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PERSPECTIVE

ADDITIVES TO INCREASE THE QUALITY OF SPERM EXTENDERS DURING CRYOPRESERVATION: A META-ANALYSIS REVIEW

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ABSTRACT

According to recent statistics, 40-50% of infertile are men have numerous extrinsic factors that play a significant role in boosting ROS, which is hazardous to sperm qualities. This meta-analysis study examines which critical and existent additives used in cryopreservation protocols are involved in sperm funtional parameters and counteract the negative impacts of ROS. We analysed the findings of 521 research papers published on PubMed, Scopus, and in various clinical studies, that investigated the effects of_ sperm extenders with different doses and durations at various in vivo and in vitro stages of sperm handling. We found that over 30-50 components were utilized as additives across multiple study trials. The main function of these additives was to reduce DFI/ROS during the freeze/thaw processes while also sustaining sperm motility and viability. The most effective natural antioxidants were found to be vitamins C and E, L-phosphatidyl choline, and an extract of the carob plant, *Ceratonia siliqua*. Overall, most studies employed *Ceratonia siliqua* as an additive/extender, resulting in maintained motility and morphology with balanced ROS/DFI levels compared to other extenders. Of the animal-derived compounds, AFP-III was more effective than other stated extenders, exhibiting significant control in semen preservation. Natural additives significantly ($p<0.05$) increase cell viability and vitality by more than 50-60% compared to commercially available extenders. Although the present trend is to use additives that are naturally sourced and cost-effective, due to their availability and affordability, concerns remain about the shelf-life of *Ceratonia siliqua* extract and AFP-III for use in sperm cells freeze/thaw procedures.

Keywords: additives; AFP-III; *Ceratonia siliqua*; motility; ROS; semen extender; vitamin E.

INTRODUCTION

Infertility is a common condition that affects roughly 12 % of the world's population, with male infertility accounting for 40% of the total (1). Male infertility has become increasingly common in recent years as a result of environmental contaminants — such as water pollutants, pesticides, and herbicides — being toxic to spermatogenesis. The ongoing population studies by various study groups have suggested that sperm counts are usually falling even though infertility has not increased much. However, the conception rate is delayed from 1 year to more than three years due to poor semen profile due to unexplained infertility. So far, the following treatment options are followed for male infertility: 1) surgery, to carefully repair a disturbed varicocele; 2) treatment of and infection-related with antibiotics, although fertility is seldom restored; 3) assisted reproductive technology (ART), involving the collection of sperm by normal discharge, surgical extraction, or from sperm donors; these sperm cells are subsequently fertilized with eggs from the female vaginal tract or utilized in IVF or intracytoplasmic sperm injection. Sperm is often cryopreserved as part of the ART therapy.

Why cryopreservation?

Sperm preservation is critical for assisted reproductive technology. Cryopreservation is currently used in clinical settings to preserve human spermatozoa. The oldest known preservation of sperm occurred around 200 years ago. Because of the massive surge in demand in the dairy business, preservation is now frequently utilized to protect bovine sperm samples (2).

Cryopreservation is the technique of exposing cells to extremely low temperatures, which causes cryoinjuries. For example, cell exposure to low temperatures can induce ice crystal development. To avoid this type of cell damage, cells are stored in a cryoprotective medium. Despite the application of cryoprotectants, post-thaw sperm still shows significant damage. Because of recent technical breakthroughs, molecular-level research reveals that the damage is often caused by ROS generation, which ultimately leads to DNA fragmentation and reduces many sperm functional parameters. Such fundamental and applied science has resulted in a better understanding of the potential harm caused by cryopreservation. Over time, semen extenders have been increasingly used as cryoprotectants. Extenders combine cryoprotectants and other additives that help reduce DNA fragmentation, osmotic stress, and ROS levels. Traditionally, sperma preservation media have four key components: 1) glycerol; 2) sugars; 3) antibiotics; and 4) a pH buffer. However, research also reveals that higher-quality sperm storage necessitates the use of extra chemicals. In this work, we investigate all potential additives for human spermatozoa.

Semen extenders and their functions

Semen extenders play a crucial role in the cryopreservation of human spermatozoa and sperm characteristics such as motility, viability, acrosome, and membrane integrity. Semen extenders typically include a medium pH buffering system (Tris, sodium phosphate, citric acid), cryo-shock preservatives (glycerin, egg yolk, soy-lecithin, milk), energy (fructose), and antimicrobials (streptomycin, penicillin, and polymixin B) (3). Osmotic stress generated by ROS generation during cryopreservation is a problem that must be addressed immediately. Antioxidants have been demonstrated to increase sperm quality (4, 5), and to be critical components of various conventional freezing techniques, advanced cryopreservation methods, and novel strategies (such as the addition of cryoprotectants, antioxidants, fatty acids, antifreeze proteins, nanoparticles, animal serum, or plant essential oils) for the protection of human and animal spermatozoa from cryo-injury. Here we review currently available semen extender chemicals that increase sperm quality after thawing.

Cryoinjuries

The cryoprotectants (CPA) are divided into two categories: permeability and non-permeability. When concentrations are high, permeable CPAs become stresssful because they disrupt osmotic balance. Non-permeable CPAs, such as egg yolk, sucrose, and albumin, operate as a barrier layer. Another concern is toxicity, which is concentration-dependent for both kinds of cryoprotectants (6). Another critical harm detected is DNA fragmentation, which is connected to elevated levels of ROS. Oxidative stress causes DNA damage (7). Other often observed metrics include motility, morphology,

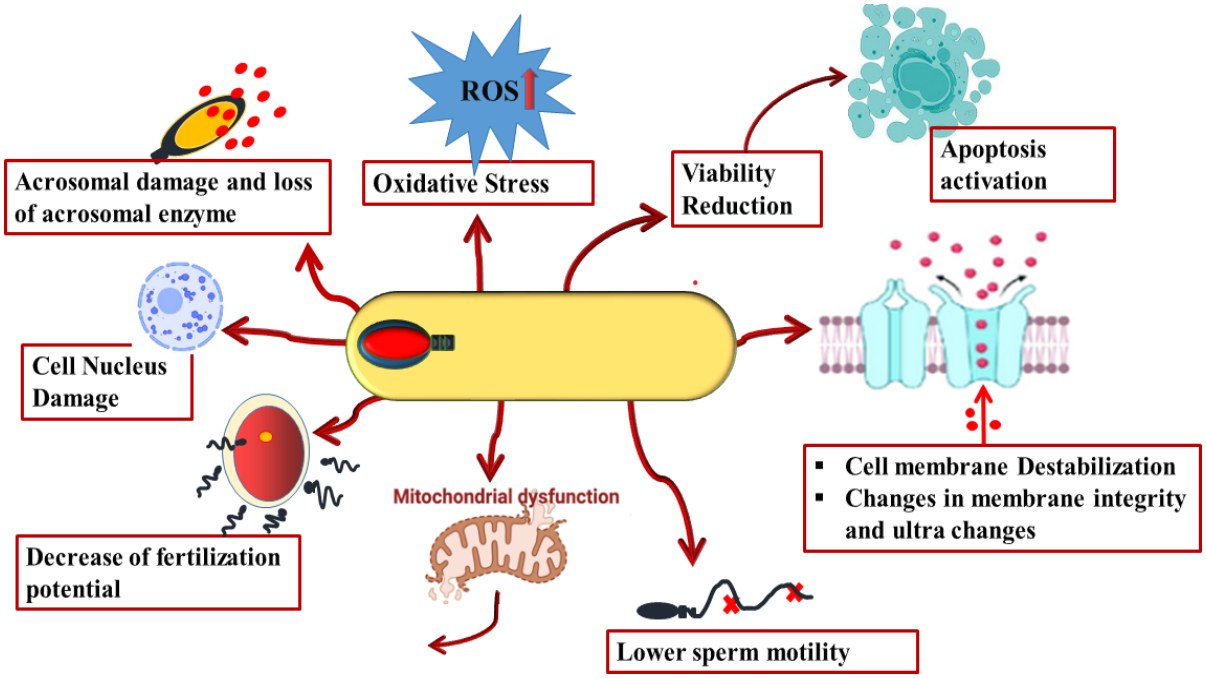

Figure 1. Cryoinjuries by various sources and its associated effects on sperm biology.

and viability, all of which have been shown to be negatively impacted by semen extenders. Antioxidants have a significant favorable influence on DNA and mitochondria integrity, as well as overall sperm quality. Figure 1 shows a graphical illustration of the numerous cryoinjuries. The following table (Table 1) summarizes cryopreservation techniques' principles, advantages, and disadvantages.

METHODS

Rationale of the meta-analysis

This work focuses on discussing the ideal semen extender alternatives/additives that are essential for the longer shelf life of cryopreserved sperms. This study's rationale is to identify and determine a perfect concoction of semen extender for human spermatozoa. The inclusion criteria were:

a) reports on the impact of antioxidants/additives on sperm parameters with a significant difference from the control group after thawing;
b) addition of additives to cryomedia before freezing;
c) assessment of the post-thaw effects of additives by the same group;
d) studies involving semen samples from healthy adult human men;
e) semen samples received after an abstinence of more than three days;
f) sperm freezing that resulted in sperm with a minimum of three of the following properties:

i. sperm volume ≥ 1.5 (ml)
ii. sperm concentration ≥15 × 10^6/mL
iii. total motility ≥ 40%
iv. progressive motility > 32%
v. viability > 58%
vi. normal morphology > 4%.

Animal studies, narrative reviews, conference papers (with insufficient or outdated data), and editorials were all excluded. All papers were screened based on their title, abstract, and full text. Data were manually retrieved from the selected publications for all of the factors investigated by each group. Furthermore, the following information was gathered from each article: Year of publication, sperm medium utilized, cryopreservation method, and mechanism of action. All retrieved data was then placed on a forest plot for comparison with each sperm parameter. Only data with significant differences ($p < 0.05$) were included in the graphic. The graph was created using the JASP program version 0.14.0.0.

RESULTS

During the initial search, 521 articles were identified using keywords from PubMed. After several screenings based on the title, followed by the abstract, and finally, through the full text, a shortlist of 25 papers was produced. In addition, 11 papers researching the same compound were also considered for meta-analysis, further enriching the depth of our research. Table 2 shows the studies that were included in the meta-analysis, covering 25 compounds.

Table 1. Approaches used for the preservation of small numbers of spermatozoa.

Cryopreservation techniques	Principle	Main advantages	Main disadvantages	Ref.
Zona pellucida	The empty zona pellucida of animals or humans is utilized to store sperm.	Identifying motile sperm takes less time, and cryoprotectants may be easily transported without losing sperm cells in the zona.	Contamination risks	(8)
Microdroplets	Small amounts of sperm cryoprotectants are applied to the dry ice's surface and then submerged in liquid nitrogen.	Avoid sperm loss by adhering to the vessel.	Droplets are difficult to handle and store in traditional freezers and liquid nitrogen tanks, increasing the risk of cross contamination	(9)
ICSI pipette	Storing spermatozoa in ICSI pipettes	Sterile and easy system	Not suitable for long-term storage; cross-contamination	(10)
Volvox globator spheres	Sperm storage in the spheres of *Volvox globator*	Significant recovery of motile sperm after thawing	Algal genetic material contamination and bioavailability	(11)
Alginate beads	Microencapsulation using alginate beads	The inert characteristic of alginate beads	Encapsulation decreases sperm motility.	(12)
Cryoloop	Individual spermatozoa are put directly on cryoprotectant film covering the nylon loop and submerged in liquid nitrogen.	Excellent vessel for vitrification; no further preparation required.	Open system: danger of cross-contamination.	(13)
Agarose microspheres	Storing sperm in agarose microspheres	Non-biological carrier	The clinical usefulness of this method has not been assessed.	(14)
Straws	Sperm and cryoprotectants placed into the mini-straw.	Sterile, simple, and convenient system	Not suited for seriously degraded samples, due to the substantial loss when cells attach to the vessel.	(14)

Table 2. Meta-analysis of the effects of various additives on semen parameters (outcome).

S. No	Compound	Conc.	Year	Effect on sperm parameter (outcome)	Ref.
1	Butylated hydroxytoluene	0.5 mM	2015	Motility, viability, antioxidant, DNA integrity	(15)
2	Caffiene	2 mM	2019	Motility, supplementation postthaw	(16)
3	Canthaxanthin	10 µM	2019	Motility, viability, antioxidant (red carotenoid), morphology	(17)
4	Catalase	200 u/mL	2012	Motility, viability, antioxidant, DNA integrity	(18)
5	Elamipretide	1 µM	2020	Motility, viability, antioxidant, DNA integrity	(19)
7	Melatonin	3 mM	2018	Motility, viability, antioxidant	(20)
8	Melatonin + caffiene	2 mM + 2 mM	2019	Motility, melatonin (prefreeze), caffiene (postthaw)	(21)
9	Mito-TEMPO	10 µM	2019	Motility, mitochondrial antioxidant	(22)
10	Myoinositol	2 mg/mL	2019	Motility, antioxidant (member of the vitamin B-complex group), morphology	(23)
11	Reduced glutathione	1 mM	2011	Motility, viability, antioxidant, DNA integrity	(24)
12	Sericin	1% w/v	2020	Motility, viability, antioxidant	(25)
13	Trolox	40 µM	2012	Motility, viability, antioxidant	(26)
14	Trolox + EDTA	200 µM/1.1 mM	2016	Motility, antioxidant	(27)
15	Vitamin C	600 mM	2018	Motility, ROS scavenger/ antioxidant, morphology	(28)
16	Vitamin D	20 µmol	2019	Motility, viability, antioxidant, DNA integrity	(29)
17	Vitamin E (N)	5 mM	2011	Motility, viability, antioxidant, DNA integrity	(30)
18	Vitamin E	5 mM	2011	Motility, antioxidant, DNA integrity	(30)
19	Quercetin	50 µM	2012	Motility, antioxidant, DNA integrity	(31)
20	*Ceratonia siliqua*	20 µg/mL	2019	Motility, antioxidant (strong), morphology	(32)
21	*Lycium barbarum* polysaccharide	1±000 µg/mL	2020	Motility, antioxidant (plant extract)	(33)
22	AFPIII	1 µg/ml	2019	Motility, thermal hysteresis antifreeze protiens	(34)
23	L-phosphatidyl-choline	3% w/v	2016	Motility, soybean extract (non-permeable preservant), morphology	(35)
24	Nitric oxide	0.01 µM	2019	Motility, sublethal nitrosative stress	(5)
25	Trehalose	50 mM	2020	Motility, viability, glycosidic linkage, morphology	(36)

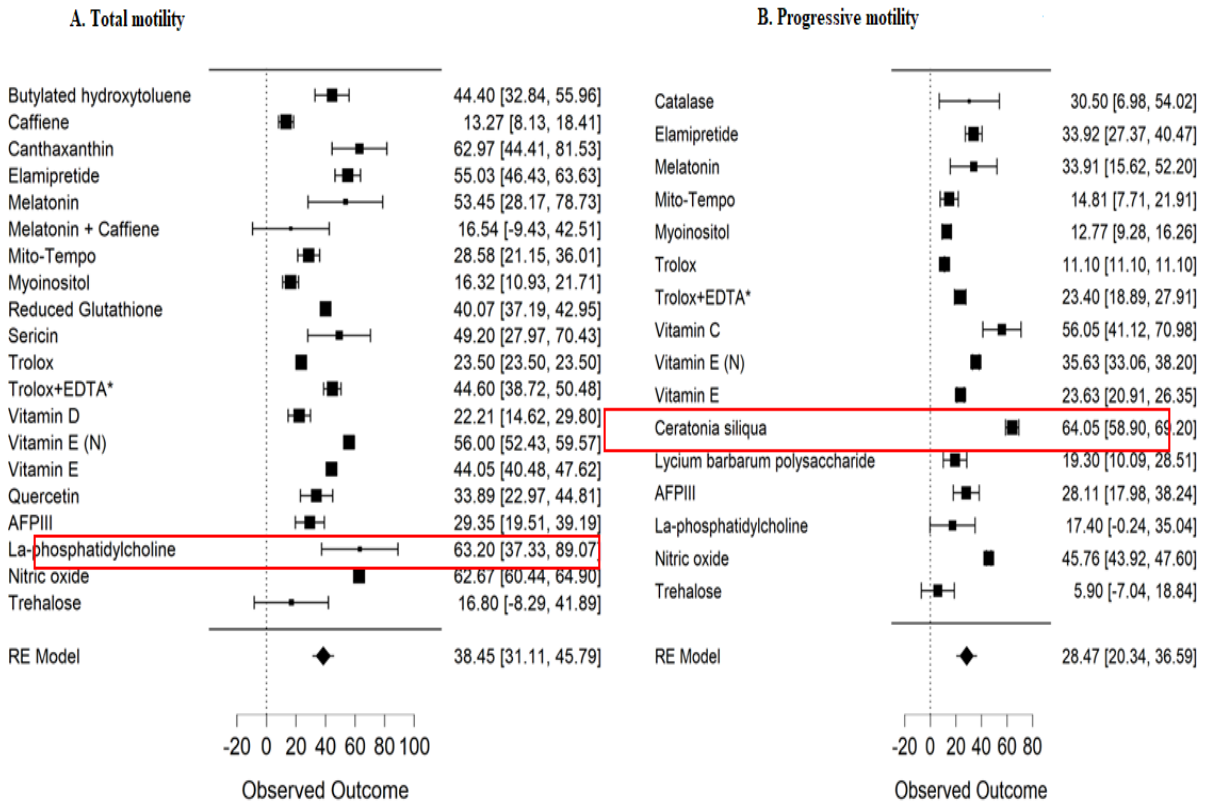

Figure 2. Results of the analysis for post-thawing sperm properties for the additives listed: (A) total motility; (B) progressive motility.

Effect on total and progressive motility

Good sperm motility is essential for active swimming along specific portions of the female tube and penetration of physical obstacles such as the uterotubal junction and ovary coverings. The proportion of motile sperm significantly determines fertility rates (37). From the meta-analytic results, phosphatidylcholine and *Ceratonia* as additives play significant effects on total and progressive motility, respectively as explained in Figure 2.

Effect on morphology

Sperm motility and morphology are inextricably related because morphologically defective sperm move slower or less effectively and are selectively excluded at various levels. Thus, the proportion of morphologically normal sperm is an excellent predictor of conception rates in humans, both in vivo and in vitro (38). The findings in Figure 3 show that trehalose has the most favorable impact, which is possibly related to glycosidic connections. The maximum activity is detected in the extract of *Ceratonia siliqua*, followed by canthaxanthin and myoinositol, which have the next closest activity.

Effect on viability

Male factor infertility has been linked to both increased DNA fragmentation and decreased viability. These two disorders are connected because DNA fragmentation is one of the penultimate steps preceding spermatozoa death, and DNA breaks are one of the significant causes of sperm apoptosis. The research has shown a definite relationship between DNA fragmentation and sperm viability, and males with high levels of DNA fragmentation are also more likely to have necrospermia. The gathered data revealed that vitamin C had the most excellent favorable effect on sperm viability, followed by elamipretide and *Ceratonia siliqua* extract. Figure 4 compares all the data retrieved to determine its influence on viability.

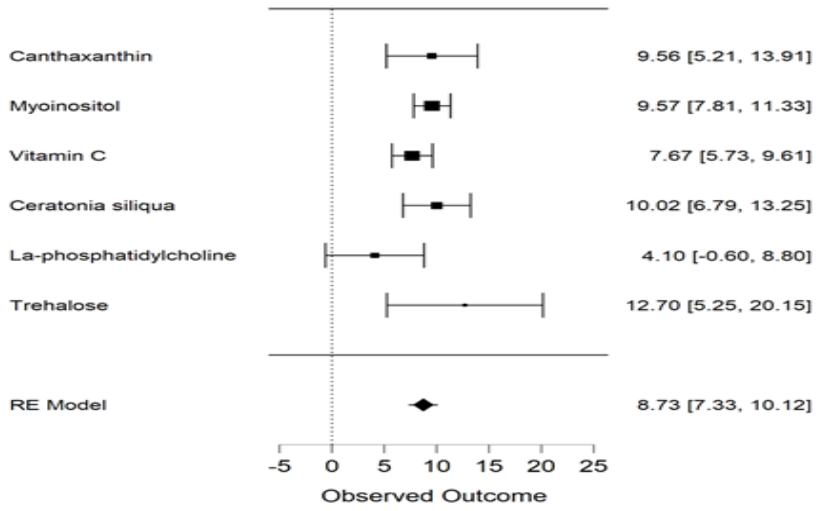

Figure 3. Sperm morphology sustainability and its comparison under different selected additives.

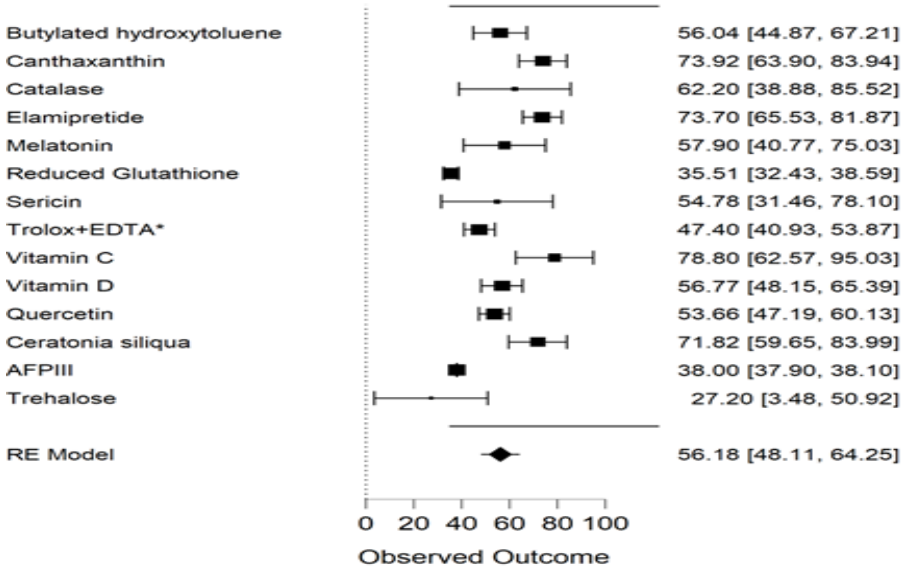

Figure 4. Sperm viability and its comparison under different selected additives.

Effect on DFI and ROS levels

Due to technical limitations, the DNA fragmentation index has historically been a less researched metric in sperm quality. However, as technology has advanced, detecting DNA damage has become more accessible. A standard metric has yet to be developed for a more straightforward comparability of data, although the top influences on DNA integrity is included in Table 3. Vitamin C is an especially remarkable substance.

Table 3. Compounds that reduced ROS levels and lower DNA fragmentation index (DFI).

Compound	Conc.	MDA		DFI	
		Sample	Control	Mean (Sample)	Mean (Control)
Butylated hydroxytoluene	0.5 mM	0.32 ± 0.04	0.35 ± 0.03	25.30 ± 7.6	49.00 ± 7.6
Elamipretide	1 µM	1.70 ± 0.22	2.31 ± 0.36	14.08 ± 2.60	19.92 ± 2.37
Vitamin D	20 µmol	6.16 ± 2.01	10.336 ± 1.2	46.77 ± 11.79	53.72 ± 7.25

DISCUSSION

Potential alternative/additives – antioxidants, cryotolerance, and antifreeze proteins

Despite the danger of cryodamage during the freezing-thawing operation, we routinely use sperm cryopreservation in male infertility situations to retain viability and concentrate motile sperm for assisted reproductive technologies (ART). Sperm cryodamage mechanisms appear complicated, with oxidative stress produced by increased generation of intracellular ROS and loss of the antioxidant enzyme system being the most critical factor in most studies. Researchers observed that adding a variety of non-enzyme antioxidants (such as vitamin E, vitamin C, cysteine, glutathione, melatonin, resveratrol, L-carnitine, and others) to cryopreservation solutions improved performance. Consequently, many organizations have placed a high value on adding antioxidants to extenders due to their capacity to battle excessive ROS formation, typically the primary or sole cause of many cell issues. According to one study, new types of mitochondria-targeted antioxidants have sparked researchers' attention due to their widespread usage, high efficiency, and low toxicity, making them perfect candidates for the protection of sperm against stress damage caused by cryopreservation (24).

Cryotolerance improvement.

As an alternative to the primary technique for shielding human sperm cells against ROS generation, the addition of antioxidants to the present cryoprotectants has been proposed. However, it has been established that this approach is not sufficient to fully ameliorate the damage caused by the freezing-melting of human sperm cells. While spermatozoa are selectively permeable and ROS can be scavenged with low levels of antioxidants, the effectiveness of antioxidants used during the critical preservation phase has been shown to diminish. Although exogenous antioxidants play a crucial role in enhancing sperm cell antioxidant capacity, the majority of these antioxidants do not penetrate sperm cells, limiting their ability to adequately remove free radicals produced within the cells.

Before freezing, animal spermatozoa show better cryotolerance after being exposed to sublethal stressors such as HHP (23), oxidative agents (11), and nitric oxide (in human cells). As a result, research groups are focused on innovative approaches to improve overall cryotolerance and address the drawbacks of traditional additives. They have greatly enhanced sperm quality. However, to increase quality, therapies such as stress pre-exposure and cryo-techniques (microdroplets, vitrification) may avoid freeze-thaw damage to sperm in those with asthenospermia, which accounts for the vast majority of male infertility cases.

Antifreeze proteins.

Zandiyeh et al. (34) researched AFP-III, which deserves special attention for its unique technique for treating cryoinjuries. Sperm cryopreservation causes many stresses that impair sperm quality. Stressors include thermal shock, osmotic damage, and the development of ice crystals. Few researchers have evaluated the use of AFPs in cryopreservation; however, (34) looked into the effects of antifreeze protein III (AFP III), specifically on human spermatozoa cryopreservation. The study tested how different AFPIII concentrations (0, 0.01, 0.1, 1, 5, 10 μg/mL) influenced metrics such as motility and viability. The study found that adding AFPIII to GEYC at 1 μg/mL enhanced motility, PMI, viability, and TAC while decreasing ROS and DNA fragmentation in cryopreserved human semen compared to the control group. AFP's method is to lower the temperature at which ice crystal formation occurs in a non-colligative way, resulting in thermal hysteresis.

CONCLUSION

In conclusion, we identified a number of notable additives, such as the *Ceratonia siliqua* extract (benefits: motility, antioxidant-strong, morphology), elamipretide (benefits: motility, viability, antioxidant, DNA integrity), and vitamin C (benefits: motility, ROS scavenger/antioxidant, morphology), have shown promising effects on various sperm parameters. Animal extracts, such as AFP-III, are also showing promise as sperm cryopreservation additions and have an advantage over other additives due to their bioavailability and compatibility.

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