Biological Relevance of Free Radicals in the Process of Physiological Capacitation and Cryocapacitation

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Abstract: Before fertilization, spermatozoa must undergo a process called capacitation in order to fulfill their fertilization potential. This includes a series of structural, biochemical, and functional changes before a subsequent acrosome reaction and fusion with the oocyte. However, low temperatures during cryopreservation may induce a premature activation of capacitation-like changes, also known as cryocapacitation, immediately after thawing, which may lead to a decreased viability, motility, and fertilization ability of cryopreserved spermatozoa. Furthermore, cryopreservation is responsible for the overgeneration of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radicals, which may result in the development of oxidative stress, cell membrane damage, and lipid peroxidation. Despite that, both capacitation and cryocapacitation are considered to be oxidative events; however, potential beneficial or detrimental effects of ROS depend on a wide array of circumstances. This review summarizes the available information on the role of free radicals in the process of capacitation and cryocapacitation of spermatozoa.

Keywords: free radicals; reactive oxygen species; capacitation; cryocapacitation

1. Introduction

Free radicals (FRs) may be described as unstable molecules with an unpaired electron in its atomic orbital. As a natural by-product of oxygen metabolism, they present with both toxic as well as beneficial properties, which are involved in the regulation of a multitude of intracellular pathways responsible for sperm capacitation, hyperactivation, acrosome reaction (AR), and fusion with the ovum [1]. On the contrary, if the production of FRs accelerates, the process may lead to the development of oxidative stress (OS). OS is a condition primarily caused by an imbalance between the generation of FRs and the antioxidant ability of cells to detoxify these highly reactive molecules [2,3]. This disproportion negatively affects the structural integrity and functional activity of a wide range of cellular components including carbohydrates, nucleic acids, lipids, and proteins [4].

Based on their backbone, FR can be divided into reactive oxygen species (ROS), reactive nitrogen species (RNS), and other nonradical reactive species [5]. The most common FRs belong to the family of ROS and include the superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^\bullet$), and hydrogen peroxide (H_2O_2). The group of RNS is presented by nitric oxide ($NO^\bullet$), nitrogen dioxide ($NO_2^\bullet$), and peroxynitrite (NO_3^-) [6,7]. In general, oxygen is necessary for aerobic metabolism of spermatozoa, but it also plays the main role in the generation of ROS, which could eventually cause a variety of anomalies such as head and acrosome defects, mid piece abnormalities, cytoplasmatic droplets, or tail dysfunction [8]. Nowadays, seminal OS is one of the main reasons for the development of male sub- or infertility [9,10].

Spermatozoa contain a high concentration of polyunsaturated fatty acids (PUFAs), which make them vulnerable to lipid peroxidation (LPO) as lipid structures of the cell

membranes are one of the primary targets for ROS. Naturally, male reproductive cells have their own defense against ROS represented by the antioxidant enzymes present in the cytoplasm; however, most of them are lost during spermatogenesis [11].

As mentioned earlier, a specific amount of ROS is important for the regulation of biological events such as sperm capacitation. The capacitation process includes a cascade of biochemical and structural transformations, which are necessary for spermatozoa to reach their full fertilization potential [12]. Under physiological conditions, capacitation begins immediately following ejaculation, or it may be induced under laboratory conditions by using a specific capacitation medium enriched with heparin or L-arginine [13,14]. After completing capacitation, spermatozoa are able to undergo AR and fertilize the oocyte. The process includes changes in the membrane fluidity and composition, a higher concentration of intracellular calcium, alkalization of cytoplasm, triggering of ion channels, and generation of a small amount of ROS, which work as molecular messengers for the initiation of protein tyrosine phosphorylation modulated by the cAMP-dependent pathway [14,15] (Figure 1).
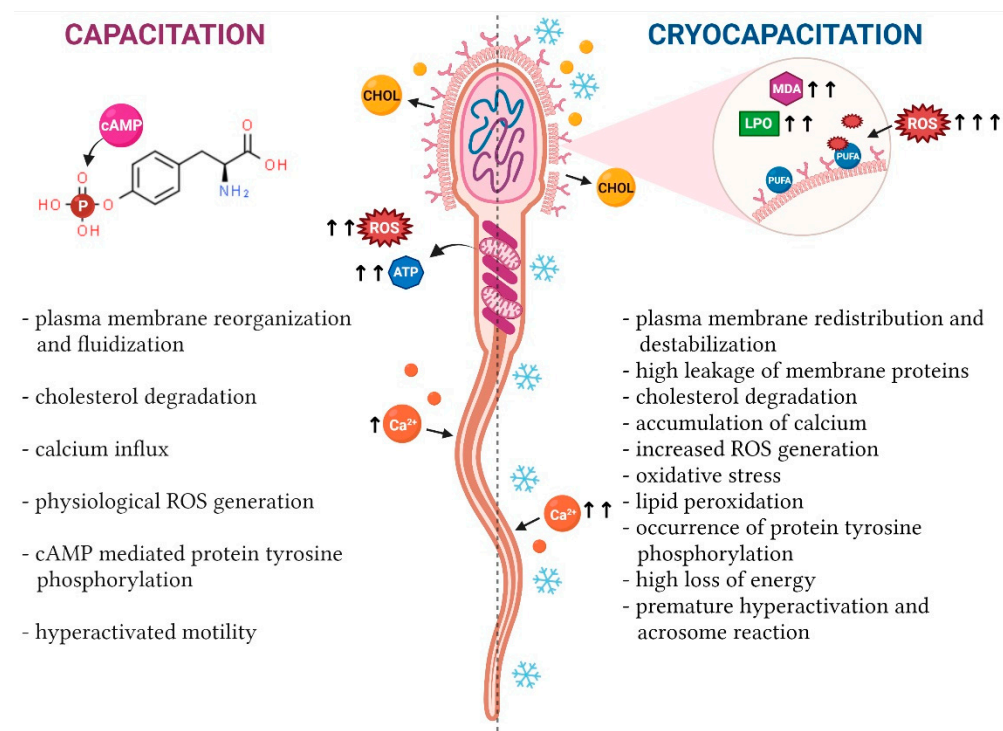

Figure 1. Differences and similarities between capacitation and cryocapacitation of spermatozoa. Created with (Supplementary: Confirmation of Publication and Licensing Rights) BioRender.com (accessed on 23 May 2022).

Over the years, cryopreservation has become a routine technique used for the long-term preservation of fertile male gametes collected not only from domestic animals [16,17] but also from humans [18,19]. Despite the benefits of cryopreservation, this technology may cause irreversible changes to cellular compounds and structures. What is more important is that cryodamage is associated with an increased generation of ROS such as $O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$ [16,17,19]. It has been reported that the cryopreservation process including dilution, cooling, and freezing/thawing protocols is responsible for the occurrence of capacitation-like changes in spermatozoa, also known as cryocapacitation [17] (Figure 1). The cryopreservation itself significantly reduces the quality of semen after thawing and is characterized by the destabilization of the cell plasma membrane accompanied by the loss of sperm movement and viability. Disruption of the membrane lipid architecture decreases

the capability of spermatozoa to reach and penetrate the oocyte, which is a commonly observed complication of premature capacitation and AR [20].

As such, the objective of this review was: (1) to summarize and evaluate the involvement of free radicals in the process of capacitation and cryocapacitation, (2) to define physiological and pathological roles of ROS in the sperm function, and (3) to describe the negative effects of cryopreservation and suggest possible prevention strategies against cryodamage.

2. The Origin of ROS in Semen

Several sources of ROS in semen have been identified such as seminal leukocytes, abnormal spermatozoa, or mitochondria. The presence of leukocytes in ejaculate is often associated with infection or inflammation [7,11]. According to Li et al., high concentrations of ROS and inflammatory cytokines strongly correlate with the quantity of leukocytes in semen collected from sub-fertile men [21]. Activated peroxide-positive leukocytes including polymorphonuclear (PMN) leukocytes and macrophages are able to produce 100 times higher levels of ROS when compared to inactive leukocytes [22]. Activated leukocytes are also responsible for NADPH oxidase catalysis of free radicals by hexose for inflammatory protection. Leukocytospermia is a condition characterized by a high concentration of white blood cells in semen. According to the World Health Organization (WHO), it is defined as the incidence of 1×10^6 leukocytes/mL in semen, which may depend on the presence and/or range of a urogenital infection in men [23,24].

Based on previous studies, a positive correlation has been established between high levels of seminal ROS and poor sperm morphology. Out of several different sperm abnormalities, cytoplasmic droplets or residual cytoplasm were identified as the most involved in ROS generation. Usually, these cytoplasmatic residues appear attached to the elongated spermatid released from the germinal epithelium and migrate away from the sperm neck toward the end of the midpiece during epididymal transport [25]. The number of immature or morphologically retarded spermatozoa is expressed by the sperm deformity index (SDI), which is a quantitative representation of the sperm morphological quality in human andrology [26–28]. Cytoplasmic droplets mediate the production of ROS by the cytosolic enzyme called glucose-6-phosphate dehydrogenase (G6PD) via two pathways. The first one is catalyzed by the nicotinamide adenine dinucleotide phosphate (NADPH), which is localized in the plasmatic membrane, while the second one is driven by the NADPH-dependent oxido-reductase present in the middle piece of spermatozoa. Indeed, plasmatic membranes of spermatozoa and mitochondria have been identified as two main producers of ROS in human semen [29,30].

Despite most of the cytoplasm being reduced during spermatogenesis, mitochondria remain in the midpiece of the sperm flagellum. The main role of these organelles lies in the production of energy in the form of adenosine triphosphate (ATP) through oxidative phosphorylation, which is elementary for the sperm movement [31]. The key to the generation of ROS is associated with the activation of the mitochondrial electron transport chain via five protein complexes (complex I–V), which can transport electrons from NADPH to oxygen (O_2). Positively charged protons and protein complex V (ATP synthase, 19 protein subunits) create the mitochondrial membrane potential, allowing the production of ATP [31,32]. However, a potential leakage of electrons from the mitochondrial electron transport chain caused by stress conditions leads to a partial reduction of O_2 into $O_2^{\bullet-}$. Following that, superoxide dismutases (SOD enzymes) will dismutate $O_2^{\bullet-}$ to H_2O_2 in the intermembrane space of mitochondria. Moreover, $O_2^{\bullet-}$ has the ability to react with nitric oxide (NO) and generate peroxynitrite ($ONOO_2^{\bullet-}$) [33,34].

On the other hand, high levels of PUFAs present in the membranes stimulate the production of lipid aldehydes such as acrolein, malondialdehyde (MDA), or 4-hydroxynonenal (4HNE), as well as lipid metabolites including lipid peroxyl or alkoxyl radicals [35]. These substances are strong electrophiles that may disrupt the functionality of the mitochondrial electron transport chain by their covalent binding into mitochondrial proteins (mainly to

succinic acid dehydrogenase) and thus dysregulate the electron flow. The result of this complex chemical event is a self-repetitive cycle where the amount of PUFAs positively corelates with the generation of mitochondrial ROS and lipid aldehydes. Moreover, 4HNE may bind to mitochondrial proteins, and subsequently induce electron leakage and ROS overgeneration [35,36].

3. Relationship between Capacitation and ROS

The process of mammalian sperm capacitation was first mentioned in 1951 by Chang [37] and Austin [38]. This molecular event combines a series of morphological and biochemical changes, which allow spermatozoa to achieve their full fertilization potential. According to Visconti et al., capacitation may be divided into fast and slow events. The fast or early activation is characterized by a vigorous and asymmetric movement of the flagellum right after ejaculation. Slow and late events include changes in the sperm motility patterns and acquisition of the ability to fertilize the ovum [39]. The initiation of capacitation is accompanied with an efflux of cholesterol from the plasmatic membrane by albumin, increasing the membrane permeability. Subsequently, membrane translocation of bicarbonate (HCO^{3-}) increases the intracellular pH and influx of calcium (Ca^{2+}). After that, activated Ca^{2+} channels and secondary messenger systems including adenylyl cyclase (AC) lead into the escalation of cAMP production and induction of protein phosphorylation, specifically tyrosine phosphorylation [39,40]. This stimulates the complexes of protein kinases and the generation of a specific amount of ROS necessary for this oxidative redox-regulated process [41,42].

Under physiological conditions, a low concentration of ROS produced by spermatozoa is required for the activation of signal transduction processes associated with capacitation. More than 20 years ago, de Lamirande and Gagnon [43] suggested that capacitation is a ROS-dependent process. According to the authors, the addition of superoxide dismutase (SOD) had a capacitation-preventive effect on human spermatozoa, while the supplementation by exogenous $O_2^{\bullet-}$ induced capacitation. SOD with other enzymes (catalase—CAT, glutathione peroxidase/reductase—GPX/GSR) is a part of a complex scavenger system provided by the mammalian seminal plasma for the protection against oxidative stress or premature capacitation [44]. ROS, and particularly their dismutated products $O_2^{\bullet-}$, H_2O_2, and hydroxyl radicals ($OH^{\bullet}$), are capable of trespassing the cell membranes and disrupting the structure of various intracellular molecules such as proteins, lipids, and nucleic acids. Nevertheless, a specific amount of ROS plays a pivotal role in the regulation of cholesterol efflux, cAMP production, and tyrosine phosphorylation [41–43]. According to de Lamirande [45], the concentration of $O_2^{\bullet-}$ reached a peak after 15–25 min of in vitro capacitation and constantly decreased for the next 1–2 h in human spermatozoa. This comes along with the theory that ROS are important mainly at the beginning of the capacitation process [40].

Spermatozoa themselves may produce ROS from sources such as NADPH oxidases (NOX) such as NADPH oxidase localized in the plasmatic membrane or NADPH-dependent oxidoreductase in the mitochondria, which plays a major role in high ATP generation. Increasing levels of $O_2^{\bullet-}$, H_2O_2, $NO^{\bullet}$, and peroxynitrite ($ONOO^-$) produced constantly during capacitation stimulate the activity of AC, cAMP, and protein kinase A (PKA), which is elementary for late tyrosine phosphorylation [46–50]. The activation of PKA phosphorylates and protein phosphatase inhibition stimulate tyrosine kinase, which accelerates actin polymerization that leads to hyperactivated sperm motility in humans [51]. Ghanbari et al. [52] demonstrated a key role of selected NADPH oxidase NOX5 in mammalian spermatozoa, which is involved in $O_2^{\bullet-}$ production. This isoform is different from other members of the NOX family. The activation of NOX5 is Ca^{2+}-dependent because of special calcium-binding sites in the N-terminal region, which means that it does not need any NADPH oxidase subunit [53]. Numerous authors proposed that NOX5-driven $O_2^{\bullet-}$ production initiates the activation of AC, the cAMP/PKA pathway, and tyrosine phospho-

rylation associated with capacitation, as confirmed in human [43], ram [53], equine [54], and rat spermatozoa [55].

It was suggested that the sperm oxidase is responsible for the production of $O_2^{\bullet-}$, right after incubation with selected inductors of capacitation. This hypothesis was supported by the fact that the generation of H_2O_2 under in vitro conditions is characteristic of the production of diphenyliodonium (DPI), which acts as an inhibitor of NADPH oxidase and a capacitation blocker. In general, $O_2^{\bullet-}$ is known for a short life-span and spontaneous dismutation into H_2O_2 by SOD. An increased consumption of H_2O_2 is associated with acrosome reaction in bovine spermatozoa, where H_2O_2 is responsible for the activation of protein kinases and phospholipase A_2 [15,55]. Nevertheless, the exact role of the sperm oxidase is still elusive; however, the presence of NADPH is crucial for the generation of $O_2^{\bullet-}$ as well as $NO^{\bullet}$. Nicotinamide adenine dinucleotide (NADH) is generated during the oxidation of lactate into pyruvate by a specific isoform of lactate dehydrogenase C4 (LDH-C4) localized in the cytosol, mitochondria, and the plasmatic membrane in spermatozoa of different species, such as humans [41], bulls [56], or mice [57]. Subsequently, pyruvate is translocated into the mitochondria and immediately converted to acetyl coenzyme A (acetyl-CoA) as a part of the Krebs cycle and ATP production. The generated NADH is then used to produce extracellular $O_2^{\bullet-}$ through cytosolic oxidases [41,42]. Previous studies reported a participation of LDH-C4 in the metabolic activity and capacitation process in bovine or mouse spermatozoa. On the other hand, low LDH-C4 activity was characterized by a limited or totally reduced sperm movement and concentration [57,58].

As mentioned before, another substantial ROS involved in the capacitation is $NO^{\bullet}$. Lefiévre et al. [59] reported that $NO^{\bullet}$ is able to regulate capacitation by protein S-nitrosylation and activation of the cAMP/PKA pathway in humans, which leads to an increase in protein phosphorylation, especially of serine, threonine, and tyrosine [60]. Another possible way by which $NO^{\bullet}$ could regulate protein phosphorylation is via soluble isoforms of guanylate monophosphate (sGC). These isoforms increase the concentration of cyclic guanosine monophosphate (cGMP) in human spermatozoa; then, cGMP stimulates the activity of cGMP-dependent protein kinase (PKG), which supports the protein phosphorylation of serine/threonine. The intracellular increase in cGMP concentration possibly ceases the degradation of cAMP by the activity of cyclic nucleotide phosphodiesterase. In the end, higher amounts of cAMP may stimulate PKA and protein phosphorylation of tyrosine, which promotes the course of capacitation [15,61,62].

It was confirmed that several proteins associated with capacitation such as protein kinase C (PKC), Ras protein, and AC undergo a process called redox signaling. The highly reactive thiol groups of cysteine sulfhydryl (SH) residues interact with ROS or RNS and create oxidative post-translational modifications. A continuous increase in SH groups is associated with the reorganization of proteins in the sperm membranes at the beginning of capacitation. The most common reversible redox signaling modifications include disulfide bridges, S-nitrosylation, S-glutathionylation, and S-sulfenation, which may be reduced back to thiols or stable oxidized products. However, a covalent sulphinylation of cysteine is irreversible and leads to the formation of tyrosine, tryptophan, lysine, and histidine. These substances may cause cell damage by the loss of a proper protein function [63,64].

4. Cryodamage and ROS Overproduction

Nowadays, cryopreservation is a powerful technique used for the stabilization of spermatozoa at cryogenic temperatures for their long-term storage. It plays a primary role in the improvement of genetic resources in farm breeding programs, preservation of rare and endangered animals, as well as management of infertility in numerous species including humans [17,18,65]. Despite numerous benefits including the preservation of sperm motility, metabolic activity, and fertility, the cryopreservation process may present with negative effects on the sperm physiology and cause irreversible changes that may possibly lead to cell death and a reduced quality of frozen-thawed spermatozoa. Cryopreservation is essentially a three-step process consisting of cooling, the addition of cryoprotectant, and

freezing/thawing. During freezing, spermatozoa are exposed to a wide spectrum of stress conditions such as cold shock, osmotic imbalance, and oxidative stress. Male gametes are protected by several membranes, which act as a natural barrier against external factors. However, for their proper function, these membranes must remain intact to secure the post-thaw viability of spermatozoa [66,67].

The generation of ROS during freezing/thawing procedures comes from mutual interactions between NADPH oxidase in the plasmatic membrane and the mitochondrial electron transport chain. Following cryopreservation, the antioxidant activity of the seminal plasma is dramatically reduced because of dilution and a decrease in enzymatic and nonenzymatic antioxidants such ascorbic acid, urate, vitamin E, and pyruvate [68].

In mammals, the plasma membrane of spermatozoa contains a high concentration of PUFAs, particularly docosahexaenoic acid (DHA), which are highly susceptible to oxidative damage. This process is also known as lipid peroxidation (LPO). Peroxidation of PUFAs is characterized as an autocatalytic self-repeating reaction, which may evolve into the loss of membrane integrity, fluidity, and functionality. The process is associated with electron removal from lipids and the production of reactive intermediates [69,70]. Bouwers and Gadella [71] observed that LPO occurs primarily in the midpiece and tail regions of the sperm flagellum, indicating endogenous ROS production by mitochondria. Peroxidation events significantly affect the mitochondria and cause an immediate decrease in the ATP production and sperm movement as reported in stallions [66] and bulls [71]. Dietrich et al. [72] suggested that specific forms of apolipoprotein A-I provide semen freezability by supporting the membranous structures of carp spermatozoa. Due to freezing, membrane proteins such as N-ethylmaleimide-sensitive fusion attachment protein α and anexin A4 were reduced in this study, which disintegrated the membrane integrity of spermatozoa in carps [72,73] and rats [74]. Other contraindications associated with membrane damage include the leakage of intracellular proteins needed for metabolism, cellular signaling, and the organization of the sperm cytoskeleton, which increase the concentration of intracellular enzymes in the extracellular space [73–75].

Due to oxidative stress, PUFAs are attacked by ROS and produce a wide spectrum of lipid metabolites, as mentioned above. Destabilization of the sperm plasma membrane is caused by the ability of lipid peroxyl radicals to take the hydrogen atoms from PUFAs. Subsequently, carbon-centered lipid radicals will react with oxygen and increase the generation of peroxyl radicals, leading to a self-propagating cycle of hydrogen removal from PUFAs and the promotion of LPO chain reactions [76,77]. These reactions will continue until one of the substrates is consumed or the case of a reaction between two radicals. This process is also responsible for the production of lysophospholipids that destabilize and disturb the microarchitecture of the plasma membrane. The changed structure and function of elemental membrane proteins has a negative effect on ATP-dependent ion pumps and voltage-regulated ion channels, which manage the motility of spermatozoa. Intensive peroxidative damage on the lipid membrane structures makes spermatozoa unable to participate in the membrane fusion processes during fertilization, including capacitation and acrosome reaction [70,76].

5. Capacitation-Like Changes of Spermatozoa during Cryopreservation

Over the years, various researchers have investigated specific changes in frozen–thawed spermatozoa that shared similar characteristics with physiological capacitation. Cryo-induced modifications or cryocapacitation destines spermatozoa to be more vulnerable to their environment because of the reorganization and redistribution of membrane phospholipids. The kinetics of cryocapacitation is not fully understood, because of numerous similarities with physiological capacitation. Following cryocapacitation, the leakage of membrane proteins from thawed spermatozoa is significantly higher in comparison to naturally capacitated spermatozoa [73]. Alterations to the sperm membrane proteins during the cryopreservation process include their segregation, inactivation of membrane-bound enzymes, and a decreased protein diffusion, all of which may eventually lead to the loss of

structural proteins and receptors located on the sperm surface [78,79]. As such, a premature capacitation may reduce the fertilization potential and viability of cryopreserved spermatozoa.

The asymmetric phospholipid architecture from the outside of the plasmatic membrane consists of phosphatidylcholine and sphingomyelin, while the inner side is made from phosphatidylserine and phosphatidylethanolamine. During capacitation or cryopreservation, the asymmetric structure of phospholipids is disturbed by scrambling phosphatidylserine and phosphatidylethanolamine, which could lead to membrane lipid disorders [19], as observed in buffalo [79], equine [80], and boar spermatozoa [81]. According to Cross [82], this disarrangement and a subsequent efflux of cholesterol following in vitro capacitation increases the permeability of the phospholipid bilayer and disrupts phospholipid packing.

Cormier et al. [13] studied the differences of tyrosine phosphoprotein profiles in heparin (HEP)-capacitated and -cryocapacitated bovine spermatozoa. The obtained results revealed the presence of two phosphotyrosine proteins 56-PP and 114-PP in the HEP-capacitated group; however, only 56-PP was found in the case of cryocapacitated spermatozoa. Cryopreserved spermatozoa presented with a characteristic tyrosine-phosphorylated state, which resembled early/fast modifications of the membrane during physiological capacitation. This hypothesis was postulated earlier by Bailey and Bérubé [83], according to who cryopreserved bovine spermatozoa exhibited the evidence of phosphotyrosine protein activity immediately after thawing. In contrary, fresh bull spermatozoa needed a minimal 4 h incubation with HEP for the detection of phosphotyrosine proteins [84].

Hyperactivation is a necessary part of capacitation, when the sperm proteins are tyrosine-phosphorylated and regulated with cAMP by activation of PKA. Several tyrosine-phosphorylated proteins have been localized in the sperm flagellum, which suggests their involvement in sperm hyperactivation. After cryopreservation, male gametes collected from buffaloes [46,79] and bulls [13,84] presented with a hyperactivated motility and weakened Ca^{2+} mechanisms, which may lead to a significant accumulation of the Ca^{2+} ion. If the intracellular Ca^{2+} concentrations reach critical levels, the acrosome reaction is activated too early, even before any contact with the receptors located in the *zona pellucida* of oocyte has occurred [13,19,78].

One of the most common signs of capacitation is the degradation of cholesterol from the sperm plasma membrane. Previous studies have recorded a decline in cholesterol concentration parallel to a higher content of phospholipids and triglycerol after freezing/thawing as a consequence of a slow diffusion from cells. The membrane of cryocapacitated spermatozoa may be characterized by disintegration and swelling, changes in fluidity and permeability, phospholipid aggregation, and a decrease in the motility, viability, and enzymatic metabolism as observed in bulls [20], stallions [66], and boars [81]. These cryo-induced membrane modifications increase the amount of capacitated and acrosome-reacted spermatozoa after thawing. While these do not affect the post-thaw motility, they decrease the longevity of male gametes and their subsequent ability to fertilize the ovum [19,78,85].

6. Future Strategies against Cryodamage

Several strategies for the reduction in the negative impact of cryopreservation on spermatozoa have been developed through the years. Cryoinjury is a result of phase changes of water at low temperatures between the intra- or extra-cellular space. It was observed that spermatozoa are mainly sensitive to a fast temperature reduction between 5 and 25 °C, which leads to cold shock [19,85]. Cryo-induced damage is often associated with osmotic rupture of the cells due to the formation of intracellular ice crystals during cryopreservation, which leads to the loss of semipermeable properties of the sperm plasmatic membrane, release of intracellular enzymes, and ion redistribution. The cryotolerance and cold susceptibility of spermatozoa depend on the lipid content of the plasmatic membrane, which is composed mainly of omega-3/omega-6 fatty acids, especially docosahexaenoic (ω-3 PUFA) and docosapentaenoic (ω-6 PUFA), which belong to the family of PUFAs [77,82]. However, a difference in the lipid profiles between species has been observed. As a supplement for the

protection of spermatozoa against cryogenic temperatures, cryoprotective additives such as antioxidants, fatty acids, antifreeze proteins, and essential oils or bioactive substances from plants may be used [86–88].

In general, cryoprotectants should have certain properties in order to secure the protection against cryoinjury such as biological accessibility, cell penetration ability, and low toxicity. Based on their ability to penetrate the cells, the cryoprotectants are divided into membrane-permeable (dimethyl sulfoxide—DMSO, glycerol)—able to replace water in the cell, and nonpermeable (egg yolk, raffinose, albumin)—unable to pass through the membranes [89].

A possible antioxidant supplementation could provide protection against the generation of ROS and subsequent OS development. The antioxidants used in freezing extenders may exhibit enzymatic or nonenzymatic activity. Glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) represent the major enzymatic antioxidants. SOD has the ability to catalyze the dismutation of $O_2^{\bullet-}$ into H_2O_2 [2,90,91]. The primary role of GPx and CAT is the conversion of H_2O_2 to water and molecular oxygen. Nonenzymatic antioxidants include vitamin C (ascorbic acid), vitamin E (α-tocopherol), vitamin B12 (cobalamin), and glutathione (GSH) [86,90,91]. This complex of free radical scavengers works as an effective defensive system, which protects the membranes of spermatozoa against ROS (vitamin E and C) [92,93] or cryodamage during the freezing/thawing process (vitamin B12) [94].

To inhibit ice crystals formation and decrease the freezing point, antifreeze proteins (AFPs) or glycoproteins are often used. The principle lies in the stabilization of phospholipids and unsaturated fatty acids in the plasma membrane, which improves the membrane integrity and osmotic resistance during the cryopreservation process [73]. According to previous findings, AFPs have significant positive effects on the motility and viability of frozen–thaw spermatozoa in a variety of species such as rams [95], rabbits [96], buffaloes [97], and chimpanzees [98]. Nevertheless, the use of AFPs depends on the type and concentration of selected proteins. Zilli et al. [99] reported that the application of AFPIII (antifreeze protein type III) preserved the sea bream sperm protein profile during cryopreservation. Interestingly, supplementation with AFPIII and AFPI (antifreeze protein type I) significantly increased the membrane integrity but had no effect on the motility in fish [75,99].

Another possibility by which to protect spermatozoa against cryoinjury lies in the application of bioactive molecules as supplements to the freezing extender. Substances such as carotenoids, flavonoids, polyphenols, phytosterols, and phytoestrogens have a natural origin and present with the ability to penetrate the cell membranes and modulate numerous intracellular and metabolic processes [100]. Tvrdá et al. suggested a possible application of various bioactive compounds such as curcumin (CUR), lycopene (LYC), or epicatechin (EPI) to prevent cryo-induced oxidative stress in bovine spermatozoa. CUR is a bright yellow phenolic compound that comes from *Curcuma longa* and is a member of the ginger family. CUR acted as an effective ROS scavenger and inhibitor of LPO. Cryopreservation medium enriched with 50 µmol/L of CUR preserved the structural and functional characteristics, as well as the oxidative profile of cryopreserved bovine spermatozoa [101]. The flavonoid compound EPI belongs to the family of catechins naturally found in green tea, cocoa, and grapes. As with CUR, EPI was able to inhibit LPO and exhibited ROS scavenging properties particularly against $O_2^{\bullet-}$ and H_2O_2. The presence of 100 µmol/L significantly decreased the generation of ROS, the amount of protein carbonyls, and the level of LPO [102]. LYC was also considered as a potential cryosupplement because of its antioxidant effects. As a natural bright red carotenoid, LYC may be found in a wide spectrum of plants such as tomatoes, carrots, or grapefruits. Following the addition of 1.5 mmol/L of LYC into a commercial semen extender, the production of ROS and intracellular $O_2^{\bullet-}$ was decreased in comparison to the control [103]. These findings confirmed the beneficial antioxidant and ROS scavenging properties of selected natural bioactive compounds (CUR, EPI, LYC),

which could at least partially eliminate oxidative damage during cryopreservation and support the vitality of male gametes after thawing.

For the inhibition of capacitation-like changes, melatonin (MEL) was also considered as a primary mitochondria-targeted antioxidant, which can detoxify cells at the mitochondrial level [104]. According to Carvajal-Serna et al. [105], ram semen samples incubated with 100 pmol/L or 1 μmol/L of MEL presented with a significantly higher amount of noncapacitated spermatozoa and a lower rate of male gametes undergoing capacitation. MEL also worked as an inhibitor of phosphatidylserine translocation, which is indicative of the initiation of apoptosis. In addition, MEL significantly decreased the level of protein tyrosine phosphorylation and cAMP in capacitated ram spermatozoa by the presence of cAMP-elevating agents in the medium [106].

7. Conclusions

In summary, we may conclude that capacitation as well as cryocapacitation are both oxidative events. The physiological concentration of ROS may promote the capacitation process. ROS are important primarily at the beginning of capacitation as regulators of cholesterol efflux, activation of the cAMP/PKA pathway, tyrosine phosphorylation, or redox signaling. However, the cryoinduced generation of ROS can accelerate OS by attacking PUFAs, which may lead to a serious membrane damage and LPO. It is clear that capacitation takes place mainly on the level of cell membranes. Meanwhile, cryo-induced capacitation in combination with high concentrations of ROS destabilizes the plasmatic membrane and reduces the fertility rate of thawed spermatozoa used for artificial insemination.

Nowadays, the application of supplements with antioxidant properties for the prevention of potential detrimental effects of ROS is very popular. These may work as effective ROS scavengers, which help to preserve the quality of cryopreserved spermatozoa in a multitude of species. Despite all of this, there are still numerous questions in the field of cryobiology, particularly regarding the process of cryocapacitation and changes associated with it. The exact course of cryocapacitation is still unclear, which is the reason why it is important to study this complex process of sperm metamorphosis from a molecular point of view.

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