effect of Antioxidant on Sperm Freezing



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ABSTRACT

A major cause of male infertility, which includes 50 causes of infertility, is oxidative stress. Aerobic respiration in the sperm cell is associated with the production of reactive oxygen species (ROS). Some reactions, such as sperm capacitation and acrosome reactions require moderate levels of ROS. However, when the amount of ROS production surpasses the natural antioxidant defense of the cell, it causes a harmful effect called oxidative stress. This results in the oxidation of lipids, proteins, carbohydrates, and nucleotides. The production of ROS is increased by various factors, including the presence of leukocytes, sperms with abnormal and immature morphology, centrifugation of samples, and changes in oxygen concentration, pH, and temperature. Sperm freezing is a method widely used in assisted reproductive technology (ART) as well as infertility treatment. The process of freezing also contributes to increased oxidative stress because it alters the fluidity of the mitochondrial membrane, leading to increased production and release of ROS. Sperm have defense mechanisms against oxidative stress caused by increased ROS production, which includes the presence of enzymatic and non-enzymatic antioxidants in semen. The utilization of supplementary antioxidants in the freezing environment is a strategy to combat oxidative stress. This review aims to summarize the current evidence on the effect of antioxidants on sperm freezing.

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## 1. Introduction

**I**nfertility is considered a common problem in today's modern societies that includes 10%-15% of couples who have been unable to conceive despite having regular, unprotected intercourse for at least one year [1, 2]. A total of 40% to 50% of causes of infertility in human is related to male infertility and almost 7% of men are affected by it [3, 4]. Recently, the rate of male infertility has increased due to factors, such as stress, unhealthy lifestyle, and air pollution [5]. Oxidative stress is a potential cause of male infertility. The semen of infertile males typically has a lower antioxidant capacity compared to fertile men. The increased production of reactive oxygen species (ROS) and subsequent increase in oxidative stress induces lipid peroxidation and DNA damage. As a result, although these sperms can fertilize, fetal growth and fertility may be disturbed [6-8]. Mammalian sperm is a cell that requires a lot of energy to function. Sperm uses two primary metabolic routes to get the energy they need, glycolysis which takes place in the main part of the sperm flagellum, and oxidative phosphorylation, a process that occurs in the mitochondria in the center of the sperm flagellum [9]. Having 50-75 mitochondria, sperms are known as one of the cells with aerobic respiration, which is related to the production of ROS. ROS are highly reactive molecules that contain oxygen with an unpaired electron in their outer layer. Some of the vital ROS include oxygen ( $O_2$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radical (OH). These molecules can cause damage to cells and tissues if their production is not properly regulated. In the context of sperm, high levels of ROS can lead to oxidative stress, which can damage the sperm cell membrane, proteins, and DNA. This can ultimately lead to reduced sperm motility, morphology, and fertility. These molecules activate the Bcl-2-associated X protein family proteins, cause the release of cytochrome C from the mitochondria, and finally activate the caspases, which results in the initiation of apoptosis or directly cause the peroxidation of bases, creating a break in one or two DNA strands and the change of deoxyribose sugar (Figure 1). ROS are produced as a result of normal cellular activity; however, when their production exceeds the cell's natural antioxidant defense system, it can lead to a destructive effect known as oxidative stress. Oxidative stress can cause the oxidation of lipids, proteins, carbohydrates, and nucleotides, leading to cellular damage and dysfunction. In the context of sperm, oxidative stress can lead to reduced sperm motility, morphology, and fertility, as well as DNA damage. Therefore, maintaining a balance between ROS production and antioxidant defense is critical for the proper functioning of cells and tissues [10-12].

## 2. Materials and Methods

A comprehensive search was conducted using the PubMed, Scopus, and Google Scholar databases to identify relevant research published between January 2000 and June 2021. The search terms included "sperm freezing", "oxidative stress", "assisted reproductive method", and "antioxidant". A total of 72 studies were included in this narrative review, which was selected based on the relevance of the studies to the subject matter and the strength of the evidence.

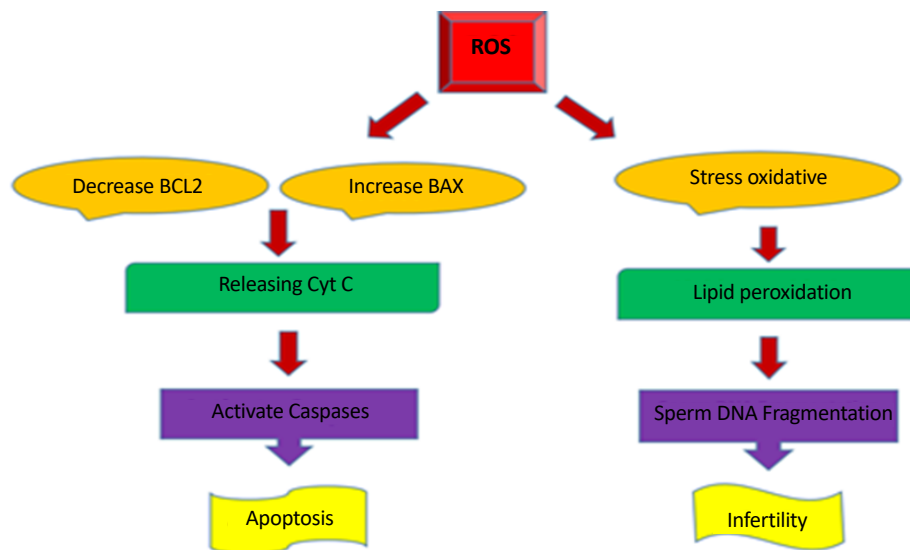
## 3. Results and Discussion

### Factors increasing ROS in semen

Destructive lifestyle conditions, such as alcohol consumption, smoking, exposure to various toxins, obesity, varicose veins, stress, and aging are conditions that usually increase seminal ROS. Leukocytes, abnormally formed spermatozoa, or immature spermatozoa containing small cytoplasmic droplets with a high level of enzymes are factors that increase the level of ROS [11, 13]. Human infertility has been recognized as a major global health problem, which has led to a significant increase in the use of assisted reproductive technology.

### The role of two types of ROS in sperm motility

ROS may have a dual role in sperm, where moderate levels of ROS are essential for certain reactions, such as capacitation, acrosome reactions, and mitochondrial stability, motility, and fertility. These reactions require a certain level of ROS to occur. However, high levels of ROS can lead to sperm DNA damage and increase the risk of miscarriage. Therefore, it is essential to maintain a balance between ROS production and antioxidant defense to ensure that the beneficial effects of ROS are maximized while minimizing the harmful effects [14-16]. Mammalian spermatozoa are special cells with the ability to live in the body regardless of where they are generated. They are special cells to deliver the parental genome into the egg cells. Following ejaculation, the sperm undergoes an evolutionary process in the female reproductive system called capacitation. For sperm to produce energy in the form of adenosine triphosphate (ATP), several cellular events are associated with capacitation, and it is debated a lot over which metabolic pathway is superior (glycolysis or oxidative phosphorylation). To produce energy efficiently, it is necessary to use oxidative phosphorylation because each glucose molecule produces 30 ATP when oxidized, but only 2 ATP molecules are produced when glycolysis occurs.



**Figure 1.** The role of reactive oxygen species (ROS) in apoptosis and infertility

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### Defense mechanism of sperm against increased production of ROS

Under normal conditions, the body has a natural antioxidant defense system to counteract the destructive and harmful effects of ROS. Seminal plasma and spermatozoa have many antioxidant defense systems against ROS that prevent intracellular damage [17, 18]. And a balance usually exists between the ROS produced and the inhibitory effects of antioxidants in the male reproductive system. Excessive ROS production or decreased antioxidant activity results in an imbalance between generated ROS and sperm antioxidant activity, leading to oxidative stress [18-20]. Sperm cell structure, plasma membrane, high number of mitochondria, low cytoplasm, and low amounts of antioxidants in the sperm cytoplasm make sperm susceptible to free radical damage [21]. Due to the destruction of sperm cytoplasm during sperm production, intracellular enzymatic and antioxidant activities, such as superoxide dismutase (SOD), peroxiredoxins (PRDX), thioredoxin (TRX), and treatment-resistant depression (TRD) remain low in sperm, and therefore seminal plasma containing antioxidants is a compensatory mechanism for this problem [22-24].

Antioxidants are the main protective factor against oxidative stress caused by free radicals. Seminal plasma contains two types of antioxidants, enzymatic antioxidants, and non-enzymatic antioxidants. Enzymatic antioxidants, also known as natural antioxidants, are glutathione peroxidase (GPx), glutathione reductase (GR), SOD, and catalase. SOD1, SOD2, and SOD3 are located in the space between the two mitochondrial membranes,

the mitochondrial matrix, and the extracellular space, respectively. The enzyme SOD transforms  $O_2$  into  $H_2O_2$ . Since  $O_2$  is a molecule with a short lifespan that cannot easily pass through lipid membranes and mitochondria, it cannot interact with many biological molecules in an aqueous environment. This molecule is produced by peroxisomal catalase and mitochondrial and cytoplasmic GPx activity. It decomposes, and one of the decomposition products is  $H_2O$ . Non-enzymatic antioxidants, also known as synthetic antioxidants or dietary supplements, include glutathione (GSH), urea, ascorbic acid, zinc, selenium, vitamin E, carotenoids ( $\beta$ -carotene), ubiquinone, taurine, and hypotaurine [25-30]. Oxidative stress can be quantified either directly by measuring ROS levels or indirectly by assessing lipid peroxidation products (malondialdehyde), protein oxidation products (carbonyl groups), and DNA oxidation [28, 29, 30].

### Lipid peroxidation (LPO)

A large amount of  $OH^-$  is a potential inducer of lipid peroxidation (LPO), which is formed as a result of two sequential reactions. The initial step is known as the Haber-Weiss reaction. This reaction involves the conversion of ferric ions ( $Fe^{+3}$ ) to ferrous ions ( $Fe^{+2}$ ) through the presence of superoxide radicals ( $O^-$ ). After this reaction occurs, a Fenton reaction follows, in which the ferrous ions ( $Fe^{+2}$ ) react with hydrogen peroxide ( $H_2O_2$ ), leading to the production of hydroxyl radicals ( $OH^-$ ) and the conversion of ferric ions ( $Fe^{+3}$ ) [31, 32]. During LPO, by-products are generated, including malondialdehyde (MDA), propanol, hexanol, and 4-hydroxynonenal (4-HNE). These by-products result from the oxidation of

polyunsaturated fatty acids (PUFAs) and can harm cellular function. For example, MDA is a highly reactive compound that can covalently modify cellular proteins and DNA, leading to cell damage and death. Similarly, 4-HNE can react with cellular proteins to form adducts that impair cell function and contribute to the development of diseases, such as cancer, Alzheimer's, and cardiovascular disease. Therefore, reducing LPO and the production of these by-products is a crucial aspect of maintaining cellular health [33]. Highly reactive molecules are generated during LPO. These molecules can attack the unsaturated fatty acids in the surrounding environment and initiate a chain of reactions that can have detrimental effects, including the destruction of membrane fluidity. By-products, such as MDA, propanol, hexanol, and 4-HNE are produced during this process and can serve as biomarkers for LPO. These by-products are indicative of the oxidative stress that has occurred and can be used to assess the extent of damage that has been done to cellular membranes and other structures. Nowadays, freezing has been a key factor in the success of assisted reproductive technology (ART) in humans and cattle. Although cryopreservation is commonly used, it is a harsh process that harms sperm motility because it increases ROS production, followed by LPO, leading to reduced sperm motility and viability [34-36].

### Sperm DNA fragmentation

Sperm DNA fragmentation (SDF) can occur when the level of (ROS) increases and the amount of antioxidants in semen decreases. ROS can cause damage to sperm DNA either directly or indirectly by activating sperm caspase and endonuclease. Sperm DNA fragmentation (SDF) occurs due to an error in chromatin compaction during the spermiogenesis process, which leads to a failure in substituting chromatin structure from histone to protamine. This damage is caused by ROS exposure after spermiation, during the movement of spermatozoa from the seminiferous tubules through the rete testis to the cauda epididymis. This issue results in the formation of oxidized guanine adducts, such as 8-OH-guanine and 8-OH-2'-deoxyguanosine, which are linked to increased DNA fragmentation and strand breaks [37].

DNA has a double-helix structure and can be fragmented in both single-stranded and double-stranded forms. Repair of DNA can only occur during specific stages of spermiogenesis and is no longer possible during nuclear condensation in the epididymis. The human oocyte is the next opportunity for single-stranded DNA break repair, which is critical for embryo development but decreases with advanced maternal age. Double-stranded DNA

breaks can lead to genomic instability and apoptosis if they are not repaired. Unrepaired single-stranded DNA breaks above a critical threshold harm embryo development and pregnancy outcomes, known as the "late paternal effect". Embryonic genome expression begins on the second day of human embryo development, making it dependent on its genome rather than maternal factors. Spermatozoa with single-stranded DNA breaks negatively affect blastulation, implantation, and pregnancy outcomes after fertilization. Oxidative stress also harms cleavage embryo development, known as the "early paternal effect". Therefore, reducing oxidative stress in semen is crucial to maintain DNA integrity and improving fertility outcomes.

Physiologically programmed cell death, known as apoptosis, occurs through various signaling and regulatory pathways. This process involves DNA fragmentation. Apoptosis can also be triggered by ROS-induced double-stranded DNA breaks. ROS can disrupt mitochondrial membranes, causing them to release cytochrome C, which activates apoptotic caspases and annexin-V binding to phosphatidylserine. In infertile patients, high levels of ROS may cause significant damage to mitochondria, leading to elevated cytochrome c levels in seminal plasma. This can result in increased apoptotic activity and DNA fragmentation, leading to reduced sperm quality and fertility. Therefore, reducing ROS levels in semen is crucial to prevent apoptosis and maintain sperm quality [37, 38].

### Sperm freezing and its effect on sperm quality and oxidative stress

The history of sperm freezing goes back more than 200 years. In 1776, Spallanzani reported that the sperms regained motility after rewarming (thawing) the semen of animals frozen in the snow. However, it was not until the 20th century that sperm freezing became a practical method for preserving fertility. In the 1950s, the first successful human pregnancy using frozen sperm was reported. Since then, the technique has been widely used in ART to help couples conceive. Sperm freezing has many advantages, including the ability to store sperm for future use, the ability to screen and select sperm with better quality, and the ability to reduce the risk of genetic diseases. Sperm freezing has become a widely accepted and routine procedure in ART clinics across the globe. It is an essential tool in the management of male infertility and has aided many couples in achieving pregnancy. This procedure involves collecting and freezing semen samples, which can be stored for extended periods. When needed, the frozen sperm can be thawed and

utilized in various ART techniques, such as intrauterine insemination (IUI), in vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI). The success rates of these techniques using frozen-thawed sperm are similar to those using fresh sperm. Moreover, sperm freezing has provided an opportunity for men undergoing chemotherapy or radiation therapy to preserve their fertility before treatment because these treatments can damage sperm and cause infertility. The sperm motility was restored after long-term storage at  $-15^{\circ}\text{C}$  and thawing. A sperm bank was later proposed for the preservation of animal and human embryos [21, 39]. In 1937, Johnel accidentally discovered that some human sperms survive at temperatures of  $-79^{\circ}\text{C}$ ,  $-196^{\circ}\text{C}$ , and  $-269^{\circ}\text{C}$ . Initial experiments with human cryopreserved cementum reported a post-thaw training efficiency of approximately 10%. However, the efficiency of sperm motility and survival is very limited in the absence of maintenance environments. Gamete freezing began in 1949 when glycerin was discovered to be a powerful antifreeze. In the same year, Polge et al. reported the first successful use of glycerol to freeze sperm [21, 39, 40]. In recent years, ART has been widely used in conjunction with infertility treatments (every 6 couples receive fertility treatment) to preserve fertility. Freezing of human sperm and fertility through ART methods has been successfully achieved.

Cryopreservation of human sperm, first introduced in the 1960s, is one such method that keeps sperm alive indefinitely [41]. Freezing is a method that helps improve fertility and preserve sperm while maintaining the functional ability of sperm so that they can be used for ART, IVF, and ICSI. Nowadays, freezing has been extensively used, for example, to preserve fertility before treatments, such as chemotherapy, radiotherapy, or before certain types of surgery, including vasectomy. It is also useful to overcome the conditions of oligospermia and azoospermia as “fertility insurance” and to store semen until HIV and hepatitis test results are negative. Men who work in industries exposed to radiation, toxic substances, and other contaminants may benefit from sperm storage. Finally, to avoid stress during sperm collection, cryopreservation is used to store and preserve sperm on the day of oocyte collection [2, 42, 43]. Sperm freezing involves cooling the semen samples and then storing them in liquid nitrogen at a temperature of  $-196$  degrees Celsius, where all the metabolic and biological processes of the cell will stop because not enough heat energy exists for the chemical reaction to occur and the aquatic environment necessary for essential metabolic activities does not exist [44].

Today, even with the use of modern protocols, the quality of live sperm decreases after freezing. Freezing results in damage, such as oxidative stress, osmotic stress, intracellular ice crystals, disruption of plasma membrane function, mitochondrial dysfunction, and DNA fragmentation. This phenomenon subsequently results in the death or severe damage of spermatozoa, reduced motility, decreased survival, and ultimately loss of fertility [18, 45, 46]. The use of cryoprotectants (such as glycerol, ethylglycone, dimethyl sulfoxide, and 1, 2-propanediol) is one of the critical innovations in sperm freezing. The low molecular weight and high permeability of these materials protect sperm from damage caused by ice crystallization during freezing. By lowering the freezing temperature, these materials reduce the content of salts and solutes in the liquid phase of the sample and lower the formation of ice crystals inside the sperm, thus protecting the integrity of the sperm membrane as well as optimizing the osmotic pressure of the external fluid. However, cryoprotection alone is not sufficient to protect cells from stress caused by the freezing process, and the process of freezing and thawing sperm affects sperm structure and function, shortens its lifespan and motility, and ultimately reduces fertility [47].

Oxidative stress is a major concern following sperm freezing. Many studies have reported a significant increase in the production of ROS after the freeze-thaw process. During freezing and thawing, increased ROS levels can result in oxidative stress, either due to increased ROS production or decreased its scavengers [10, 48]. One of the primary manifestations of damage induced by ROS in sperm is caused by the oxidative damage of methylene bis-allylic groups of sperm phospholipids attached to polyunsaturated fatty acids (PUFAs), resulting in LPO. Due to the peroxidation of membrane lipids, membrane permeability, and fluidity changes, leading to irreversible loss of sperm movement, intracellular enzyme leakage, sperm DNA damage, and defects in oocyte penetration and sperm-to-oocyte binding [49]. The production of ROS during freezing and thawing has been proven, and data have shown that freezing and thawing sperm causes an increase in the production of superoxide radicals, and a sudden burst of nitric oxide radicals has also been observed during thawing [35]. In samples with detectable levels of ROS at baseline, these levels increase significantly after freezing and thawing. Moreover, in samples with undetectable ROS, ROS can be detected when exposed to freezing and thawing [34]. Oxidative stress by ROS during freezing causes membrane lipid peroxidation and damage, which in turn reduces sperm motility. Loss of SOD activity causes lipid peroxidation [50, 51]. Among the most dangerous prod-

ucts of lipid peroxidation are MDA and 4-hydroxynonal, which can cause severe DNA damage and cell proliferation inhibition [52]. ATP is synthesized by mitochondria during oxidative phosphorylation in eukaryotic cells. As part of their function, mitochondria in sperm also produce ROS required for capacitation, intracellular movement, survival, and the normal function of sperm [53-55]. The effect of freezing is to increase the potential of the mitochondrial membrane by changing its fluidity, causing ROS to be released. In cold environments up to 4°C, ROS are produced by sperm and seminal leukocytes, causing DNA damage and fragmentation, loss of integrity of the plasma membrane, deactivation of enzymes, and eventually sperm dysfunction [55-57]. Culture medium, oxygen concentrations, lighting degrees, pH, and temperature are factors that can affect sperm quality and increase ROS levels when they change during preparation [58].

#### The role of antioxidants in sperm freezing

Antioxidants can improve sperm quality by decreasing free radicals and the negative effects of oxidative stress, improving sperm parameters, and thus increasing the chances of conception. Therefore, they have major clinical importance about nutrition [59]. Given the role of free radicals in inducing apoptosis and producing oxidative stress states, compounds that inhibit free radicals in semen during the freeze-thaw process, reduce oxidative stress, and improve fertility in ART are of great interest, and many studies have focused on the use of such antioxidants in sperm freezing environments to reduce the negative effects of ROS on sperm [60, 61].

Dietary supplements as well as in vitro antioxidants are responsible for reducing ROS in seminal fluid. Sperm media prepared by adding different concentrations of vitamins C and E have shown significant reductions in H<sub>2</sub>O<sub>2</sub> [62]. Many antioxidants have been shown to have a positive effect on improving sperm motility. Vitamin C is one of the main antioxidants in seminal plasma that protects lipoproteins from peroxy radicals. Data show that vitamin C increases and improves sperm motility in a dose-dependent manner. Evidence shows that vitamin C enhances and improves sperm motility in a dose-dependent manner. For example, exposure of sperm to 800 µmol of vitamin C for 6 hours improved sperm motility, while concentrations above 1000 µmol decreased sperm motility [63]. Vitamin E, another vitamin antioxidant, effectively maintains sperm motility and morphology by inhibiting LPO [64]. The effects of vitamins E and C on sperm motility were dose-dependent. Although, vitamins E and C have a positive effect, such as anti-H<sub>2</sub>O<sub>2</sub>, high

concentrations of these vitamins induce LPO and increase damage in normospermia and asthenozoospermia [65]. Vitamins C, E, and selenium have been shown to secure sperm DNA from H<sub>2</sub>O<sub>2</sub>-induced harm in both normozoospermic and asthenozoospermic samples [58, 66].

A variety of antioxidants have been used in recent years to protect spermatozoa during the freezing and thawing processes, most focusing on biological and chemical antioxidants. Melatonin is also one of the secretions of the pineal gland that is effective in regulating some physiological phenomena. In a study of 400 infertile couples with a low IVF rate of about 30%, ICSI was found to significantly improve fertility in these individuals, resulting in decreased sperm function and permeability. Mitochondria are vital in the normal functioning of spermatozoa. The study found a problem with the mitochondrial microstructure in these men's sperm. Three-nitro propionic acid (3-NPA) is a substance that inhibits succinate dehydrogenase. It significantly reduces oxygen consumption in complex II of the mitochondrial electron transport chain and increases the production of mitochondrial ROS, leading to mitochondrial oxidative stress. Researchers found that melatonin reduces oxidative stress, improves mitochondrial respiratory capacity, increases sperm-to-egg permeability, and improves IVF outcomes. Based on a study of 13 fertile men with sperm densities above 20 million per mL, melatonin with different concentrations is effective for improving survival, and motility, and reducing ROS and MDA levels, and the most effective dose is 25 mg [67].

Several studies have shown that the addition of melatonin to fresh and frozen semen from animals also has antioxidant properties. For example, in cattle, the addition of melatonin at concentrations of 2 and 3 mM in a frozen medium preserves sperm viability and motility and reduces lipid peroxidation in the post-thaw phase [68]. The addition of 1 mM melatonin to ram frozen medium has been shown to enhance embryonic division as well as have beneficial effects on sperm motility, viability, and DNA health [69]. Turmeric, whose scientific name is *curcuma longa*, belongs to the Zingiberaceae family. Curcumin is an active ingredient and polyphenol in the rhizome of the turmeric plant. According to the World Health Organization, in studies of men between the ages of 30 and 42 years with healthy sperm parameters, curcumin at doses of 2.5, 5, 10, and 20 µM was detected by adding curcumin to frozen medium, while parameters, such as sperm motility and viability were improved and ROS and DNA fragmentation were reduced. These positive effects were more pronounced at a curcumin concentration of 20 µM [70]. Curcumin has been shown to have antioxidant effects and increased antioxidant en-

zyme activity, and previous studies have determined that curcumin's antioxidant and free radical-inhibiting activities are related to its phenoxyl structure and conjugated double bonds. It can inhibit and scavenge free radicals, such as hydroxyl radicals. In addition to the molecular mechanisms based on the direct elimination of free radicals, curcumin can enhance the activity of intracellular enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, which act as antioxidants [71, 72].

#### 4. Conclusion

Factors increasing ROS in the sperm negatively affect sperm parameters. However, the existence of defense systems against ROS, including enzymatic and non-enzymatic antioxidants, is a way by which the sperm is protected from damage. Moreover, sperm freezing also enhances the level of ROS in sperm; however, the application of supplementary antioxidants during freezing attenuates the negative effect of ROS on sperm parameters.

#### Ethical Considerations

##### Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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##### Authors' contributions

Methodology: Maryam Zamani, Fariba Zafari, Farzad Rajaei and Amir Hosseini; Writing—original draft preparation: Maryam Zamani and Amir Hosseini; Validation: Maryam Zamani, Amir Hosseini and Fariba Zafari; Data gathering: Maryam Zamani; Writing—review and editing: Vahid Najafzadeh, Amir Hosseini and Mana Kamranjam; Conceptualization, project administration and funding acquisition: Amir Hosseini.

##### Conflict of interest

The authors declared no conflict of interest.

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