Review

Compounds with Antioxidant Activity as a Supplement of Media Used for Human Semen Cryopreservation: A Narrative Review

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Sperm cryopreservation is an important technique in preserving male fertility. Several methods for semen and sperm cryopreservation are available; however, the quality of thawed spermatozoa is poor and this is due to different mechanisms during the freezing-thawing process, including temperature changes, crystal ice formation, osmotic stress and oxidative stress (OS). OS is the result of an overproduction of reactive oxygen species (ROS) that, if present in high concentration, can damage the cellular structures and impair sperm function. Modulation of OS is an important issue in human sperm freezing. A large group of antioxidant molecules is used in cryopreservation processes as a pharmacological strategy to counteract the oxidizing effects of preservation procedures and thus protect sperm quality. The main body of the review comprises the analysis of different studies, starting from 2000 up to the present, dealing with the effects of different antioxidant compounds, including natural extract, used as supplement of cryopreservation media. Many studies have reported several beneficial effects of antioxidants that are added during freezing-thawing protocols on sperm cryo-damages; however, these improvements are not always evident. Over the past decade, the attention has been mainly focused on the phytoextracts and natural extracts. Phytoextracts can be obtained by waste products, which are a rich source of compounds with strong antioxidant activity. Because these by-products can be used in the industrial, cosmetic, nutraceutical, and human and animal reproductive fields, this topic of research is worthwhile implementing. The freezing and thawing protocols still have many pitfalls and the quality of thawed spermatozoa is not satisfactory. For this reason, new strategies to minimize cyodamages and to increase sperm cryostability are advisable to guarantee better sperm function and survival, permitting successful future clinical application.

Keywords: antioxidants; phytoextracts; oxidative stress; sperm cryopreservation

Introduction

Infertility is a global health problem. It is defined as the inability to conceive after 12 months or more of regular, unprotected sexual intercourse [1]. It is estimated that 8%–12% of couples worldwide have fertility problems [2] and assisted reproduction technologies (ART), by which gametes and embryos are handled, represent the treatment of choice for these couples. In this scenario, the cryopreservation of spermatozoa is a common, important, and effective method for managing and preserving male fertility in humans and animals [3,4]. Sperm cryopreservation is recommended in many circumstances, for example for autologous use before treatment with chemotherapy or radiotherapy, for men undergoing vasectomy and in case of severe oligozoospermia. Finally, sperm from healthy donors can be cryopreserved for heterologous insemination programs and sperm banking is now a common worldwide procedure [5]. Since the first attempt in human sperm cryopreservation, many improvements have been made in terms of efficacy of the cryoprotectants used [6], and cooling and thawing protocols [5,7]. However, despite the use of modern freezing protocols, the quality of thawed spermatozoa is not still satisfactory [8]. One of the issues with freezing sperm is the ongoing lack of a cryopreservation medium that completely prevents sperm damage during freezing, so the ability of frozen sperm to fertilize is lower than that of fresh samples. Although spermatozoa are differentiated cells with a negligible amount of cytoplasm, they are highly susceptible to freezing/thawing procedures [5]. The main damages occurring during freezing-thawing protocols affect sperm motility, vitality, membrane integrity, DNA fragmentation, and mitochondrial activity [3].

During the freezing process, different factors such as sudden temperature changes, ice crystal formation, osmotic stress, and enhanced production of reactive oxygen species (ROS), which causes oxidative insults, have been suggested as reasons for post-thaw poor sperm quality [9,10]. It is
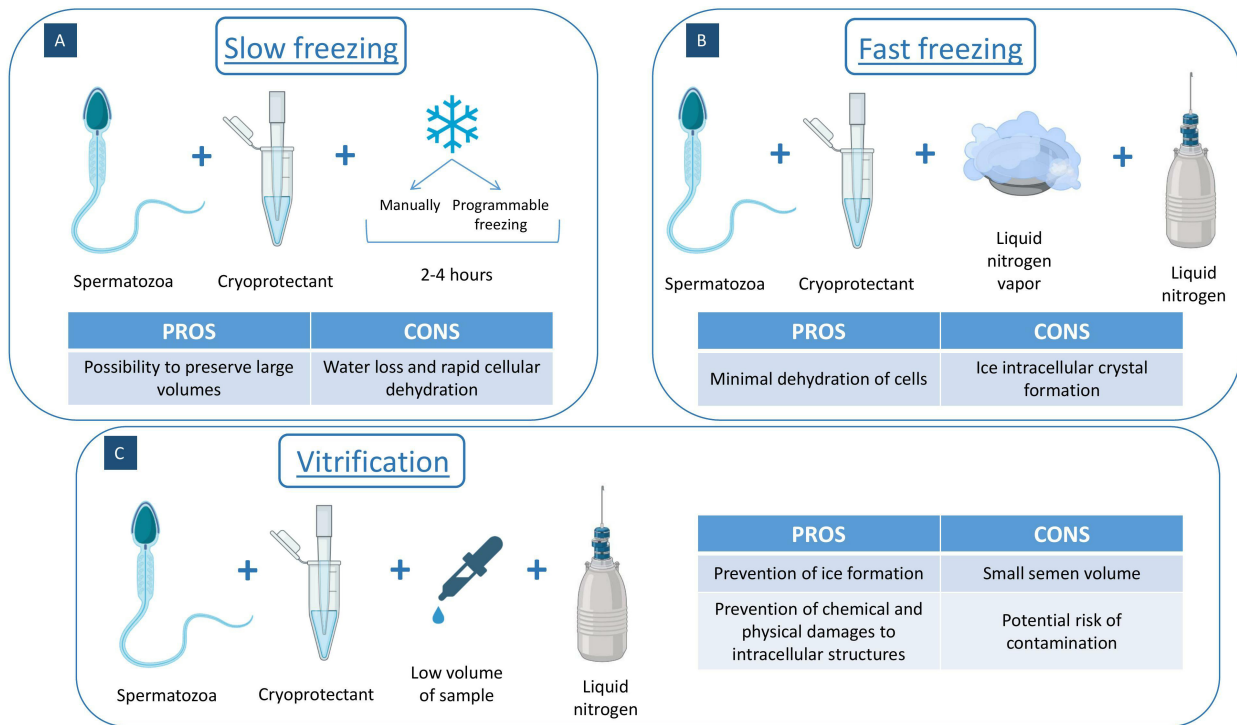
Fig. 1. Available methods for semen cryopreservation. In the figure, pros and cons of the three available methods for semen cryopreservation are shown. In (A) slow freezing method, in (B) fast freezing method and in (C) vitrification method are represented. The picture was assembled with PowerPoint (Microsoft Office Professional Plus version 2016, Microsoft Corporation, Redmond, WA, USA) and Adobe Photoshop (version 2021, Adobe Incorporated, San Jose, CA, USA).

recognized that ROS overproduction damages the cellular structures and impairs sperm function [11], including the membrane, particularly rich in polyunsaturated fatty acids (PUFA), and molecules such as DNA and proteins [12,13].

To minimize sperm cryoinjuries, different strategies have been proposed including the use of different cryoprotectants, as well as antifreeze proteins and glycoproteins, supplementation of media with antioxidants, application of mild stress before freezing that induces a reaction and adaptation of spermatozoa showing a better post-thawing quality [3,14]. This review focuses on the effects of antioxidants used as a supplement of media used during human semen cryopreservation to minimize oxidative stress (OS).

The Cryopreservation of Human Semen

ART represents a pool of techniques that help couples with fertility problems. Today, the requests for ART interventions are increasing and parallel improvements in cryopreservation techniques are required [15]. Cryopreservation is a process of freezing cells and tissues and storing them in liquid nitrogen at –196 °C: the cryopreservation of sperm, oocyte and embryo represents one of the most important and common procedures applied to the assisted reproduction field. The storage in liquid nitrogen at –196 °C guarantees a very low thermal energy and the cell metabolism ceases.

The first report of "semen cryopreservation" dates back to 1776 when Lazzaro Spallanzani observed that sperm frozen in the snow maintained motility. Scientists were fascinated by the behavior of spermatozoa at low temperature and, since then, many scientific advances have been made [16].

Several methods for semen and sperm cryopreservation are available [7]; the most used methods are manual and automated slow freezing, rapid freezing, and vitrification [17] (Fig. 1).

The differences among these techniques reside in the velocity of freezing and thawing, and in the concentration of cryoprotectant [18]. In any case, all these methods aim to obtain the highest possible percentage of sperm survival. Slow freezing and rapid freezing methods are the conventional cryopreservation methods. Specifically, in the slow freezing procedure (Fig. 1A) spermatozoa are cooled over a period of at least 2–4 hours manually or by a programmable device, whereas during the rapid freezing (Fig. 1B), the semen, treated with cryoprotectant, is first exposed to liquid nitrogen vapor phase and then plunged into liquid nitrogen [3]. Both the slow and rapid freezing can cause sperm damages.

The freezing temperature is crucial to prevent cryoinjuries: if the velocity is too slow, the cells lose water rapidly and dehydrate; if the velocity is too fast, water is not removed quickly enough and forms intracellular ice crystals,

which damage the cell [17]. For cell survival, neither of the two strategies is very efficient: too-low or too-high cooling speed can damage the cells. The vitrification (Fig. 1C) is a process to solidify liquid into an amorphous or glassy state, and may represent a faster alternative method of sperm cryopreservation with significant benefits concerning simple equipment and applicability to fertility clinics [19,20]. Vitrification techniques are also useful to store selected spermatozoa without seminal plasma and this appears to be one of the most effective ways to maintain sperm function [19]. This technique can prevent chemical or physical damages to the intracellular structures and reduces the injury to the plasma membrane [19]. However, also sperm vitrification has limitations: a small volume is plunged into liquid nitrogen to achieve high cooling rate; large volumes of semen cannot be cryopreserved and, in addition, the direct contact with liquid nitrogen increases the potential risk of contamination [20]. The most crucial aim in the freezing process is to find solutions to reduce the formation of ice crystals in the cytoplasm of the cells in order to minimize the damages [5]; however, both too-low or too-high cooling rates can damage cells. Therefore, finding an "optimal cooling temperature" should be one of the open research areas to achieve optimal results in cryopreservation techniques.

An additional important task regards the culture media used. Cryoprotectants are low-molecular-weight compounds used for minimizing the problems derived from freezing and thawing processes, in particular the damages due to ice crystal formation. Cryoprotectants decrease the freezing point of intracellular and extracellular water, interact with cytoplasmic components, and can form a protective layer around the cell membrane. They can be distinguished in permeable and non-permeable agents, according to their mechanism of action. Permeable agents show low molecular weight, and they easily cross the cell membrane, and replace water via simple diffusion or by facilitated diffusion via channels [17]. They can be toxic if used at high concentration, inducing a significant decrease in fertility potential [3,21]. Among penetrating agents, glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol can be mentioned. They generate an osmotic gradient to limit the formation of ice and stabilize the lipid bilayer. Non-permeable agents do not enter the cells and act on the extracellular environment generating an osmotic gradient that causes dehydration. Based on their molecular weight, this type of cryoprotectants can be grouped in low (monosaccharides, disaccharides, trisaccharides) and high molecular weight (polyvinyl pyrrolidone, polyethylene alcohol) cryoprotectants [17]. They contribute to water leakage from cytoplasm and protect membrane integrity of the cells [5]. Finally, although the thawing process represents a critical step, it has received less attention compared to the cooling process. Common theory holds that cells should be warmed rapidly to prevent recrystallization of ice [22]. In fact, it has been noted that the warming process after cryopreservation is a critical moment since the formation of ice crystals may occur at specific temperature ranges: between $-15\,°C$ and $-60\,°C$ or between $-10\,°C$ and $-20\,°C$. This problem could be controlled by increasing heating rates, thereby reducing possible lethal consequences [23]. The World Health Organization manual for semen evaluation suggests a fast thawing at $37\,°C$ [1].

ROS Production and Sperm Damages during Cryopreservation

Spermatozoa are potentially perfect candidates for cryobiology compared to other cells because they are small cells with a large surface area [3]; however, the harmful effects of cryopreservation on sperm function (Fig. 2) are well known. They affect sperm motility, vitality, DNA, and chromatin integrity, particularly in severely oligozoospermic patients where the sperm quality is already compromised in the basal semen before cryopreservation [4,24].
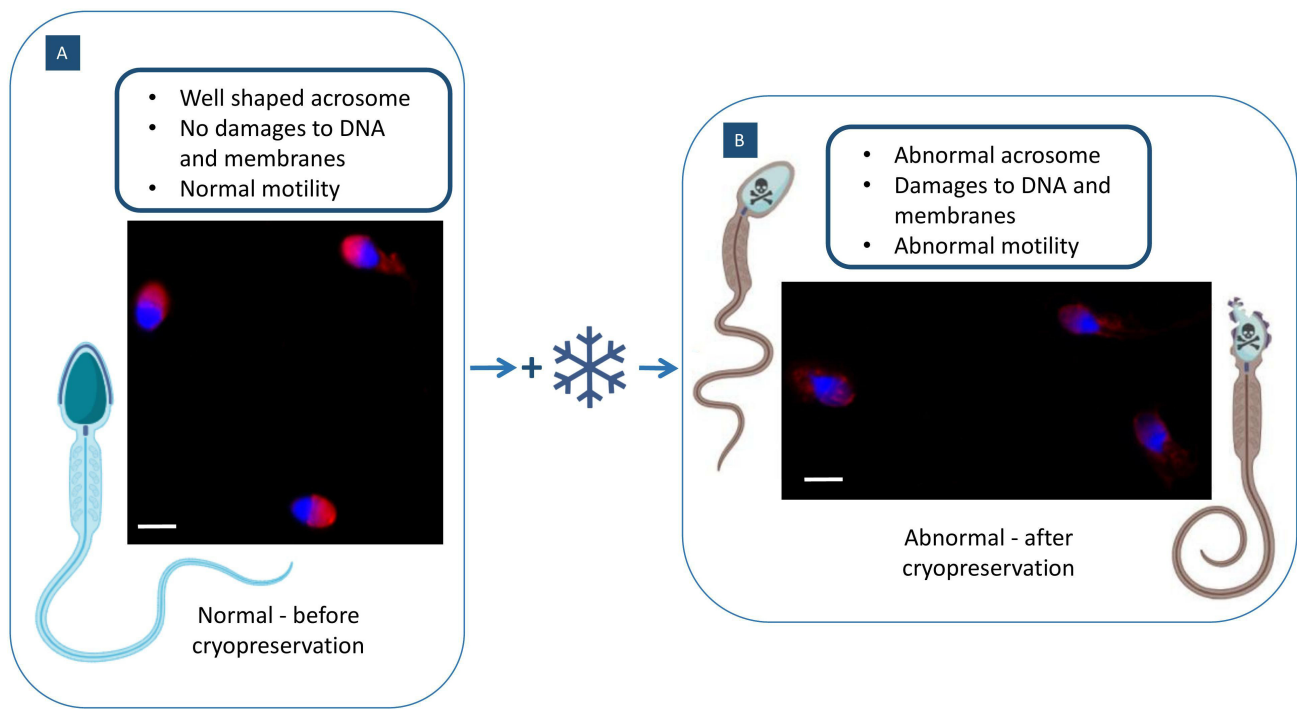
The ultraviolet (UV) micrographs (Fig. 2) represent spermatozoa before cryopreservation and spermatozoa after thawing, both treated with *Pisum sativum* agglutinin (PSA) tetramethylrhodamine isothiocyanate (TRITC)-conjugated, a lectin that label the acrosome.

The mechanisms behind cryodamage may be linked to the formation of intracellular ice crystals, cold shock, osmotic stress, and the over-production of ROS, which can cause OS. The term OS, 'oxidative stress', was applied to define a severe pro-oxidant/antioxidant imbalance, in favor of pro-oxidant species, potentially able to cause biological damage [25]. ROS are oxidants, partially reduced metabolites of molecular oxygen, generated by several different cellular processes and metabolic reactions [26,27]. ROS include superoxide anion ($O_2^{·-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), nitric oxide and peroxynitrite [28].

Below a certain threshold, ROS are relevant in signal transduction pathways and cellular physiological processes. In particular, in spermatozoa ROS are necessary for sperm motility, capacitation, acrosome reaction, and oocyte interaction [29,30]. On the contrary, ROS overproduction in spermatozoa and the consequent antioxidant imbalance can damage the cell structure [11], as during freezing-thawing procedures.

Sperm Membrane

Even though cryoprotective media and membrane-stabilizing agents can partially protect spermatozoa from cryodamages, the lipid composition of plasma membrane is one of the most critical factors that influences the sperm cryotolerance. Sperm membranes are extremely susceptible to ROS action due to the high content of PUFA [30]. In particular, the fatty acid profile and omega 3/omega 6 ratio in sperm from different species and in fertile/infertile subjects cause a different sperm tolerance to cryopreservation procedure [31]. PUFA peroxidation in the sperm membrane

Fig. 2. Harmful effects of cryopreservation on sperm structure and function. In panel (A), a normal spermatozoon and ultraviolet (UV) micrograph of spermatozoa before cryopreservation treated with *Pisum sativum* agglutinin (PSA) tetramethylrhodamine isothiocyanate (TRITC)-conjugated are represented. The normal acrosome is visible as a red cap in the sperm head. In panel (B), abnormal spermatozoa and UV micrograph of spermatozoa treated with PSA after cryopreservation treatment are represented: abnormal acrosomes are clearly visible. Sperm nuclei were stained with $4',6$-diamidino-2-phenylindole (DAPI). Bars: 6 μm. The picture was assembled with PowerPoint and Adobe Photoshop 2021.

leads to cellular dysfunction due to the loss of membrane fluidity and integrity, necessary for the successful sperm-oocyte fusion. The unconjugated double bonds of PUFA in the sperm membrane are attacked by ROS, producing aldehydes [32].

These highly reactive products damage sperm from many points of view: they react with proteins and DNA, they alter the proteins of the electron transport chain causing electron leakage, and they cause a decrease in mitochondrial membrane potential (MMP) and ATP production. These damages affect motility and increase mitochondrial ROS production in a self-perpetuating manner [4,33]. Integral membrane protein functions can also be damaged by lipid peroxidation (LPO) that alters plasma membrane microarchitecture [29].

Sperm Motility and Vitality

The most evident damage of the freezing-thawing process on spermatozoa regards motility and vitality (Fig. 2). These parameters are of paramount importance since they have a strong impact on fertilization outcome. The decrement of motile sperm percentage ranges from 30% to 50% and depends on the initial semen quality and on the cryopreservation protocol used [5]. It is known that the loss of sperm quality is more evident in patients with low basal sperm parameters before cryopreservation [34]. Obviously, motility and vitality are closely related, and it is easy to understand that one of the main reasons for decreased motility is the loss of vitality [35]. In addition, sperm motility decrement involves alterations of mitochondria that are damaged in their structure and function by cryopreservation. Ultrastructural studies by transmission electron microscopy (TEM) showed alterations of mitochondria, including swollen cristae and the presence of vacuoles [36] and by scanning electron microscopy revealed an increased percentage of sperm with bent and coiled tails, mostly due to osmotic changes during the process, respect to the basal samples [35]. To justify the decrease of sperm motility after thawing, a direct damage of flagellar and cytoskeletal proteins such as tubulin, the major component of microtubules, and others cannot be excluded [37–39]. Concerning sperm vitality, Shah *et al*. [40] were able to obtain a percentage of viable sperm around 70% simply modifying at 42 °C the temperature used to warm cells during thawing procedure.

Sperm Acrosome

Acrosome is a Golgi-derived vesicle covering 2/3 of the sperm head and contains proteolytic enzymes needed for fertilization. For this reason, acrosome should be intact until it reaches the oocyte; however, the freezing-thawing

process can affect this organelle (Fig. 2). Probably, the problem is related to the ROS increase and to the consequent change of membrane fluidity [17]. At TEM level, acrosomes of post-thawed spermatozoa showed wrinkling of plasma membrane, acrosomal swelling, and acrosome reaction [35], probably due to an increased Ca^{2+} load caused by the freezing procedure itself [41]. An increase in the spontaneous acrosome reaction can compromise the fertilizing ability when thawed sperm are used for intrauterine insemination or *in vitro* fertilization, procedures where the acrosome plays an important role.

Sperm DNA

Many studies agree that cryostorage causes DNA damage; in particular, DNA fragmentation is the most frequently studied anomaly. DNA fragmentation is reputed to be one of the most critical factors in the success of both *in vivo* and *in vitro* reproduction [42] and sperm DNA of infertile men is more prone to cryodamage with respect to that of the fertile population [5]. One of the most important mechanisms through which the cryodamage is caused includes the ROS overproduction concomitant with a decrement of antioxidant defenses. In this way, ROS can induce nucleotides modifications causing mutations and genome instability, because spermatozoa have limited DNA repair mechanisms [43]. In addition, some studies, revised by Tamburrino *et al*. [5], reported that ROS produced during sperm cryostorage could influence epigenetic pattern, raising many concerns since cryopreservation is a widely used technique in ART laboratories.

Antioxidant Systems in Human Semen and Spermatozoa

Under normal conditions, both spermatozoa and seminal plasma have different antioxidant defenses. Spermatozoa undergo morphological changes during maturation and one of the most important is the loss of cytoplasm. For this reason, the amount of cytoplasmic antioxidants is limited in sperm cells and the seminal plasma contains the greatest part of antioxidant buffering capacity to prevent the OS damaging effects [44]. This should be considered when spermatozoa are manipulated *in vitro* and the cryopreservation is applied to sperm populations selected by density gradient centrifugation or *swim up* method, which eliminates altered spermatozoa and seminal leukocytes, the main ROS producers in semen. These techniques also remove the seminal plasma and, with it, all the protective antioxidants contained in the semen [4,45].

Antioxidants can be grouped as enzymatic and non-enzymatic ones [10,44,46]. The enzymatic antioxidant system in semen is composed of endogenous molecules such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) that participate in semen natural antioxidant defenses and work in a synergic manner. SOD converts two-superoxide anions into oxygen and hydrogen per-

oxide; CAT aids the decomposition of hydrogen peroxide to water and oxygen [47], GPx catalyzes the reduction of organic and inorganic hydroperoxides using reduced glutathione as an electron donor [48].

There are two types of non-enzymatic antioxidants: endogenous and exogenous; among the non-enzymatic antioxidants, albumin, β-carotenes, L-carnitine, reduced glutathione (GSH), pyruvate, taurine, hypotaurine, ubiquinones, ascorbic acid (AA), α-tocopherol, zinc, selenium can be mentioned [47]. One of the most important antioxidants in intracellular protection is the GSH, a tripeptide formed by cysteine, glutamate, and glycine that acts as a cofactor for GPx and reacts directly with ROS by its sulfhydryl groups. Total GSH scavenges excess ROS and it is oxidized to GSSG -oxidized glutathione-, which is then converted back to reduced GSH by glutathione reductase or via AA, in a cycle that includes the dehydroascorbic acid (DHA).

AA, a major water-soluble antioxidant, acts as a scavenger for a wide range of ROS; AA is a powerful electron donor that reacts with superoxide, peroxide, and hydroxyl radicals to form DHA [49].

As described before, *in vitro* manipulation of sperm and semen cryopreservation induces ROS production and can decrease the concentration of several antioxidants [39]. For example, in human sperm cells after cryopreservation a decrease of SOD1 concentration was described [50,51] and the same trend was observed in other animal spermatozoa [39]. In addition, Ďuračka *et al*. [52] recently reported that the prediction of semen cryotolerance is more likely based on antioxidant rather than pro-oxidative markers.

In this scenario, the addition of compounds with antioxidant activity to semen extenders could represent a defensive and controlled strategy to minimize the sperm damages induced by OS generated during cryopreservation.

Antioxidants as a Supplement during Human Semen Cryopreservation

In the last decades, most research was focused on methods and strategies to improve the freezing efficiency of human and animal semen. As already mentioned, human semen is conserved for assisted fertilization or sperm banking. In animals, the semen cryopreservation is even more widespread; it contributes to the genetic improvement through artificial insemination, and supports the preservation of endangered species and the conservation of biodiversity [53]. In both cases, even though the cryopreservation can supply many benefits, it impairs sperm function and one of the most popular strategies to overcome this problem is the development and supplementation of incubation media with antioxidants. Within this wide topic, we limited our focus to the literature on human semen treated with antioxidants during cryopreservation protocols, starting from the year 2000 up to the present.

Tables 1,2 show the effects on human spermatozoa of several molecules used alone (Table 1) and in combination (Table 2) as a supplement of cryopreservation media.

One of the first compounds, still used today, was pentoxifylline employed at a concentration ranging from 1.8 mM to 5 mM in the different considered studies (Tables 1,2). Pentoxifylline had a positive effect on sperm motility, in particular, if added after thawing [54,55] and in a cryopreservation medium used to freeze sperm recovered directly from the testis [56] and from normozoospermic men [57] (Table 2). In contrast, Esteves *et al*. [58] (Table 1) observed that pre-freezing treatment of poor-quality human sperm with pentoxifylline did not improve post-thawing motility and vitality; however, this compound increased the ability of thawed sperm in undergoing the acrosome reaction (Table 1) in response to calcium ionophore.

More recently, a study compared the effect of pentoxifylline (3.6 mM) and papaverine (93 μM) as a supplement during a freezing-thawing protocol of epididymal and testicular sperm (Table 2) and found that papaverine was more efficient than pentoxifylline in protecting motility [59].

Pentoxifylline is a methylxanthine derivative that increases intracellular cyclic adenosine monophosphate-cAMP-concentration and nitric oxide level, which, in turn, stimulates the guanylate cyclase pathway and may affect human sperm respiration, motility, and acrosome reaction. In addition, the inhibition of xanthine oxidase, which lowers the amount of intracellular ROS and LPO, is a contributing factor to pentoxifylline's antioxidant activities [56,60].

Zinc (100 μM), used as supplement during semen cryopreservation [61], improved many sperm parameters (Table 1) and, administered as nanoparticle formulation [62], reduced malondialdehyde (MDA) levels, a well-known marker of oxidative damage. As a possible mechanism of action, zinc is able to inhibit *in vitro* superoxide anion generation [63].

Supplementation of freezing media with vitamin E [64], trolox [65,66], as well as vitamin C [67,68] showed a general relevant improvement of sperm motility and MMP as well as DNA integrity, in particular in infertile patients [69] (Table 2) and decreased phosphatidylserine (PS) externalization (Tables 1,2). It is known that possible cryodamages are due to lipoperoxidation of membrane lipids. For example, vitamin E, a lipid-soluble molecule, can block lipid oxidative chain donating its hydrogen to a lipid radical (making it inactive) and forming the vitamin E radical. Therefore, vitamin E increases and maintains membrane fluidity by protecting oxidizable lipids [70]. Among the family of vitamin E isoforms, α-tocopherol is the most widely investigated compound in different human conditions; however, Zerbinati *et al*. [71] (Table 2) observed that cryo-supplementation with γ-tocopherol induced a higher post-thaw human sperm vitality and motility than those observed in samples treated with α-tocopherol.

In addition, vitamin E has a role in a variety of enzymatic processes, including those of phospholipase A2 and lipoxygenases, which are essential for the biosynthesis of lipid mediators, and it can also modulate protein kinase C activity, a crucial regulator of signaling pathways [70].

Other compounds (Tables 1,2) showed protective effect during cryopreservation protocols improving sperm motility and vitality as L-proline [72], glutathione [73,74], catalase (Table 2) [68,75], L-carnitine [57,76,77]; most of them had a positive effect on DNA and membrane integrity (Table 2) and reduced OS.

Furthermore, the treatment during cryopreservation with two different molecules such as L-carnitine and coenzyme Q [78] (Table 2), used in oligozoospermic semen, or SOD and catalase, used semen with normal parameters [79] (Table 2), showed a significant beneficial effect on sperm parameters, DNA and chromatin, lowering ROS level. In particular, the combination of SOD and catalase [79] appeared particularly effective because of the simultaneous action of the two enzymes on superoxide anion and hydrogen peroxide.

Brugnon *et al*. [80] described a protective effect of hypotaurine on the percentage of live sperm with PS externalization, hypothesizing a protective effect on the apoptotic process (Table 1). The supplementation with N-acetyl-L-cysteine [77,81] and alpha lipoic acid, administered alone or in combination [81], decreased sperm cryodamage (Table 2) and increased the expression of nuclear factor erythroid 2-related factor 2 (*NRF2*) gene. NRF2 is a redox-sensitive transcription factor involved in regulating cellular mechanisms against oxidative damage through the KEAP1-NRF2 pathway by regulating cytoprotective gene expression [82]. In addition, the role of NRF2, as a regulator of anti-ferroptotic genes, is well known with ferroptosis described as a cell death triggered by iron and LPO [83]. NRF2 also plays an important role in spermatozoa to counteract OS. Significant evidence has been presented in this regard. For example, abnormal *NRF2* mRNA levels have been found to be correlated with low sperm motility [84], functional *NRF2* polymorphisms appeared to be associated with the risk of oligoasthenozoospermia [85], and a relationship between *NRF2* promoter polymorphism and sperm DNA damage has been demonstrated in male infertility [86]. Furthermore, in terms of reproductive toxicity in males, microplastics and cadmium were shown to induce LPO and ferroptosis due to inhibition of the KEAP1-NRF2 pathway [87]. Considering the antioxidant signaling pathways controlled by NRF2, the expression of *NRF2* was measured to evaluate the beneficial effects of additives to the sperm freezing medium during cryopreservation [81] (see also Tables 1,2).

Antioxidant compound	Dose	Subjects	Step of supplementation	Results
Pentoxifylline	3 mmol/L [54]	24 normozoospermic	During and after cryopreservation	= sperm motility, vitality (during cryo); > sperm motility (after cryo)
	5 mM [58]	16 oligoasthenozoospermic	During cryopreservation	> AR = sperm motility, vitality
	3.6 mmol/L [55]	30 asthenozoospermic	After cryopreservation	> sperm motility = chromatin/DNA integrity
	2.4 mmol/L [56]	68 azoospermic Sperm recovered from testis	During cryopreservation	> sperm motility = sperm vitality
Zinc	100 μM [61]	58 normozoospermic 51 abnormal semen parameters	During cryopreservation	> sperm motility, intact DNA, mitochondrial function, sperm capacitation and AR
	100 μg/mL zinc oxide nanoparticle [62]	40 normozoospermic	During cryopreservation	< chromatin damage, MDA levels = sperm motility
Vitamin E	5 mM [64]	38 normozoospermic 59 asthenozoospermic	During cryopreservation	> sperm motility, DNA integrity (in both patients' groups)
Trolox	20, 40, 80 μM [65]	20 healthy fertile	During cryopreservation	> VCL, VSL, VAP (40 μmol)
	20, 40, 80 μM [66]	20 normozoospermic 20 oligoozoospermic	During cryopreservation	> MMP < SDF, PS externalization
Vitamin C	600 μM [67]	40 normozoospermic	During cryopreservation	> sperm motility, vitality, normal morphology < chromatin damage, AR
L-proline	1, 2, 4 mmol/L [72]	30 normozoospermic	During cryopreservation	> sperm motility, vitality, TAC < MDA, ROS levels, chromatin damage (4 mmol/L)
Glutathione	1, 5 mM [73]	24 semen samples	During and after cryopreservation	> sperm motility, vitality > sperm motility, vitality < ROS levels (1 or 5mM after cryo) = chromatin integrity (1 mM during cryo);
	5 mM [74]	20 pooled semen samples	During cryopreservation	> sperm vitality < LPO, SDF, H_2O_2 and $O_2^{·-}$
Hypotaurine	50 mM [80]	64 oligoasthenoteratozoospermic	During cryopreservation	> PS externalization in live sperm
Catalase	200 umL^{-1} [75]	50 fertile	During cryopreservation	> sperm motility, vitality < DNA damage
L-carnitine	0.5 mg mL^{-1} per 5×10^6 cell mL^{-1} [76]	22 infertile	During cryopreservation	> sperm motility, vitality = DNA oxidation levels

Table 1. Continued.

Antioxidant compound	Dose	Subjects	Step of supplementation	Results
Myo-Inositol	1 mg/mL [88]	41 infertile	During cryopreservation	> cryo sperm survival rate
	2 mg/mL [89]	40 healthy	During cryopreservation	> sperm motility, normal morphology, TAC < MDA levels, SDF = ROS levels
	2 mg/mL [90]	40 oligoasthenoteratozoospermic	During cryopreservation	> sperm motility, TAC < SDF = ROS levels
	20 mg/mL [91]	25 normozoospermic	During and after cryopreservation	> sperm motility, vitality (after cryo) < carbonyl groups levels (after cryo)
	2 mg/mL [92]	25 asthenozoospermic	During cryopreservation	> sperm motility, vitality, normal morphology, SDF < TAC, MDA levels
Melatonin	0.001, 0.005, 0.01, 0.05, 0.1, 1 mM [93]	43 fertile	During cryopreservation	> sperm motility, vitality < ROS, MDA levels (0.005, 0.01, 0.05, 0.1, and 1 mM)
	0.001, 0.01, 0.1, 1 mM [94]	20 men	During cryopreservation	> sperm vitality, membrane integrity, *NRF2*, *BCL-2*, *HSP90* mRNA levels < ROS levels, LPO (0.1 mM)
	3 mM [95]	33 healthy	During cryopreservation	> sperm motility, vitality, AKT phosphorylation < intracellular ROS levels, caspase-3 activity
Curcumin	2.5, 5, 10, 20 μM [101]	60 healthy	During cryopreservation	> sperm motility, *GPx4* mRNA levels < intracellular ROS levels, SDF (20 μM)
	10 μM [102]	23 normozoospermic	During cryopreservation	> sperm motility, chromatin condensation, DNA integrity
Alpha Lipoic Acid	0.05, 0.1, 0.2, 0.4, 0.8, 8 mM [99]	30 men	During cryopreservation	> sperm motility < DNA damage, LPO (0.2 mM)
Vitamin B12	0.5, 1, 2,2.5 mg/mL [100]	30 normozoospermic	During cryopreservation	> sperm motility, vitality (1, 2 mg/mL); < SDF (2 mg/mL)

Annotation: AR, acrosome reaction; MDA, malondialdehyde; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; MMP, mitochondrial membrane potential; SDF, sperm DNA fragmentation; PS, phosphatidylserine; TAC, total antioxidant capacity; ROS, reactive oxygen species; LPO, lipid peroxidation; *NRF2*, nuclear factor erythroid 2-related factor 2; HSP90, heat shock protein 90; *GPx4*, glutathione peroxidase 4; $O_2^{\cdot-}$, superoxide anion; H_2O_2, hydrogen peroxide.

Table 2. Papers reporting the effects and doses of two antioxidant compounds used alone (OR) or in combination (AND) during freezing-thawing protocols in human spermatozoa of men with different seminal conditions.

Antioxidant compound	Dose	Subjects	Step of supplementation	Results	
Pentoxyphilline (PTX) OR Papaverine [59]	3.6 mM PTX; 93 μM papaverine	13 non-obstructive 3 obstructive (sperm surgically obtained)	After cryopreservation	> sperm motility (papaverine)	
Pentoxifylline OR L-carnitine (LC) [57]	1.8 mM PTX OR; 1.8 mM LC	30 Normozoospermic	Pre-treatment and cryopreserved	> sperm motility = membrane integrity (PTX);	> plasma membrane, acrosomal integrity, non-capacitated sperm (LC) = sperm motility
Alpha- OR Gamma-Tocopherol (TOH) [71]	120 μM α-TOH; 120 μM γ-TOH	15 Normozoospermic	During cryopreservation	> sperm motility, vitality (γ-TOH)	
Ascorbic Acid OR Catalase [68]	300, 600 μM ascorbic acid; 200 ,400 IU/mL catalase	30 Normozoospermic	During cryopreservation	> sperm motility, vitality, MMP < ROS levels, PS externalization (300 μM ascorbic acid);	> sperm motility, vitality, MMP < ROS levels, PS externalization (200 and 400 IU/mL catalase)
Ascorbic Acid OR Resveratrol [69]	10 mM ascorbic acid; 10 mM resveratrol	10 fertile 10 infertile	During cryopreservation	< DNA damage in infertile patients (ascorbic acid);	< DNA damage in both patients' groups (resveratrol)
N-acetyl cysteine (NAC) OR L-carnitine (LC) [77]	5 and 10 mM NAC; 1 and 10 mM LC	20 Normozoospermic	During cryopreservation	> sperm motility, MMP, plasma membrane integrity < ROS levels (NAC OR LC at all concentrations)	
N-acetyl-L-cysteine AND/OR Alpha Lipoic Acid (ALA) [81]	1 mg/mL NAC; 0.5 mM ALA	30 astheno-teratozoospermic	During cryopreservation	> sperm motility, vitality, MMP, expression of *NRF2* gene < SDF (NAC AND ALA)	
Catalase AND/OR Superoxide Dismutase (SOD) [79]	200 U/mL catalase; 200 U/mL SOD; 100 U/mL + 100 U/mL catalase + SOD	25 Normozoospermic	During cryopreservation	= sperm motility (catalase OR SOD);	> sperm parameters (catalase AND SOD)
Acetyl-L-carnitine (ALC) AND/OR Progesterone [104]	1 and 10 μM progesterone; 2.5, 5, 10, and 20 mM ALC; 1 μM + 5 mM progesterone + ALC	35 Subfertile	During cryopreservation	= sperm motility, plasma membrane integrity, cryosurvival rate (ALC AND/OR progesterone)	
L-Carnitine (LC) AND/OR Coenzyme Q10 (CoQ10) [78]	100 μM LC AND/OR CoQ10	30 oligozoospermic	During cryopreservation	> sperm motility < ROS levels, SDF, protamine deficiency (LC AND/OR CoQ10)	
Coenzyme Q10 (CoQ10) AND/OR Curcumin (CUR) [103]	25 μM CoQ10; AND/OR 0.25 mM CUR	40 Healthy	During cryopreservation	> sperm motility, MM structure, TOC > TAC, TOC = sperm vitality, acrosome integrity (CoQ10)	> MM structure, TAC, TOC = sperm vitality, acrosome integrity, (CUR + CoQ10) SDF (CUR)
Melatonin AND/OR Caffeine [96]	2 mM melatonin AND/OR 2 mM caffeine	30 normozoospermic	Melatonin during cryopreservation, caffeine after cryopreservation	> sperm motility (caffeine; melatonin + caffeine) > mitochondrial activity (melatonin + caffeine) = SDF, ROS	

Annotation: ROS, reactive oxygen species; MMP, mitochondrial membrane potential; PS, phosphatidylserine; *NRF2*, nuclear factor erythroid 2-related factor 2 gene; SDF, sperm DNA fragmentation; TOC, total oxidant capacity; TAC, total antioxidant capacity; MM, mitochondrial membranes.

Recently, the most commonly used substances as a supplement in freezing protocol applications have been myoinositol [88–92] and melatonin [93–96]. The effects of these compounds are reported in Tables 1,2. Myoinositol is the most biologically important form of inositol, a component of the vitamin B complex. At the plasma membrane level, it is involved in signal transduction as a precursor of second messengers. Myoinositol can raise Ca^{2+} levels in the cytosol and, as a result, in the mitochondria. This event activates the oxidative phosphorylation and ATP production, improving the sperm mitochondrial function, preventing apoptosis, and facilitating chromatin condensation [97]. Ponchia *et al*. [91] reported that, after sperm thawing, myoinositol causes an increase in oxygen consumption and a relevant decrease in the content of carbonyl groups (Table 1), the main structural changes that take place in the OS conditions.

Melatonin was found in seminal plasma and its receptor in sperm plasma membrane. The mechanism of action of melatonin is based on its powerful antioxidant properties and includes the direct scavenging of free radicals, the supporting action on SOD and GPx, the stimulation of glutathione production, and the protection against OS [98]. In addition, melatonin plays a role in stimulating mitochondrial respiration and ATP synthesis, as well as increasing the activity of respiratory chain complexes I and IV (oxidative phosphorylation) [96].

The supplementation with melatonin [93–95] (Table 1) played a positive role in increasing sperm quality, the expression of heat shock protein 90 and NRF2 [94], and decreasing apoptotic markers [95]. A synergic positive effect of melatonin and caffeine [96] (Table 2) on sperm motility and mitochondrial activity was observed in samples supplemented during a sperm freezing protocol.

In Table 1 are also reported other compounds such as alpha-lipoic acid [99], vitamin B12 [100], and curcumin [101,102] that protect human spermatozoa during cryopreservation protocols. For this purpose, Tas *et al*. [103] supplemented cryopreservation media with curcumin and coenzymeQ10, alone and in combination, and observed that sperm morphology and motility were better preserved in the presence of coenzymeQ10 (Table 2) whereas DNA fragmentation was significantly increased in samples to which curcumin was added.

Finally, Duru *et al*. [104] using different doses of progesterone and acetyl-L-carnitine, alone or in combination, did not observe improvement in cryodamage assessed by motility changes or membrane integrity in human spermatozoa of subfertile men.

Table 3 reports studies in which molecules of natural origin, mostly polyphenols, have been used as a supplement during cryopreservation of human spermatozoa. The use of polyphenols and molecules of natural origin for cryopreservation has gained popularity over the past 20 years. Fruits, vegetables, nuts, seeds, tree bark, and alcoholic beverages like wine, beer, and tea all contain polyphenols. There are several subclasses of polyphenols, including non-flavonoid polyphenols like resveratrol and flavonoid polyphenols like quercetin, epigallocatechin-3-gallate, genistein, and naringenin [105]. Flavonoids are known to have a dual effect on the homeostasis of ROS. Under normal circumstances, they act as antioxidants, but they can also have pro-oxidant activity, which is why they can cause apoptosis of cancer cells [106]. The biological activity of polyphenols on animal cells has been explained by several mechanisms. Due to the presence of hydroxyl groups, polyphenols directly scavenge ROS and chelate metal ions, and they can show specific actions depending on the particular structural and chemical characteristics of the different compounds. These indirect effects include the activation of antioxidant enzymes, suppression of pro-oxidant enzymes and others [105].

Resveratrol and quercetin are the two molecules most often employed as supplements for cryopreservation media. Resveratrol [69,107–109] has been used at different concentrations (5 µM–10 mM) and its effects are reported in Tables 2,3. Essentially, resveratrol, added during cryopreservation, reduced DNA damage in post-thawed spermatozoa [69,109], LPO, ROS levels, and apoptosis [108] and had no effect on sperm motility and vitality [107,108]. The cryoprotective effects of resveratrol also included an increase in AMPK (energy sensor and metabolism regulator)—activated protein kinase phosphorylation and in key paternal transcripts (Table 3) of cryopreserved human spermatozoa [109].

Supplementation of the cryopreservation medium with 50 µM quercetin [110–112] induced a significant improvement in post-thaw sperm parameters in terms of motility, vitality, and DNA integrity (Table 3); an increase of antioxidant enzyme activity and a decrease of OS-induced damages and ROS levels were also evident [111,112].

A decrease of ROS, membrane lipid disorder and DNA damage, and a slight improvement in the sperm parameters have been observed when the isoflavone genistein (10 µM, Table 3) was added to cryopreserved sperm samples [113]. Whereas gallic acid [114] (Table 3), added to cryopreservation medium, had a positive effect on sperm motility, vitality, DNA integrity and lipoperoxidation at a dose 50 µg/mL, but not at 100 µg/mL, chlorogenic acid [115] protected sperm motility, vitality, mitochondria and DNA integrity by reducing ROS at a concentration of 100 µM. Procyanidin [116], used during cryopreservation, was able to reduce sperm DNA fragmentation, which was also a target of cyanidin-3-glucoside [117], effective only at low doses (50 µM and 100 µM, not at 200 µM); it also improved sperm vitality and motility and decreased ROS levels. The supplementation of sperm freezing media with 1 µM astaxanthin, a red-orange carotenoid pigment, improved sperm motility and vitality, reduced ROS levels, and prevented PS externalization [118].

Table 3. Effects and doses of antioxidant compounds of natural origin used during freezing-thawing protocols in human spermatozoa of men with different seminal conditions.

Antioxidant compound	Dose	Subjects	Step of supplementation	Results	
Resveratrol	0.1, 1, 10 mM [107]	20 infertile 10 fertile	During cryopreservation	< LPO = sperm motility (0.1, 1, 10 mM)	
	5, 15, 25 µM [108]	22 normozoospermic	During cryopreservation	> AMP-activated protein kinase phosphorylation (25 µM) > MMP < ROS levels, apoptosis (15 µM) = sperm motility and vitality (5, 15, 25 µM)	
	15 µM [109]	22 normozoospermic healthy	During cryopreservation	> AMP-activated protein kinase phosphorylation, protamines (1 and 2) and ADD1 mRNA levels < SDF	
Quercetin	50 µM [110]	9 normozoospermic	During cryopreservation	> sperm motility, vitality and DNA integrity = caspase 3 activation	
	50 µM [111]	25 asthenozoospermic	During cryopreservation	> sperm motility, vitality, and normal morphology, antioxidant enzyme activity < MDA levels, DNA damage	
Quercetin AND/OR Tempol [112]	10 µM quercetin 5 µM tempol	58 semen samples	During cryopreservation	> sperm motility, vitality < SDF (quercetin OR tempol)	< ROS levels (quercetin) (quercetin AND tempol)
Genistein [113]	1, 10 µM/L	20 subjects for infertility screening	After cryopreservation	> sperm motility < ROS levels (10 µM/L);	< membrane lipid disorder, DNA damage (1 and 10 µM/L)
Gallic Acid [114]	50, 100 µg/mL	30 Healthy	During cryopreservation	> sperm motility, vitality and normal morphology < SDF, LPO (50 µg/mL);	= sperm parameters (100 µg/mL)
Chlorogenic Acid [115]	100 µM	8 men	During cryopreservation	> sperm motility, DNA integrity, MMP, phospho-AMPKα labelling in sperm head < MDA levels	
Procyanidin [116]	20 µM	25 infertile	During cryopreservation	< SDF	
Cyanidin-3-O-glucoside [117]	50, 100, 200 µM	39 normozoospermic	During cryopreservation	> sperm motility, vitality < ROS levels (50 µM, 100 µM);	= sperm quality (200 µM); < SDF, HDS (in all groups)
Astaxanthin [118]	1 µM	25 healthy	During cryopreservation	> sperm motility, vitality < ROS levels, PS externalization, LPO	

Annotation: LPO, lipid peroxidation; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; ADD1, adducin 1 alpha; HDS, high DNA stainability; MDA, malondialdehyde; SDF, sperm DNA fragmentation; PS, phosphatidylserine.

Table 4. Effects and doses of natural extracts used during freezing-thawing protocols in human spermatozoa of men with different seminal conditions.

Antioxidant compound	Dose	Subjects	Step of supplementation	Results	
Ceratonia siliqua [119]	20, 30, 40 µg/mL	20 normozoospermic	During cryopreservation	> sperm motility, vitality, PMI (20 µg/mL);	> MMP < ROS levels, AR (20, 30, 40 µg/mL)
Opuntia ficus-indica [125]	5 mg/mL	15 normozoospermic	During cryopreservation	< SDF	
Opuntia ficus-indica Mill. [120]	50 µL in the medium	5 normozoospermic	After thawing	> sperm motility, vitality < SDF, OS levels	
Origanum vulgare [121]	100 µg/mL	20 healthy fertile	During cryopreservation	> sperm motility, vitality < MDA and ROS levels	
Tea polyphenol (*T. arjuna* bark extract) [122]	/	42 infertile smokers 28 fertile	During cryopreservation	> sperm motility, normal morphology < DNA damage	
Tribulus terrestris [123]	20, 40, 50 µg/mL	80 Fertile	During and after cryopreservation	> sperm vitality (40 µg/mL after);	> sperm motility (40, 50 µg/mL after)
Holotheria parva coelomic cavity extract [124]	250, 500 µg/mL	50 Healthy	During cryopreservation	> sperm motility (250 µg/mL);	> sperm vitality < DNA damage, ROS levels (250, 500 µg/mL)

Annotation: PMI, plasma membrane integrity; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; AR, acrosome reaction; SDF, sperm DNA fragmentation; OS, oxidative stress; MDA, malondialdehyde.

In recent years, interest in using natural extracts, such as phytocomplexes, as supplements for sperm-freezing media (Table 4) has increased. Based on the compounds' synergistic effects and higher bioavailability in *in vivo* experiments, a phytocomplex can be preferred over its isolated constituents [105]. Table 4 lists the studies reporting the use of plant extracts or natural products in supplementing sperm-freezing media during cryopreservation protocols.

Even in these cases a general beneficial effect was observed. In particular, the extracts reported in Table 4 had a positive effect on motility and vitality of post-thawed spermatozoa [119–124] and on DNA integrity [120,122,124] reducing ROS levels and LPO [119–121,123,124]. Except for *Opuntia ficus-indica* extract that was used at a concentration of mg/mL [125], the supplementation of the other extracts was in the order of μg/mL.

It is noteworthy that the quality of frozen-thawed human sperm was also improved by using the coelomic cavity extract of *Holotheria parva*, a marine organism (Table 4). This extract, rich in antioxidants, showed ROS-scavenging activity and cryoprotective effects on oxidative stress [124].

Finally, also sericin [126], a water-soluble globular glycoprotein derived from the silkworm *Bombyx mori,* can represent a feasible cryoprotective supplement for freezing media in human spermatozoa.

Conclusion

In this narrative review, we focused on the effects of different antioxidants used in cryopreservation media on sperm quality. Although the techniques of cryopreservation of human semen were improved, several critical issues are still present and interventions to optimize the post-thaw sperm quality are advisable. Cryopreservation is a process that can generate ROS and consequently can cause oxidative damage to spermatozoa. In particular, the knowledge acquired so far on sperm structural and functional cryodamages, which concern sperm motility and vitality, DNA, and membrane integrity, should represent the basis for protocol optimization.

The supplementation of media with antioxidant molecules or extracts during freezing-thawing procedures seems to be the most often employed approach at present, because it represents a strategy for minimizing oxidative damage.

Despite many studies having reported several beneficial effects of antioxidants added during freezing-thawing protocols on sperm cryodamages, these improvements are not so evident. The cause of this observation can reside in the use of semen with different characteristics and qualities and in the possible pathologies affecting the different patients. Future studies should ideally focus on finding individualized and tailored solutions.

Over the past decade, phytoextracts and natural extracts have attracted more attention. Phytoextracts can be obtained from waste products, which are a rich source of compounds with strong antioxidant activity. Considering that these by-products can be used in the industrial, cosmetic, nutraceutical, and human and animal reproductive fields, this topic of research is worth implementing. Recently, a particular research line involved the use of nanoparticles, liposomes, and exosomes to minimize the cryoinjuries due to semen cryopreservation in animal models; it is possible that in the near future, these biological devices could be used also in the field of human gamete handling and cryopreservation.

As a conclusion, we would like to propose an interesting approach that starts from an opposite point of view: instead of using a defensive strategy, some research projects proposed controlled "offensive strategies" to improve sperm quality. Sub-lethal stress can cause broad adaptability and greater resistance to numerous future shocks in spermatozoa; in other words, this method represents a sort of training that spermatozoa undertake before cryopreservation. Mild treatment with hydrostatic or osmotic pressure, heat or oxidative agents before freezing has been tested in human and, mainly, in animal spermatozoa. These moderate offensive strategies seem to induce the biosynthesis of stress-related proteins such as heat shock proteins and intracellular antioxidants that protect spermatozoa against cryoinjury. Further research in this field would help in understanding the cellular mechanisms involved in the application of sub-lethal doses of stress before and after freezing.

Abbreviations

ART, assisted reproduction technologies; ROS, reactive oxygen species; OS, oxidative stress; PUFA, polyunsaturated fatty acids; LPO, lipid peroxidation; MMP, mitochondrial membrane potential; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GSH, reduced glutathione; AA, ascorbic acid; DHA, dehydroascorbic acid; MDA, malondialdehyde.

Availability of Data and Materials

The data generated and analysed during this study are included on this published article and are available from the corresponding author.

Author Contributions

EM, CS, GC, LS designed the research study. RC and LL performed the bibliographic research, assembled the tables and the graphical section of the manuscript. EM, GC, CS, RC, and LL wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

Luciano Saso is serving as one of the Editorial Board members/Guest editors of this journal. We declare that Luciano Saso had no involvement in the peer review of this article and has no access to information regarding its peer review.

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