Chapter 7

The Roles of Antioxidants and Fatty Acids in Sperm Cryopreservation

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Additional information is available at the end of the chapter

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Abstract

Despite research developments in the area of sperm storage, it has become inevitable to realize a marked reduction in the quality of fresh semen following cryopreservation. As a result, research has continued and will also continue in the future looking forward for a much better and improved methods of sperm cryopreservation along with better understanding of the physical and biochemical challenges that the sperm has to face to survive during freezing. Among the various attempts made to improve the cryopreservation process and subsequently result in superior quality of sperm after thawing include manipulating the composition of semen extenders by addition of exogenous products including antioxidants and fatty acids. While fatty acids are added to strengthening plasma membrane stability, Antioxidants are incorporated to compensate the reduction in the endogenous antioxidants level of seminal plasma due to dilution as well as to combat with the excess reactive oxygen species (ROS) production during freezing. In this chapter, the roles of antioxidants and fatty acids in mammalian sperm cryopreservation, both from endogenous and exogenous perspectives, will be discussed with reference to the latest research findings.

Keywords: antioxidants, fatty acids, sperm cryopreservation

1. Introduction

Demand for livestock products are increasing throughout the world, due to the shifting of consumption patterns toward livestock products and increase in human population. For example, meat and dairy consumption over the last decade increased at a rate of 3–5% annually in Asian countries [1]. To fulfill the increasing demand for animal products, there is a need to increase animal production by improved reproductive technologies. One of the reproductive

technologies that have tremendously contributed to the genetic improvement and development of animal production especially, in the dairy cattle industry is artificial insemination (AI).

AI is a process of depositing sperm manually into a female reproductive tract (usually, uterus, or cervix) for the purpose of achieving viable pregnancy through *in vivo* fertilization using a method other than natural mating. The first documented history of successful use of AI backdates to 1780 by L. Spallanzani who experimented in a bitch that subsequently gave birth to three pups. Later in about 1900, research on AI continued in farm animals and subsequently E.I. Ivanoff who initially studied in horses became the first to successfully inseminate cattle and sheep [2]. Since then, AI has undergone tremendous advances in techniques and applications in a wide variety of species of animals and human.

AI provides a lot of advantages over natural breeding. It maximizes the lifespan reproductive potential of a given male as a single semen ejaculate can be diluted and used to inseminate several females. Other prominent advantages of using AI in farm animals include improvement of genetics through more accurate evaluation of breeder males and greater use of superior germplasm, control of sexually transmitted diseases, improved record keeping, and it avoids the cost and necessity of keeping breeder males in the farm. Although the need to detect females on estrus is one of the prominent disadvantages of AI considered, with the development and advances in other assisted reproductive techniques (ART) such as estrus synchronization and timed AI as well as heat detection aids, this disadvantage of AI is dwindling.

AI can be performed using either fresh or cryopreserved (frozen-thawed) semen. Although the use of fresh semen in AI results in a higher success rate than using cryopreserved semen, it requires keeping males for semen collection in nearby place and immediate shipment of the semen for insemination; otherwise, the semen quality will quickly deteriorate. However,

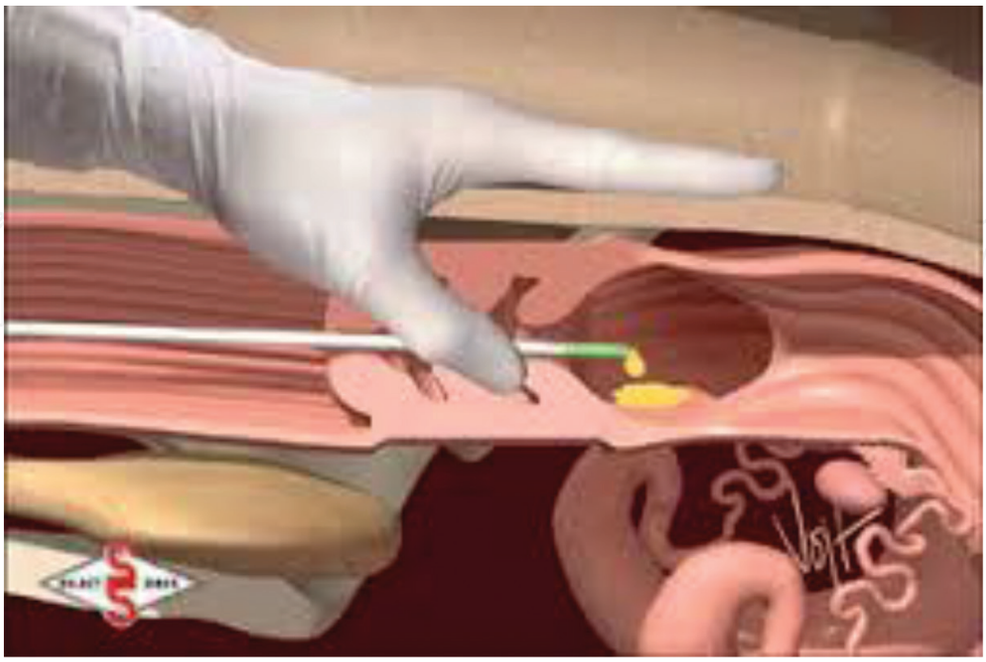

Figure 1. Proper placement of insemination gun to deposit semen in the body of the uterus [61].

cryopreservation provides the opportunity to store the semen for a longer period of time as well as an easy global shipment of superior germplasm to be used for AI. According to Turner [3], it has been documented that AI was estimated to be used on over 110 million breeding cattle (including buffaloes) globally by the turn of the twenty-first century, with the largest number of AIs (50 million) being carried out in the Far East (mainly China, India and Pakistan). There were 1600 cattle semen banks globally and the cattle industry produced over 260 million doses of bull semen from a relatively small number of 41,000 bulls at 648 collection centers worldwide. There was an international trade in bull semen (most of it Holstein), with over 19 million doses recorded as exported globally. The proportion of all cattle inseminations that were carried out by AI rather than by natural mating accounted for about 61% in Europe and about 25% in North America and the Far East. These reflect the significant contribution of semen cryopreservation to the global application of AI in cattle for genetic merit as well as the impact of the quality of postcryopreserved semen that could have on the successful outcome of AI (**Figure 1**).

2. Semen cryopreservation

Semen cryopreservation is a reproductive biotechnology used to preserve and store sperm at a low-freezing temperature for a short or long period of time for various purposes such as in assisted reproduction technologies (ART), species or breed conservation and fertility treatment as in clinical medicine. As discussed earlier, this technique has played a significant role in the livestock industry by overcoming space, distance, and time limitations for the transport of genetically valuable sperm globally and use of AI. Sperm cryopreservation is also an integral component of human reproductive medicine, recognized as an efficient procedure for management of male fertility before therapy for malignant diseases, vasectomy, or surgical infertility treatments, to store donor and partner spermatozoa before assisted reproduction treatments and to ensure the recovery of a small number of spermatozoa in severe male factor infertility [4].

The observation made by Spallanzani in 1803 stated that sperm cooled with snow was not killed but rendered motionless until exposed to heat, after which they became motile for several hours, and the successful cattle insemination in the 1900s could be considered the initial triggers toward the discovery of the cryopreservation procedure. It was in 1940, A. S. Parkes and C. Polge developed a successful method for sperm freezing and storage at low temperatures (-79°C) using dry ice [2]. The same researchers were able to identify glycerol, which is commonly used as a cryoprotectant up to now, as an important factor that helps to protect fowl sperm during the freezing and thawing process. Later in 1957, the freezing mechanism transformed from the use of dry ice to the use of liquid nitrogen contained in a large stainless steel or aluminum vacuum containers, pioneered by the American Breeders Association [2]. Packaging sperm using plastic tubes (straws) or glass ampules were also important adjunct discoveries in the history of development of successful cryopreservation protocol. From the onset of using frozen semen until about the 1970s, glass ampoules were used almost exclusively for packaging, while the straws (0.5 ml—medium straw; 0.25 ml—mini straw) have been the package of choice from the 1970 up to now. As shown in **Figure 2**, liquid nitrogen tank is used to freeze and store the sperm.

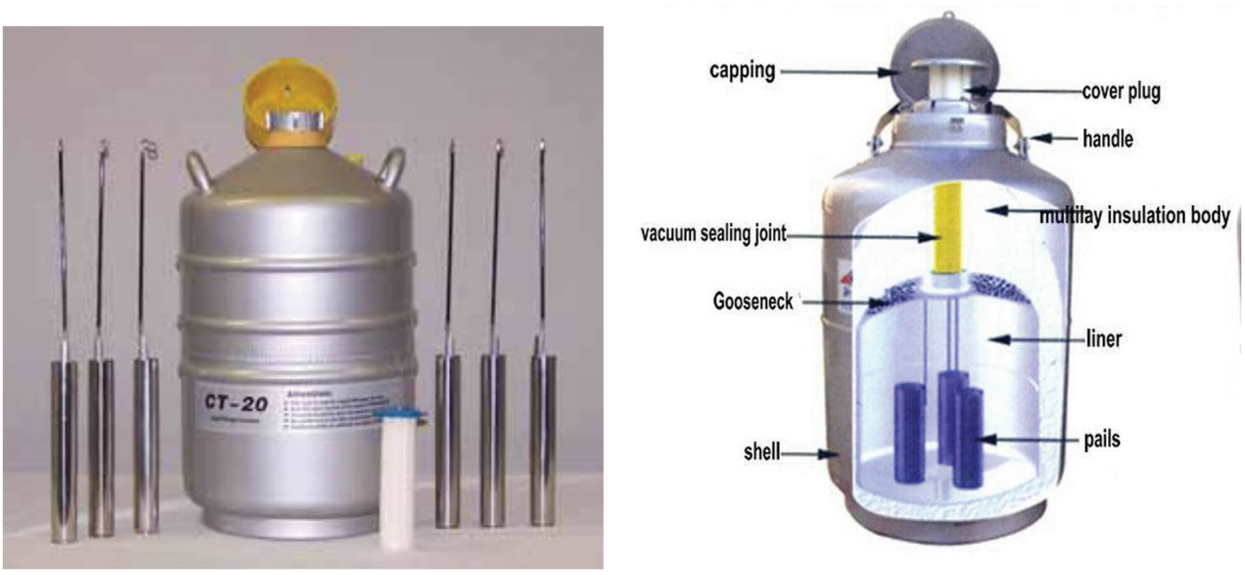

Figure 2. Liquid nitrogen tank used to freeze and store semen in straws (left) and a cross section of a typical semen storage unit (right).

2.1. Semen cryopreservation media

A cryopreservation media, which is also known as semen diluent or extender, plays a crucial role in the quality of sperm after thawing and consequently, it affects significantly the success of AI in the livestock industry. This is because, the survival of the sperm after going through all the physical and biochemical challenges during cooling, freezing, and thawing is dependent mainly on the protective roles provided by the different components of the semen extender used. There are a number of ready-to-use commercially available semen extenders for the production of animals, which include Bioxcel®. Semen extenders such as tris-egg-yolk can also be prepared freshly in the laboratory provided that the components to be mixed are available. In spite of some variations among extenders in their composition, the ultimate objectives intended to be achieved are usually common. These objectives, which have been described as properties that a good semen extender should have [2], include:

1. To have the same free ion concentration or to be isotonic with semen.
2. To have a buffering capacity to maintain pH by neutralizing acid produced by sperm metabolism.
3. To protect the sperm from cold shock injury during cooling from body temperature down to 5°C.
4. Provide nutrition for the sperm metabolism.
5. Control microbial contaminants.
6. Provide sufficient protection to the sperm from damage during freezing and thawing.
7. Preserve the life of the sperm with minimum drop in fertility.

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Despite vast research that has been conducted in the area of semen extender's composition and sperm cryopreservation, and remarkable improvements, yet it has remained inevitable for a big portion of the fresh semen to be damaged during cryopreservation and subsequently resulting in a significant decrease in its quality. This might conclude that the above-mentioned properties of a good semen extender are not good enough and the presence of many other unknown damaging factors that need to be identified and addressed as well. Among the other challenges that the sperm has to face during cryopreservation that appears to be overlooked is oxidative stress (OS). Traditional semen extenders lack specific components targeted to deal with oxidative stress or protect sperm from oxidative damage. As a result, recent studies have focused on finding supplements to semen extenders to protect sperm from oxidative stress as well as other supplements that would help to protect sperm damage in structure and function. In this chapter, we focus on antioxidants and fatty acids as supplements for sperm cryopreservation.

3. Oxidative stress and antioxidants

3.1. Oxidative stress (OS)

Before discussing about antioxidants and their role as supplements to protect sperm from oxidative damage, it is also important to review on what oxidative stress mean and evidence that shows sperm quality is indeed affected by oxidative stress. Oxidative stress (OS) refers to a disturbance in the balance between the production of reactive oxygen species (ROS) (free radicals) and the antioxidant defense that helps to counteract or detoxify their harmful effects. As discussed earlier, cryopreservation of sperm is a routine practice especially in cattle breeding industries for the purpose of artificial insemination. The freezing-thawing procedures of cryopreservation are known to produce ROS in sperm samples. Exposure of semen to cold shock and atmospheric oxygen during cryopreservation increases the susceptibility to lipid peroxidation (LPO) due to higher production of ROS [5, 6].

Oxidants, such as reactive oxygen species (ROS) which are produced physiologically in living cells during respiration as well as by abnormal or dead sperm and phagocytic cells of both the ejaculate and female reproductive tract, affect the quality of postthawed sperm in animals [7, 8]. These ROS can inhibit sperm motility, capacitation, and acrosome reaction mediated by lipid peroxidation (LPO) of sperm membrane. Lipid peroxidation has been correlated with exposure of spermatozoa to ROS and it has been demonstrated that spermatozoa undergoing freeze-thaw cycles produces ROS [9]. A lipid peroxidation rate can be assessed by measuring malondialdehyde (MDA) level in the semen [10] which is one of the final products of polyunsaturated fatty acids (PUFAS) peroxidation in the cell and considered to be an oxidative stress biomarker.

Oxidative stress, which occurs when oxidants outnumber antioxidants in tissues or cells causing pathological effects, is known to play a significant role in the pathophysiology of infertility in human [11]. Factors causing oxidative stress such as ROS are known to be involved in multiple physiological processes from oocyte maturation to fertilization, embryo development,

and pregnancy [11]. According to Agarwal et al. [12], OS is also considered to be one of the key causes of defective gametes and non- or poorly developing embryos in assisted reproductive techniques (ART). A poor fertilization rate, impaired embryo development, and higher rates of pregnancy loss associated with increasing OS in male germ cells are among the adverse effects recorded [13]. A number of other studies have also confirmed significant pathological effects of OS on gametes, embryos, and subsequent implantation resulting in poor pregnancy outcomes. Sperm DNA damage [14] implicated as the cause of increased incidence of abortion [13], loss of plasma membrane fluidity that leads to decrease in vigor and ultimate immobilization, and decrease in mitochondrial potential that leads to apoptosis are among the pathological effects of OS on sperm reported [15]. While reduced quality, early developmental block, and retardation of embryos [11], high fragmentation, and lower blastulation rate that lead to a lower pregnancy rate [2] are among the pathological effects of OS reported on embryos. Considering these adverse effects of OS on reproduction, ameliorating strategies such as *in vivo* and *in vitro* supplementation of antioxidants have been suggested and implemented with improved results [15, 16].

3.2. Antioxidants and their role in sperm cryopreservation

Antioxidants can be classified as enzymatic and nonenzymatic antioxidants. Enzymatic antioxidants are also known as natural antioxidants; they neutralize excess ROS and prevent it from damaging the cellular structure. Enzymatic antioxidants are composed of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase (GR), which also cause reduction of hydrogen peroxides to water and alcohol [6, 11]. Nonenzymatic antioxidants are also known as synthetic antioxidants or dietary supplements. The antioxidant system in the body is influenced by dietary intake of antioxidants, vitamins, and minerals such as vitamin C, vitamin E, zinc, taurine, hypotaurine, and glutathione [6, 11].

In mammals, seminal plasma contains a number of antioxidants which include superoxide dismutase, catalase, glutathione peroxidase, free radical scavengers such as vitamins C and E, hypotaurine, taurine, and albumin [17]. The presence of these antioxidants in semen helps to counteract with the oxidants and protect the spermatozoa from damage. Semen dilution using extenders for the purpose of having more doses from a single ejaculate and cryopreservation, however, decreases the concentrations of natural components of antioxidants. The decrease in the components of antioxidants due to dilution coupled with an increase in production of ROS during cryopreservation exacerbates the condition of spermatozoa and further degrades its postthaw quality and fertilizing ability. To minimize the effect of oxidants on a diluted semen, researchers have tested the impact of adding antioxidants into extenders in many species of animals including bull and have observed improvement in the quality of postthaw spermatozoa compared with controls based on conventional andrological tests [8, 17].

There are numbers of antioxidants tested as supplements to mammalian sperm cryopreservation, but perhaps the most frequently studied antioxidant is alpha-tocopherol form of vitamin E. Vitamin E is a fat soluble vitamin that may directly quench the free radicals such as peroxyl and alkoxy (ROO•) generated during ferrous ascorbate-induced LPO; thus, it is suggested as major chain breaking antioxidant and a protectant of LPO and polyunsaturated fatty acids

(PUFAS) in cell membranes from oxidation [6]. Addition of natural antioxidants such as alpha-tocopherol and ascorbate has been reported to have protective effect on metabolic and cellular viability of cryopreserved bovine spermatozoa [18, 19]. More recently, butylated hydroxyl toluene (BHT), a synthetic analogue of vitamin E, has been tested for its antioxidant effect on bulls [8] and buffalo bull spermatozoa [17]. These studies investigated the impact of adding BHT into semen extenders on postthaw semen quality based on conventional andrological tests such as motility and viability. Results of their study indicated improvement of semen quality following cryopreservation compared to untreated controls. A concentration range of BHT added between 0.5 and 1 mM for bull spermatozoa [8] and between 1 and 2 mM for buffalo sperm [17] was reported to be optimum for cryopreservation and better postthaw sperm quality. BHT is a synthetic analogue of vitamin E that controls the auto-oxidation reaction by converting peroxy radicals to hydroperoxides [17]. BHT has also been successfully tested to preserve liquid semen in other species of animals, such as turkey, to minimize cold shock damage in ram, boar, and goat spermatozoa [17]. Recently, BHT has also been tested in our laboratory as a supplement to both lecithin-based and egg-yolk-based extenders for bull sperm cryopreservation [10]. Findings showed that supplementation of BHT improved general motility, progressive motility, morphology, acrosome integrity, DNA integrity, and oxidative stress level of sperm at 0.5 mM/ml for lecithin-based Bioxcel® and at 1–1.5 mM/ml of BHT for tris- and citrate-egg-yolk extenders compared with their controls. However, higher concentrations of 2.0 and 3.0 mM/ml of BHT had a detrimental effect compared with the control of all extenders evaluated and it was concluded that BHT supplementation at lesser concentrations (0.5–1.5 mM/ml) could improve frozen-thawed bull sperm quality by reducing oxidative stress produced during the freezing-thawing procedures in either lecithin or egg-yolk-based extenders.

4. Fatty acids and their role in sperm cryopreservation

Fatty acid is the composite of a hydrocarbon chain, a methyl group and a carboxylic acid group. The hydrocarbon chains vary in length between 14 and 24 carbon atoms and have difference in the number and position of carbon-carbon double bonds. Saturated fatty acids (SFA) contain no double bonds as all carbon atoms are saturated with hydrogen atoms. This gives the general formula of (CH_3 (CH_2) n COOH) where n is the number of hydrocarbon chain [20]. Fatty acids are classified as essential fatty acids (EFA) and nonessential fatty acids (nEFA) in mammals. Essential fatty acids cannot be synthesized by the body and must be included in the diet due to a lack of desaturase enzymes and the inability to synthesize fatty acids [21].

Fatty acids are vital components of phospholipids and diglyceride and triglycerides, where they are attached to a glycerol molecule, with an additional polar, organic molecule adjoined to the glycerol molecule of the phospholipid. Phospholipids and diglycerides contain two fatty acid chains while triglycerides contain three. When these fatty acids are not part of a larger structure, they are known as free fatty acids.

Fatty acids contain hydrophobic tail of long carbon chains and a hydrophilic head containing a negatively charged phosphate group [22]. The roles of phospholipids are primarily as constituents of biological membranes [23].

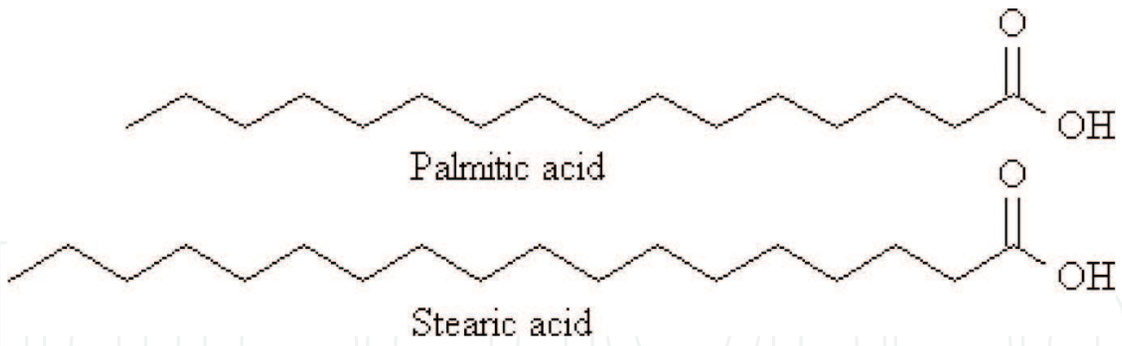

Figure 3. Structure of palmitic acid (upper) and stearic acid (lower). Both have COOH groups to the end with methyl (CH_3) end to the left, C and H atoms not marked.

4.1. Types of fatty acids

Fatty acids are broadly classified as saturated and unsaturated fatty acids.

4.1.1. Saturated fatty acids (SFAS)

Saturated fatty acids (SFAs) are considered as they do not have double bonds in their hydrocarbon chain. The most common SFAs in sperm are palmitic acid (16:0) and stearic acid (SA; 18:0) [24, 25] (**Figure 3**). Because of the absence of double and triple bonds, saturated fatty acids are crowded tighter in cell membranes, therefore, reduce membrane fluidity. Membrane fluidity increases as levels of membrane unsaturation increases [26].

4.1.2. Unsaturated fatty acids

Unsaturated fatty acids are further divided as monounsaturated fatty acids (MUFA) or polyunsaturated (PUFAs). Monounsaturated fatty acid (MUFA) components contain only one double bond and PUFAs contain two or more double bonds. MUFAs and PUFAs are then further classified into three families, omega 3, 6, and 9 (*n*-3, *n*-6, and *n*-9) unsaturated fatty acids according to the distance of the first double bond from the methyl terminal [26].

4.1.2.1. Omega 3, 6 and 9 fatty acids

Omega 3 fatty acid is a group of fatty acids in which the first double bond is located on the third carbon-carbon bond from the methyl end of the hydrocarbon chain. The first double carbon-carbon bond of omega 6 fatty acid is located on the sixth carbon-carbon bond from the methyl end. Omega 9 fatty acids have the first double bond on the ninth carbon-carbon bond from the methyl end group. Omega 3, 6, and 9 fatty acids can also be denoted as *n*-3, *n*-6, and *n*-9 or ω-3, ω-6, and ω-9 fatty acids, respectively. Omega 3 fatty acids in many species of sperm include ALA (18:3), DHA (22:6), DPA (22:5), and eicosapentaenoic acid (EPA; 20:5) (**Figure 4**). Arachidonic acid (AA; 20:4) and LA (18:2) are two *n*-6 fatty acids in sperm (**Figure 5**) while oleic acid (OA; 18:1) is *n*-9 fatty acid family (**Figure 6**) [27].

Animals, such as bull and boar, and humans cannot manufacture fatty acids with carbon chains more than 18 carbons, because of deficiency in the desaturase enzymes at

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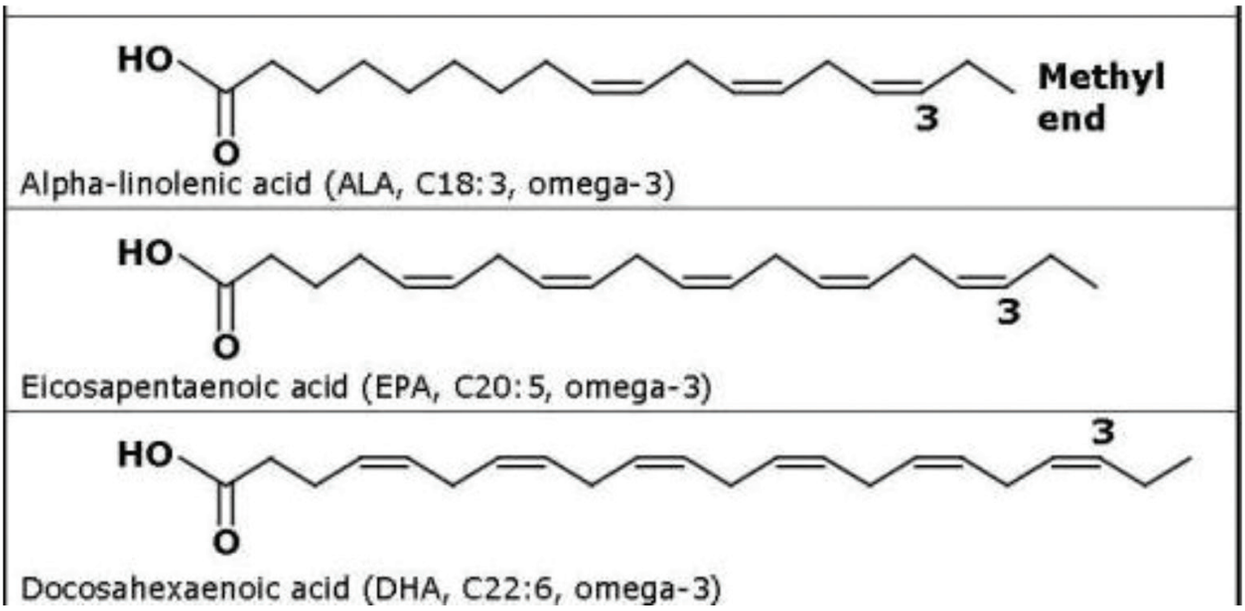

Figure 4. Structure of *n*-3 polyunsaturated fatty acids (PUFAs), alpha-linolenic acid, docosapentaenoic acid, docosahexaenoic acid and eicosapentaenoic acid.

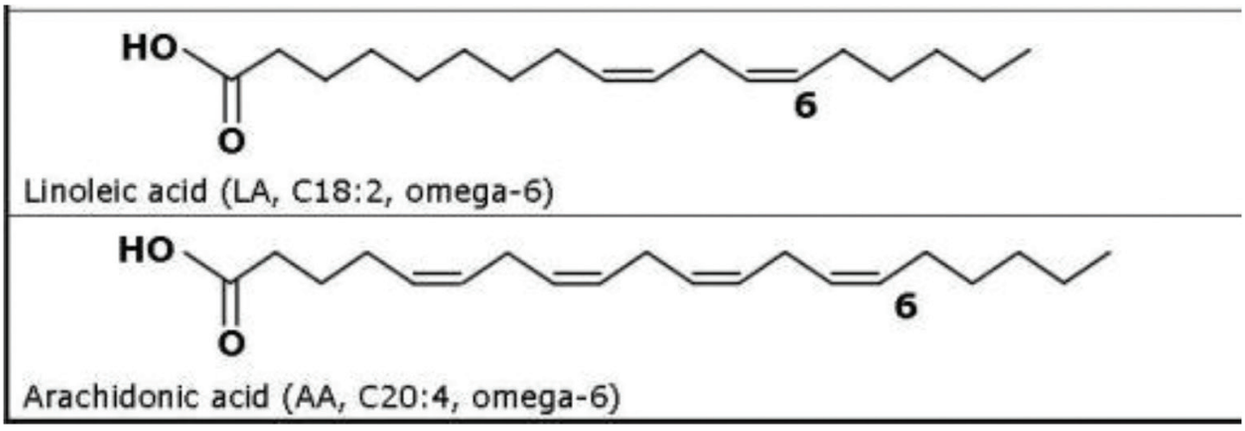

Figure 5. Structure of *n*-6 polyunsaturated fatty acids (PUFAs), arachidonic acid and linoleic acid.

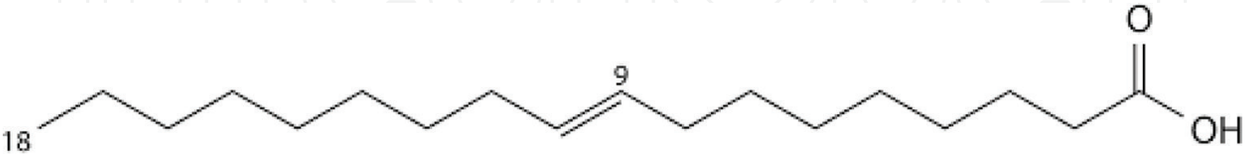

Figure 6. Structure of *n*-9 monounsaturated fatty acids (MUFA) and oleic acid.

Δ1-desaturase, Δ2-desaturase, and Δ3-desaturase enzymes, which could form ALA, LA, and OA, whereas these animals contain Δ4-desaturase, Δ5-desaturase, Δ6-desaturase, and Δ9-desaturase, the number indicating the location that the desaturase enzyme places the double bond in the carbon chain [27]. *n*-3, *n*-6, and *n*-9 fatty acids are commonly known

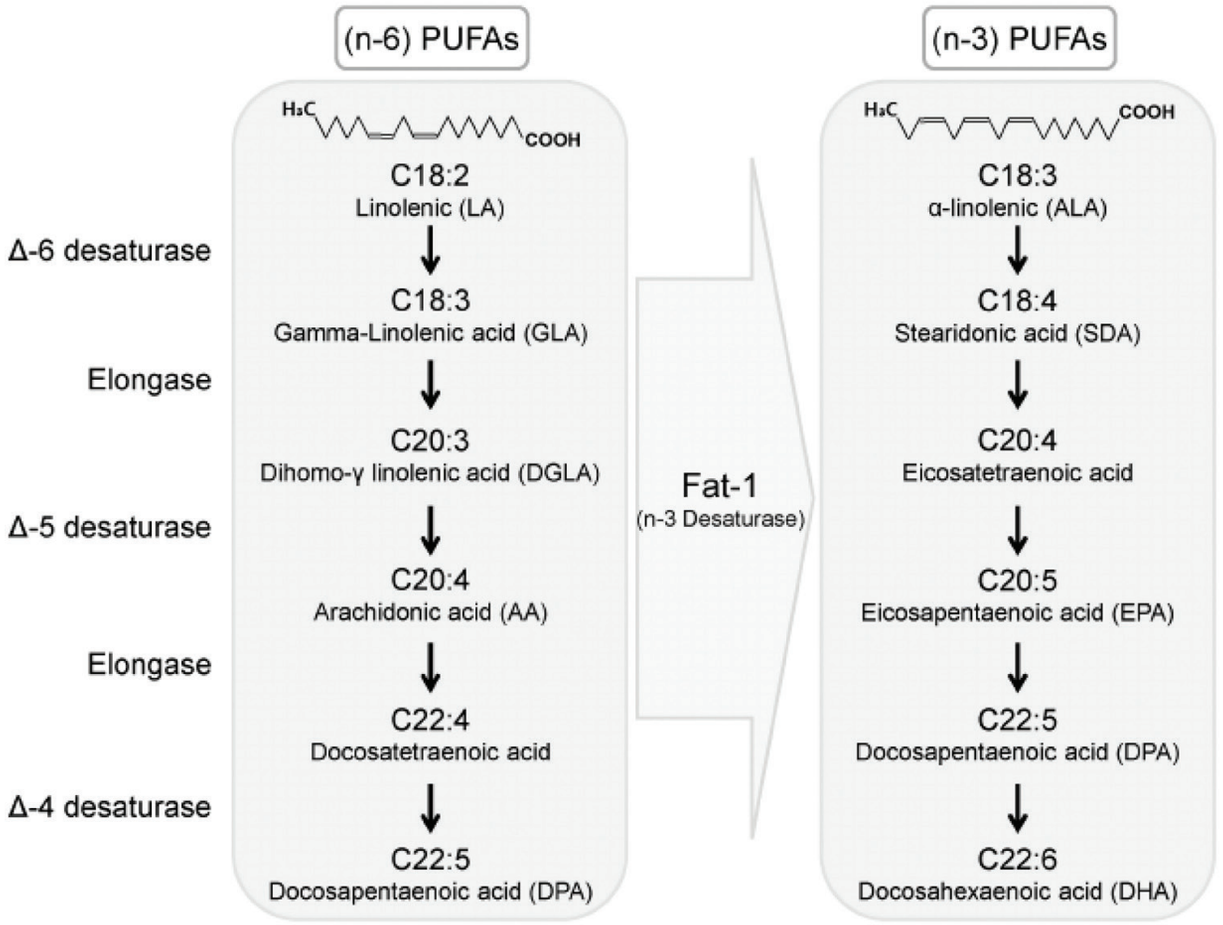

Figure 7. Metabolism of parent fatty acids ALA (*n*-3) and LA (*n*-6) into longer carbon chain fatty acids with relevant enzymatic reactions to form the fatty acids [26].

as the parent fatty acids. These fatty acids are usually found in the diet. Longer chain fatty acids are manufactured by the process of elongation and desaturation reactions generally named as *de novo* synthesis of fatty acids [26, 27] (**Figure 7**).

4.2. Fatty acid compositions of sperm

Epididymis is the store house of sperm where sperm undergo the process of maturation and remodeling of the plasma membrane also occurs. During remodeling, secreted epididymal glycoproteins uptake takes place, consumption of phospholipids from the membrane bilayer and relocation of protein and lipid constituents are restructured during maturation [28] and sperm acquire progressive motility and the ability to fertilize an oocyte [29]. Particularly, bull sperm lose half of their phospholipid and major phospholipid [30]. Fatty acids, as a major component of phospholipids, also undergo a major reduction during epididymal stage [30]. Retention of fatty acids (SFA, MUFAs, and PUFAs) is an indication of immature and defective sperm [25, 31] who studied bull sperm heads and tails described that sperm tails retained more *n*-3 PUFAs than the sperm head while *n*-6 PUFAs were more concentrated in sperm heads than in the tails. Same patterns of *n*-3 and *n*-6 by were found in human sperm. A higher

Type	Name	Carbon chain length
Saturated fatty acids	Myristic	C14:0
	Palmitic	C16:0
	Palmitoleic	C16:1
	Stearic	C18:0
Unsaturated fatty acids	***n-9 PUFAs;***	
	Oleic acid	C18:1*n*-9OA
	n-6 PUFAs	
	Linoleic acid	C18:2*n*-6
	Gamma linolenic acid (GLA)	C18:3*n*-6
	Arachidonic acid (AA)	C20:4*n*-6
	n-3 PUFAs	
	Eicosapentaenoic acid (EPA)	C20:5*n*-3
	Alpha-Linolenic acid (ALA)	C18:3*n*-3
	Docosapentaenoic acid (DPA)	C22:5*n*-3
	Docosahexaenoic acid (DHA)	C22:6*n*-3

Table 1. Fatty acids found in sperm of different animals [35].

percentage of *n*-6 fatty acids (28%) were found in the total bull sperm than *n*-3 fatty acids (23%) in both bull and human sperm. Poulos et al. [30] reported that DHA was one of the main fatty acids of caudal and ejaculated bull sperm and human sperm, respectively. Lenzi et al. [26] has suggested that up to 60% of PUFA in normal human sperm consists of DHA; however, Zalata et al. [32] also reported the same.

Palmitic acid and stearic acid have been identified as the most saturated fatty acids of whole human sperm [25, 26]. Human sperm fatty acid from asthenozoospermic (low motility and viability) males differed from normospermic (normal) males in composition. The former showed lower of DHA but higher OA levels [32]. While unsaturated fatty acids, as a whole, were reduced in the asthenozoospermic males compared to normospermic males [33]. However, infertile human males were found to have higher levels of *n*-6 PUFAs, which decreased sperm concentration, decreased motility, and higher abnormal count [34]. High levels of *n*-3 PUFAs (ALA, DHA, DPA, and EPA) were linked with sperm development, improved motility, and morphology and cryogenic resistance [34]. The superabundance of unsaturated fatty acids, leave sperm extremely susceptible to reactive oxygen species (ROS) attack, oxidative stress (OS), and lipid peroxidation (LPO) [24, 25] (**Table 1**).

4.3. Roles of fatty acids in sperm cryopreservation

Adenosine triphosphate (ATP) produces anaerobic and aerobic respiration and provides energy within the cells for sperm functions. ATP produced through glycolysis (anaerobic

respiration) is a major source of ATP in sperm. Glycolysis occurs in the cytosol of sperm; hence it distributes ATP uniformly in sperm. Mitochondria present in sperm midpiece produce 15 times more ATP by oxidative phosphorylation or aerobic respiration. The aerobic respiration requires oxygen (O_2) and carried out through electron transport chain (ETC) is an effective energy production method. ROS is the by-product of the ETC by leaking of electron and formation of superoxide during respiration [36]. The mitochondrial ETC is composed of four (Complex I–IV) multiprotein complexes and many electron carriers, i.e., flavoproteins, iron-sulfur proteins, ubiquinone, and cytochromes [37]. Electrons are uptaken on ETC by complex I and complex II. Complex I carries electron from nicotinamide adenine dinucleotide (NADH) and nicotinamide and complex II from succinate ($C_4H_6O_4$) [37]. Succinate is a flavin adenine dinucleotide (FAD) linked substrate, which acts as a coenzyme in redox reactions in the body, and later electrons move through carrier to complex III by coenzyme Q (CoQ) or ubiquinone and after that by cytochrome C transport electrons to complex IV [37]. During transport at ETC, electrons escape and form superoxide, which are then transformed to ROS, i.e., hydroperoxyl, hydrogen peroxide, and hydroxide radicals Superoxide are formed at complex I and complex III and O_2 is fully reduced to water (H_2O) at the end of the ETC [37].

Nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) are reducing agents and provide protons within a cell. NADPH and NADH also are the source of increased ROS by supplying electrons for the formation of free radicals, by the reduction of oxygen to superoxide [38]. NADPH is present at the residual cytoplasmic droplet and triggers a NADPH oxidase (NOX) system in the human sperm plasma membrane [39]. According to the previous studies, human sperm generate ROS using the NOX5 enzyme with increase in calcium ions (Ca^{2+}) [40]. NADPH oxidases are plasma enzymes that catalyze the production of ROS by electron flow from NADPH to surrounding cell membrane to molecular oxygen, in order to form superoxide by reduction [41].

The supplementation of exogenous NADPH human sperm encouraged superoxide generation [39] and exogenous NADPH stimulate ROS effectively, necessarily, is to penetrate the sperm membrane and results that sperm damaged membranes showed a higher tendency to absorb NADPH and as a consequence form ROS. NADPH production in the cytoplasm is named as the monophosphate shunt, regulated by enzyme glucose-6-phosphate dehydrogenase. This enzyme also controls the glucose efflux rate and the presence of this enzyme itself is an indicator for immature human sperm [38].

Many studies have been conducted to determine effect of fatty acids particularly polyunsaturated fatty acids on cooled, chilled, and frozen-thawed semen quality in different species of animals. Some of the testimonies are discussed below.

4.3.1. Bulls

Kiernan et al. [42] determined that ALA maintained sperm motility at 100 μM and viability at 10 and 50 μM in citrate-based extender in bull semen chilled for 7 days. Palmitic acid and oleic acid maintained motility and viability at 50 and 100 μM. Takahashi et al. [43] reported that addition of linoleic acid improved frozen-thawed spermatozoa motility and viability of bull semen. Nasiri et al. [44, 45] added DHA improved sperm quality of frozen-thawed quality of

bull sperm. Dietary ALA improved the plasma membrane integrity, acrosome integrity, and DNA integrity of frozen-thawed spermatozoa [46]. In feed also resulted in improved motility in fresh semen of bull [47, 48]. Kaka et al. [35, 49, 50] reported that individual addition of ALA and DHA in tris and bioxcell extender improved cooled and frozen-thawed quality of bull semen while combination of ALA and DHA decreased semen quality after freezing. Kandelousi et al. [51] and Abavisani et al. [52] also reported that omega-3 PUFAs did not improve motility, progressive, morphology, and viability in citrate extender in frozen-thawed quality of bull semen.

4.3.2. Goat

In vitro addition of omega-3 increased the quality of frozen-thawed spermatozoa in goats [53]. Supplementation of egg-yolk DHA rich in citrate extender also improved the total motility, progressive motility, viability, and morphology of frozen-thawed goat spermatozoa [54].

4.3.3. Sheep

Samadian et al. [55] and Towhidi and Parks [45] tested omega-3 fatty acids and reported the improved frozen-thawed quality of semen in rams.

4.3.4. Buffalo

Fatty acids such as arachidonic acid improved postthawed spermatozoa motility, membrane integrity, acrosome integrity, viability, and DNA of buffalo bull spermatozoa [7].

4.3.5. Human

Omega-3 fatty acid is higher in fertile men than in the infertile so that omega-6 fatty acid is important for sperm quality (Saferinajad et al., 2010). The existence of surplus of unsaturated fatty acids in defective human spermatozoa may increase the oxidative stress which reduces in male fertility [34].

4.3.6. Boar

Boar spermatozoa motility, viability, and acrosome integrity were also improved following addition of linoleic acid, oleic acid, and arachidonic acid [56–58]. Chanapiwat et al. [59] and Kaeoket et al. [60] added that DHA improved motility, membrane integrity, and acrosome integrity, viability, and DNA integrity in boar sperm when used alone and in combination with *L*-cysteine in lactose-egg-yolk extender.

5. Conclusion

As research studies show, it is evident that supplementing semen extenders, with some antioxidants, such as vitamin E and its synthetic analogue, BHT, and fatty acids such as alpha linoleic acid, is beneficial to the sperm to endure the physical and biochemical changes and challenges faced during cryopreservation and results in a superior postthaw quality available

for AI. However, it is worth noting that supplements work best at a particular optimum concentration that varies with the type of extender used, as well as the species of the animal from which the sperm has come from. Hence, prior use of a particular antioxidant or fatty acid as a supplement, proper investigation needs to be conducted to determine their optimum concentration to be added into a specific semen extender type.

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