

## PERSPECTIVE

# ENCAPSULATION NANOTECHNOLOGY IN SPERM CRYOPRESERVATION: SYSTEMS PREPARATION METHODS AND ANTIOXIDANTS ENHANCED DELIVERY

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### Abstract

Sperm cryopreservation promotes the storage and transport of germplasm for its use in artificial insemination (AI) and other advanced reproductive technologies. However, sperm cryopreservation causes several stresses including thermal shock, osmotic damage, and ice crystal formation, thereby reducing sperm quality. Supplementing cryoprotectant media with antioxidants has been reported to be positive in different species. It has been widely suggested to combine antioxidants with nanotechnology, to maximize therapeutic activity and to minimize undesirable side effects. In this review, we discuss the role of different antioxidants in sperm cryopreservation and their improved therapeutic effect through their formulation using nanotechnology. In addition, we report the different nano-systems preparation methods present in literature. Whilst the use of nanotechnology in animal production is still in its infancy, encouraging results from nutrition, biocidal, remedial, and reproductive studies are driving further investigations.

**Keywords:** antioxidants; nano-systems; preparation methods; sperm cryopreservation.

### INTRODUCTION

Sperm cryopreservation has an essential impact on the long-term preservation of genetically superior males of the species (1). In humans, sperm cryopreservation becomes an important issue in preserving male fertility particularly before medical treatment like radiotherapy or chemotherapy, which may lead

to ejaculatory dysfunction or testicular failure (2, 3, 4). In addition cryopreservation is also very relevant for the reproductive management of domesticated animals (5, 6), as it allows the breeding of animals of immense commercial value by collecting spermatozoa after castration or necropsy, and subsequent use in breeding programs (7, 8). Moreover, cryopreservation facilitates the distribution of semen over

distance. Which has contributed considerably to the development of reproductive technologies such as artificial insemination and in vitro fertilization worldwide (9).

Cryopreservation aims to keep sperm viability and functionality by a sequential process of reduction in temperature (10), dehydration of the cell, freezing, storage and then thawing (11). Throughout the freezing-thawing process, spermatozoa are subjected to several negative effects, including a drop in temperature, ice crystal formation, and various types of stresses (physical, chemical, osmotic, and oxidative). These effects can critically compromise sperm quality and fertility (1, 12, 13, 14). Thereby, the post-thawing quality of the spermatozoa is significantly reduced, with motility loss that can be in the region of 40%-60% (15).

Cold shock is associated with the lipid composition of the membrane bilayer (14). The decrease in temperature during cooling produces a phospholipid transition that affects the membrane rigidity and fragility, leading to lipid phase separation (16, 17). In addition, oxidative stress results from the formation of large amounts of reactive oxygen species (ROS) or molecules that contain free radicals (RL) (18). As a result there is the loss of the pro-oxidant/anti-oxidant equilibrium (19).

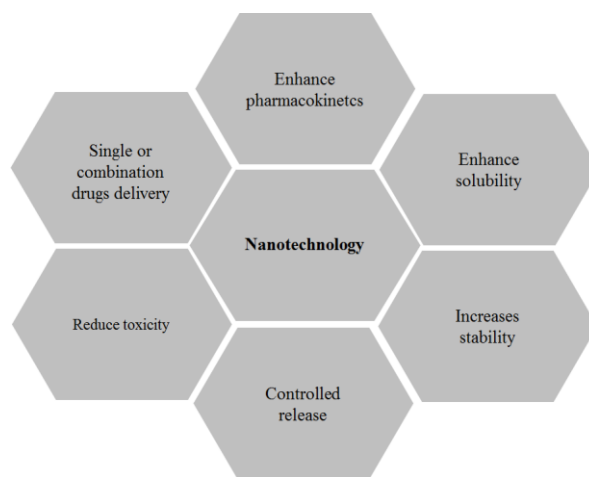
Different strategies are used to ensure that free radicals are scavenged and post-thaw sperm function is improved; in particular, the addition of antioxidants and cryoprotective agents (20-23). Antioxidants are the main defence factors against oxidative stress (24). There are two types of antioxidants, based on their chemical structure: enzymatic antioxidants, such as glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase (25, 26); and non-enzymatic antioxidants (27), including reduced glutathione (GSH), urate, ascorbic acid, carotenoids ( $\beta$ -carotene) and vitamin E ( $\alpha$ -tocopherol) (28).

However, antioxidants present some limitations, like the low durability to harsh conditions and poor solubility and stability in aqueous media (29). Encapsulation nanotechnology can be applied to protect antioxidants from degradation by direct contact of external factors such as light, oxygen, chemicals, heat, and pressure. This technology can also increase antioxidant solubility and stability giving a better bioavailability in biological fluids (30).

In this review, we discuss the role of different antioxidants in sperm cryopreservation and their improved therapeutic effect through their formulation using nanotechnology. In addition, we report the different nano-systems preparation methods currently reported in the literature.

## NANOTECHNOLOGY FOR ANTIOXIDANTS DELIVERY IN SPERM CRYOPRESERVATION

Encapsulation nanotechnology advances have contributed to the design of novel nano-antioxidant compounds that possess protective properties in sperm cryopreservation. The nanotechnology is able to transport the drug to a specific destination (nanocarriers) to perform its therapeutic activity with maximum safety, improve delivery of poorly water-soluble protectants, and co-delivery of two or more drugs for combination therapy (31, 32) (Figure 1). The following section presents the different examples of nano-systems that have been reported in the literature in relation to sperm cryopreservation.



**Figure 1.** Nanotechnology advantages in drug delivery.

### *Liposome*

The liposomes are artificial vesicles of spherical shape, consisting of two or more layers of lipids with an internal aqueous cavity (33). Liposomes are composed of phospholipid for biocompatibility and cholesterol for stability, they enable entrapment of water-soluble drugs

that would otherwise not pass through the bilayer membrane easily, and are able to load lipophilic drugs in the lipid layers to make them dispersible in aqueous media (34, 35). There are several advantages in applying liposomes including: (33, 36)

- Ease of synthesis;
- Pharmacokinetics and pharmacodynamics are easily manoeuvrable;
- Applicable to drugs with different properties;
- High biocompatibility and similarity with the biological membrane;
- Enhanced therapeutic index;
- Biodegradable;
- Producing on a large scale.

Today, liposomes are used in several areas such as vaccine delivery, cosmetic formulations, nanomedicines, and sperm cryopreservation (37). Recent studies have stimulated interest in using liposomal formulations as preservation diluents, reducing the risk of egg yolk contaminant, and escalated the values of semen quality through better protection of sperm from damage (38, 39).

### ***Cyclodextrins***

Cyclodextrins (CDs) represent a family of non-toxic cyclic oligosaccharides, obtained by enzymatic degradation of starch. They have six, seven, or eight glucose units connected by a -1-4 bonds, named respectively  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CD. Various modified CDs functional groups could be joined to native CDs, leading to a modification in their solubility and/or stability (40, 41). They have a shape like to a cone; with a hydrophilic outside surface, which makes CDs water-soluble. A non-polar internal cavity is formed, enabling the formation of inclusion complexes of lipophilic guest molecules (42). Due to their capacity to change the physicochemical properties of drugs and other compounds, CDs are generally indicated as enabling pharmaceutical excipients. One or more drug molecules can form a complex with one CD molecule and one or more CD molecules can form a complex with one drug molecule. Usually, one drug molecule creates a complex with one CD molecule (43). It was established that treating diverse species sperm with cyclodextrins pre-loaded with an appropriate drug molecule (antioxidants, essential oil) before cryopreservation, can improve sperm quality after freezing-thawing process (44, 45).

### ***Polyethylene glycol***

Poly (ethylene glycol) is a neutral non-toxic polymer with the  $\text{HOH}_2\text{C}(\text{CH}_2\text{OCH}_2)_n\text{CH}_2\text{OH}$  structure and different molecular weights. Polyethylene glycol (PEG), is presently one of the most used polymeric materials in drug delivery (46). Further, PEG can modify the pharmacokinetics and usually toxicity of bioactive molecules (47). Moreover, PEG may improve the lifetime of the “drug-carrier” assembly, thus enabling the administration of lower concentrations of the “drug-carrier” composite and consequently reducing toxicity (48). PEG covers a large-scale of beneficial properties that include high solubility in aqueous media as well as organic solvents, making it simple for end-group modification (49). It is also widely employed for modification of carriers used in therapeutics (50). The use of PEGs in sperm cryopreservation has a positive effect. Amokrane et al. (2020) demonstrated that treating rabbit semen with vitamin E dispersed in PEG 6000 (PEG/Vit E) is effective in protecting sperm cells during chilling at 4°C (51).

## **METHODS OF PREPARATION**

### ***Solvent injection***

It is a common method regarding the displacement of solvents. The principle of this method is dissolving the lipid in a water-miscible solvent such as ethanol, acetone, or isopropanol and injected the obtained solution into the aqueous phase using an operating syringe. The solvent migrates rapidly in the water and lipid particles precipitate in the aqueous phase (52). The solvent injection technique has a lot of advantages, such as the ability of using pharmacologically acceptable organic solvents (Table 1) (52).

### ***Solvent emulsification evaporation technique***

The double emulsion solvent evaporation method can be described as the dispersed phase including one or more types of a smaller dispersed phase in it. This technique involves a double emulsion of water-in-oil-in-water emulsion. A water-in-oil emulsion (w/o) is first provided by adding a drug containing aqueous solution into a polymer and lipid-containing an organic solvent. Then, the emulsion is joined into another aqueous phase to form a water-in-oil-in-water emulsion (w/o/w). The organic

solvent is immediately removed by evaporation (53, 54).

**High-pressure homogenization technique**

This technique is used on a large scale. It includes two approaches: cold homogenization method; or hot homogenization technique. By the application of high pressure through very high shear, stress on the lipid is applied and it is forced through a specifically designed homogenization valve to form suspended particles with a uniform size distribution. It is important to know that both elevated temperature or below room temperature may be used depending on the nature of the drug and excipients (55, 56).

**Microemulsions**

The microemulsion technique is defined as creating clear, thermodynamically stable, isotropic liquid mixtures between a nonpolar liquid (oil) and a polar liquid (aqueous) and surfactant, generally in combination with a cosurfactant. Ultrafine emulsion droplets are formed as a result of microemulsion breakage, which immediately crystallizes; further, the excess water is removed by lyophilization (57).

**Melting dispersion technique**

The dispersion technique consists of a dispersed of one or more active components in an inert solid-state carrier or matrix prepared by melting (fusion), solvent or melting solvent

**Table 1.** Advantages and drawbacks of numerous nanocarrier system preparation methods.

Methods of Preparation	Advantage	Drawback(s)
Solvent injection	<ul style="list-style-type: none"> <li>• Robust, simplest and versatile method.</li> <li>• Method involving the displacement of solvents.</li> <li>• Use of pharmaceutically acceptable organic solvents. (94)</li> </ul>	<ul style="list-style-type: none"> <li>• Population is heterogeneous.</li> <li>• Use organic solvents and high temperature (95, 96).</li> </ul>
High-pressure homogenization	<ul style="list-style-type: none"> <li>• Narrow particle size distribution.</li> <li>• Use of water-based technology. (97).</li> </ul>	<ul style="list-style-type: none"> <li>• Energy-intensive process.</li> <li>• Biomolecule damage.</li> <li>• Low efficiency of the process. (98).</li> </ul>
Solvent emulsification/evaporation	<ul style="list-style-type: none"> <li>• Avoidance of heating.</li> <li>• Drug delivery controlled. (62)</li> </ul>	<ul style="list-style-type: none"> <li>• The complexity of controlling the evaporation rate. (99).</li> </ul>
Membrane contactor technique.	<ul style="list-style-type: none"> <li>• The facility of use.</li> <li>• Control of particle size. (63).</li> </ul>	<ul style="list-style-type: none"> <li>• The presence of membrane barrier between the two phases introduces an extra resistance to the overall mass transfer process.</li> <li>• Membranes clogging (100)</li> </ul>
Micro-emulsion	<ul style="list-style-type: none"> <li>• Spontaneous formation.</li> <li>• Thermodynamic stability.</li> <li>• Improvement of drug solubilization and bioavailability. (99).</li> </ul>	<ul style="list-style-type: none"> <li>• High water content, difficulties in the excess water removal.</li> <li>• The uses of a high concentration of surfactants and co-surfactants. (101).</li> </ul>
Ultra-sonication	<ul style="list-style-type: none"> <li>• Reduced shear stress (102).</li> </ul>	<ul style="list-style-type: none"> <li>• Physical instability like particle growth upon storage. (103).</li> </ul>
Melting dispersion technique	<ul style="list-style-type: none"> <li>• A low temperature is required for the evaporation of organic solvents.</li> <li>• Increased efficiency of the drug. (104)</li> </ul>	<ul style="list-style-type: none"> <li>• Limited to drugs with a low therapeutic dose.</li> <li>• The number of polymers that can be used by this method is limited. (105).</li> </ul>

**Table 2.** Utilization of nanotechnology in the delivery of antioxidants for the protection of sperm during cryopreservation.

Active molecule	Nano-technology type	Function during cryopreservation	Sperm species	Ref
Ellagic acid	Liposomes	Improved total motility, membrane functionality and viability.	Rooster	(93)
Soy lecithin and cholesterol	Liposomes	Replace the chicken egg yolk from human semen cryopreservation media without compromising post-thaw outcome.	Human	(80)
Lycopene	Liposomes	Improve the quality spermatozoa after freeze-thawing	Rooster	(106)
Vitamin E	Liposomes	Improve sperm motility	Bovine	(72)
Cholesterol	Cyclodextrin	Stabilizing sperm plasma membrane. Improve parameters progressive motility, rapid sperm percentage and acrosomal membrane integrity. Decreasing levels of superoxide anion formation.	Ram	(107)
Cholesterol and vitamin E	Cyclodextrin	Effective on post thawing sperm motility.	Dog	(108)
Vitamin E	Cyclodextrin	Effect on sperm motility and gamete integrity.	Ram	(109)
Cholesterol and vitamin E	Cyclodextrin	A powerful protection in cryopreserved semen to fight simultaneously against cold shock and oxidative stress.	Bovine	(110)
Cholesterol	Cyclodextrin	Membrane protects it against H <sub>2</sub> O <sub>2</sub> or cryo-induced oxidative damage.	Ram	(77)
Cholesterol and vitamin E	Cyclodextrin	Protecting concomitantly against cold shock and oxidativestress.	Ram	(111)
Cholesterol and vitamin E	Cyclodextrin	Improved significantly the post-thaw kinematic parameters.	Rabbit	(73)
Cholesterol	Cyclodextrin	Improve sperm cryosurvival	Goat	(112)
Cholesterol	Cyclodextrin	Protect against cryodamage. Reducing the ryocapacitation	Bull	(113)
Cholesterol	Cyclodextrin	Improved sperm cryopreservation, low freezability.	Ram	(114)
Cholesterol	Cyclodextrin	Improve quality of frozen-thawed ram sperm.	Ram	(115)
Cholesterol	Cyclodextrin	Increasing cryopservability of stallion spermatozoa.	Marwari Stallion*	(9)
Cholesterol	Cyclodextrin	Improves sperm resistance to seminal plasma-mediated injury and protects sperm quality.	Goat	(116)
Cholesterol	Cyclodextrin	Improved sperm viability, motility, and acrosome integrity.	Goat	(117)
Vitamin E	Poly Ethylene Glycol	protecting sperm cells during chilling at 4°C	Rabbit	(51)

\**Equus ferus caballus*

method. The melting method includes the formation of a physical mixture of a drug and a water-soluble carrier and heating it immediately

until it is melted. The melted mixture is then solidified rapidly in an ice-bath under energetic

stirring. Finally, solid mass is crushed, pulverized and sieved (58).

#### ***Ultrasonication technique***

This technique was initially used to prepare solid lipid nanodispersions. These methods do not use organic solvents, a large amount of surfactants or any additives. The method involves heating a solid lipid at a temperature of 5 to 10°C above its melting point and dispersing into previously heated aqueous surfactant solution under high-shear homogenization conditions, followed by ultrasonication using a sonicator; the resulting emulsion is cooled to room temperature (59).

#### ***Supercritical fluid technique***

The supercritical fluid method is mostly applied to carbon dioxide. CO<sub>2</sub> is considered as environmentally friendly and has been identified as a prime candidate to develop alternative clean processes for the preparation of drug-loaded polymeric matrixes. Matrix and drug are dissolved and sprayed with lower pressure and then the particles are formed. The adiabatic expansion of the composite results in its rapid cooling (60).

#### ***Microwave-assisted technology***

Microwave-assisted technology offers uniform heating and yields low polydispersity particles with a small size. This process consumes little energy and is based on a microemulsion-technique. After completion of the microemulsion synthesis, the hot (o/w) microemulsion is dispersed rapidly into cold water and under magnetic stirring (61).

#### ***Solvent evaporation technique***

The solvent evaporation method was the first one developed for the preparation of nanoparticles. This method consists of two steps. First, dissolving, dispersing, or emulsifying the drug into an organic solvent, which is then emulsified into an aqueous or oil phase. Second, the solvent is evaporated by increased temperature and reduced pressure with continuous stirring to form microspheres. The solvent method offers the advantage of prevention from thermal decomposition of drugs or carriers because of the low temperature required for the evaporation of organic solvents (62).

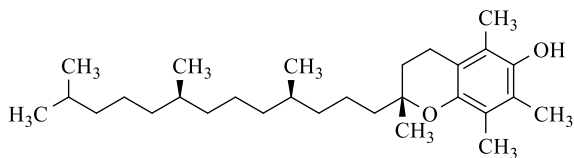
#### ***Membrane contractor technique***

This method is based on melt dispersion, using a low energy process. The membrane contractor allows the lipid phase to be introduced through the membrane pores at the melting temperature of the lipid, into another phase (aqueous phase) which flows tangentially to the membrane surface and sweeps away the droplets forming at the pore outlets. During cooling at room temperature forms the particles form (63).

### **UTILIZATION OF NANOTECHNOLOGY IN THE DELIVERY OF ANTIOXIDANTS FOR PROTECTION DURING SPERM CRYOPRESERVATION**

#### ***Vitamin E***

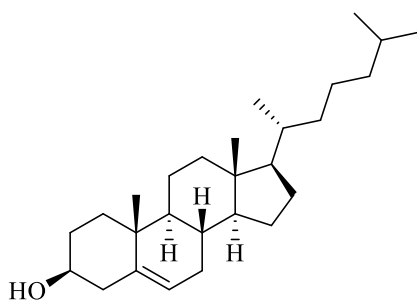
Vitamin E (Figure 2) has been identified as a potent fat-soluble (non-enzymatic) antioxidant through extensive research. Alpha-tocopherol is the biologically most active form of vitamin E. Vitamin E, is a chain-breaking antioxidant with the capability of inhibiting oxidative degradation (64, 65, 66). This vitamin was approved as an important dietary supplement for growth, reproduction, immune function, disease prevention and tissue integrity (67). Several studies have reviewed the importance of an adequate vitamin E status for protection from cryopreservation damages (65). It was also established that the vitamin E levels in seminal plasma have a direct relationship with the percentage of motile spermatozoa in semen. Moreover, in the semen of infertile men, low levels of vitamin E are observed (68, 69). In a study on the vitamin E impact on semen characteristics, it was observed that tocopherol promoted progressive motility and viability of spermatozoa both before and after cryopreservation (70). However, there are a number of challenges associated with incorporating vitamin E into the cryoprotectant medium due to its chemical instability, poor water solubility, and variable bioavailability (71). Alternatively, it needs to be incorporated or encapsulated into a suitable delivery system. An important antioxidant effect resulting in better semen cryopreservation has been observed when vitamin E was preloaded in liposomes, or cyclodextrins, or polyethylene glycol (51, 72, 73).



**Figure 2.** Alpha-tocopherol.

### **Cholesterol**

Cholesterol (Figure 3) is a lipid-type molecule and it is one of the most important structural components of cell membranes (74). Cholesterol has a great influence on cell membrane fluidity and permeability by interacting with both the hydrophilic head groups and the hydrophobic tails of phospholipids (75, 76). These molecules have several applications, such as in drug delivery, bioimaging, as liquid crystals, as gelators, as anticancer agents and in antioxidant agents (77, 78). Cholesterol plays an important role in sperm membrane protection and sperm capacitation. It was hypothesized that increasing sperm cholesterol content, increases the lifespan of cryopreserved sperm cells (79). Cholesterol-loaded cyclodextrin and liposomes technology have been widely studied in human and various animal species, to reduce sperm capacitation, increase preservation of sperm membranes and increase sperm motility after cryopreservation (77, 80).

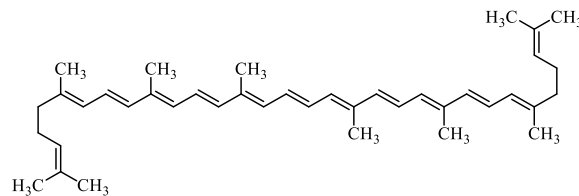


**Figure 3.** Cholesterol.

### **Lycopene**

Lycopene (Figure 4) is a natural pigment, from the carotenoid family (81). Chemical and in vitro cell studies have revealed that this hydrophobic antioxidant molecule (82) can limit diseases caused by oxidative stress, such as specific cancers, cardiovascular diseases (83, 84) and infertility (85). Several studies have reported that the addition of lycopene to semen extenders positively affects ram semen cryopreservation

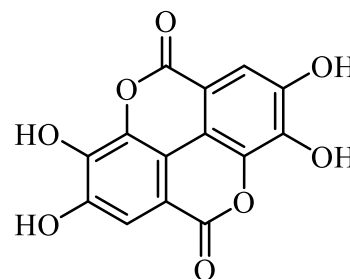
(86). It also raises the survivability of turkey spermatozoa, by reducing lipid peroxidation and partially rescuing the structural damage incurred to the spermatozoa during refrigeration and cryopreservation (87). Najafi et al (2018) revealed that lycopene could improve the quality of rooster spermatozoa after freeze-thawing, and lycopene entrapment with nanoliposomes seems to be promising (106).



**Figure 4.** Lycopene.

### **Ellagic acid (EA)**

Ellagic acid (Figure 5), is a polyphenolic antioxidant of poor water solubility (88). It possesses multiple pharmacological properties such as anti-inflammatory (89), anti-mutagenic, anti-clastogenic, anticarcinogenic (90) and neuroprotective effects (91). Recently, the antioxidant effect of EA was assessed during the semen ram preservation. The results showed that EA supplementation has a potential effect on sperm quality and protection from oxidative stress during the cryopreservation (92). Najafi et al (2019) studied the antioxidant effect of EA loaded in liposomes during post-thawing of rooster spermatozoa. It was found that the association of EA with the liposome significantly improves sperm parameters and increases the sperm protective effect of EA (93).



**Figure 5.** Ellagic acid.

## FUTURE PERSPECTIVES AND CONCLUSION

Antioxidant supplementation in semen extender improves post-thaw semen quality and fertility. Nanotechnology might convey advantages by protecting and delivering the antioxidants to the cells and enhancing these effects. In this review, several antioxidants with different therapeutic benefits in sperm cryopreservation have been cited and discussed. Moreover, several studies focusing on the application of nanotechnology in the delivery of these antioxidants have already shown promise in enhancing their therapeutic benefits in sperm cryopreservation. Several strategies can be developed to create multifunctional nanoencapsulation systems with active targeting incorporation to enhance uptake in specific cells, or surface charge modification and attachment of specific ligands for target tissue. Besides, the understanding of mechanisms by which these nanoencapsulation systems are taken up by the spermatozoa cells and their intracellular fate is of primary importance to optimize their intended functions. This approach can be successfully used to treat male infertility, especially in farm animals, and enhance testicular functions. In addition, more information is required regarding the fate, toxicity, kinetics, and physical-chemical properties of different types of nanoencapsulation systems in biological systems and environments. This may require multidisciplinary cooperation to assess the release, transportation, transformation, accumulation, toxicity, and uptake of engineered nanoencapsulation systems from the biological systems into the environment.

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