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Review article

Supplementation of ram semen extender to improve seminal quality and fertility rate

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ABSTRACT

In sheep, artificial insemination serves as an important technique for breed improvement. In this context, genetic material from a small number of superior sires can be used in a large number of females. During this process, the storage of ram sperm may influence the efficiency of artificial insemination. Two main methods are currently used for ram semen storage: liquid storage and cryopreservation. The oxidative stress during the storage process can injure ram sperm and in some cases this leads to irreversible damage at the cellular level. To reduce such negative effects, different preservation protocols, extenders and protective components have been tested to improve ram sperm quality and to achieve greater fertility rates. This review provides an overview of the recent progress in extender supplementation using antioxidants and other compounds to improve ram semen quality parameters and fertility rates. It will emphasize on enzymes, vitamins, amino acids, proteins, some plant extracts and other compounds such as sugars, seminal plasma and fatty acids that can be used to supplement the extenders to reduce the formation of oxidants in ram semen and maintain its quality and enhance its fertility. It will also stress on how these supplements act, what were the tested levels giving beneficial effects on motility, viability, plasma membrane integrity and DNA fragmentation in liquid, cooled and post-thawing semen?

1. Introduction

The process of artificial insemination (AI) in sheep (*Ovis aries*) occurs as a result of a combination of several actions. One of the most important steps in this process is semen preservation in both liquid and frozen forms. Handling semen generally causes a gradual decrease in both quality (sperm motility, viability and functional integrity of ram sperm membranes; Maxwell and Salamon, 1993; Salamon and Maxwell, 1995, 2000; De Lamirande et al., 1997; Gillan et al., 1997; Azevedo, 2006; Maia, 2006, 2009; Rodello, 2006; Mahfouz et al., 2010) and fertility (Maxwell and Salamon, 1993; Vishwanath and Shannon, 1997). Sperm are extremely susceptible to low temperatures during the cooling or freezing process. This has been attributed to the high concentrations of polyunsaturated fatty acids in the plasmatic membrane of ram sperm (Jones and Mann, 1976; White, 1993; Buhr et al., 1994) that renders the cells sensitive to cold shock as well as to lipid peroxidation in the presence of reactive oxygen species (ROS; Alvarez and Storey, 1983; Alvarez et al., 1987; Griveau et al., 1995; Holt, 2000; Watson, 2000; Samadian et al., 2010). Sperm generate ROS as a normal consequence of oxidative metabolism and a low concentration of ROS has an important role in mammalian sperm functions, like, capacitation, the

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acrosome reaction, and stabilization of the mitochondrial capsule for the midpiece (Alvarez and Storey, 1984; Aitken and Fisher, 1994; Kodama et al., 1996; Griveau and Le Lannou et al., 1997; Baumber et al., 2000). To maintain proper physiological activities a fine balance between ROS production and recycling around sperm cells is essential. Any imbalance can impair sperm function through oxidative stress, leading to increased rates of lipid peroxidation and consequently to loss of motility during prolonged storage (Aitken, 1995; Gibb and Aitken, 2016). During processing, ram sperm produce large amounts of hydrogen peroxide (Maia et al., 2010; La Falci et al., 2011), which decreases post-thawing sperm motility (Maia et al., 2014).

Successful sperm storage (liquid and frozen) requires slowing of the cell metabolism and thereby prolongs viability (Maxwell and Salamon, 1993; Yoshida, 2000; Gibb and Aitken, 2016). To achieve this goal and to improve sperm quality, the use of a suitable extender and cryoprotectant and an appropriate cooling/warming process (Fiser, 1991) are necessity. For this purpose, various components have been added to extenders to maintain motility and fertilization capacity and to preserve the integrity of the sperm membrane (Sarlos et al., 2002; Riha et al., 2006; Bucak and Tekin, 2007; Maia et al., 2009; Coyan et al., 2010; AminiPour et al., 2013). In most cases, these protectants have antioxidant activity and either reduce the process of oxidation (Pietta, 2000), or regulate, suppress or prevent the formation of ROS (Sikka, 2004; Maneesh and Jayalekshmi, 2006). Supplementation with antioxidants and other compounds, therefore, may reduce the negative effect of oxidative stress caused by ROS on ram sperm during the preservation process (Maxwell and Stojanov, 1996; Upreti et al., 1997; Bucak et al., 2007; Coyan et al., 2010; Forouzanfar et al., 2010). This review provides an overview of the recent progress in extender supplementation using antioxidants and other compounds to improve ram semen quality parameters and fertility rates.

2. Antioxidants

Mammalian sperm have an antioxidant defense system that includes superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione reductase (GR), as well as non-enzymatic antioxidants such as methionine, ascorbic acid and α-tocopherol (Mann and Lutwak-Mann, 1981; Kantola et al., 1988; Aitken, 1995; Mara et al., 2005; Bucak et al., 2012). The biosynthetic capacity of sperm, however, is limited (Aitken, 1995), and the concentration of the antioxidants present in the semen can be reduced by dilution, and as a result decreasing the beneficial effect of this endogenous antioxidative defense. Thus, the addition of antioxidants, even in small concentrations, can improve sperm function during preservation.

2.1. Enzymes

Enzymatic antioxidants are macromolecules that protect cells against ROS (Barreiros et al., 2006). The enzyme superoxide dismutase (SOD) in the cytoplasm (Cu, Zn - SOD) and in the mitochondria (Mn-SOD) is responsible for combining two molecules of the superoxide anion (O_2^-) into one of hydrogen peroxide (H_2O_2), and catalase (CAT) and glutathione peroxidase (GSH-Px) converts of H_2O_2 to H_2O and O_2 (Nordberg and Arnér, 2001; Amidi et al., 2016). This enzyme has been detected in ram semen (Marti et al., 2003; Kasimanickam et al., 2006; Bucak et al., 2008; Marti et al., 2008). Its antioxidant capacity, however, changes with semen quality (Kasimanickam et al., 2006) as well as during the freezing-thawing process (Bucak et al., 2008; Marti et al., 2008). Such changes in enzyme activities in semen as well as the relationship (positive or negative) with sperm quality can be due to oxidative stress, because enzymes are utilized in excess to protect or maintain sperm quality, or because the enzymes do not have the capacity to maintain sperm quality (Bilodeau et al., 2001; Kasimanickam et al., 2006).

In fresh semen, the SOD is the main active enzyme while the glutathione reductase (GSR) and GSH-PX are low (Kasimanickam et al., 2006; Marti et al., 2008). Furthermore, Kasimanickam et al. (2006) reported that GPx activity in ram sperm was greater in ejaculates with poor sperm quality while SOD activity did not vary with seminal quality. After incubation at 15 °C for 6 h, there was no reduction in the activity of SOD and GSH-Px (Marti et al., 2003). After a freeze-thawing cycle, however, the activity of GSR and GSH-Px remained stable, while SOD activity was about half that observed in fresh and refrigerated semen (Marti et al., 2008). In frozen ram semen, Bucak et al. (2008) detected lesser activity of GSH-Px and CAT.

Controversial results have been reported regarding the inclusion of SOD in extenders. Maxwell and Stojanov (1996) and Forouzanfar et al. (2013) reported that addition of 800 U/mL or 150 μM of SOD to the extenders provided greater protection to ram sperm cells submitted to refrigeration. However, Silva et al. (2011) did not found any enhancement in sperm kinematic parameters when adding SOD (25, 50 and 100 U/mL) to the freezing extender, but ultrastructural analysis revealed that addition of 100 U/mL of SOD preserved the integrity of the acrosome, and enhanced preservation of the mitochondria.

Catalase (CAT) has been used in extenders to improve the antioxidant capacity of semen and preserve sperm function (Maxwell and Stojavone, 1996; Upreti et al., 1998; Maia et al., 2009). Maia (2006) observed that there was a greater percentage of intact plasma and acrosome membranes when ram semen was cryopreserved with a Tris-hydroxymethyl aminomethane (Tris) egg yolk extender containing 50 μg/mL of catalase. Moreover, the inclusion of 100 and 200 U/mL of catalase in diluents can prevent the harmful effects of cooling on total motility (Câmara et al., 2011a) and on survival of ram sperm (Maxwell and Stojanov, 1996) during liquid storage at 5 °C. Nevertheless, concentrations of catalase greater than 200 U/mL were toxic to the sperm.

Most enzymatic systems for the control of cellular peroxide concentrations consist of glutathione peroxidases and several ancillary enzymes required for the synthesis and reduction of glutathione (GSH). Amid et al. (2016) reported in a review that glutathione peroxidase (GSH-Px, GPx) acts upon GSH to reduce hydrogen peroxide to H_2O and lipoperoxides to alkyl alcohols. The GSH subsequently can be regenerated from its oxidized form (GSSG) by glutathione reductase (GSR), the activity of which is inducible upon oxidative stress.

In fresh semen, the addition of GSH also had no effect on the kinematic parameters of ram sperm (Câmara et al., 2011b).

However, in a frozen-thawed ram sperm, Bucak et al. (2008) observed that adding 5 mM of GSH or GSSG to semen extender resulted in an increase in the GSH concentration and GSH-Px activity, but had no effect on lipid peroxidation or sperm motility and viability. Silva et al. (2011) also found no effect of the addition of GSH (2.5 and 7 mM) on the kinematics of the post-thaw ram sperm, except with regard to progressive motility, such that use of 7 mM GSH led to lesser values. Thus, the addition of enzymes to the cooling/freezing extender may or may not improve ram semen quality after in vitro storage

2.2. Vitamins

The presence of vitamins in semen has an important role in sperm quality and at least four vitamins having and important effect. Among these are, alpha-tocopherol, one of the primary sperm antioxidants, which is abundant in the sperm membrane (Aitken, 1995). Vitamin E is the main lipophilic antioxidant that protects polyunsaturated fatty acids in tissues against peroxidation. Vitamin E is a potent peroxyl radical remover (LOO·) and probably the most important inhibitor of the lipoperoxidation chain reaction in animals (Halliwell and Gutteridge, 1999). The addition of vitamin E in different forms (Trolox, α-tocopherol) to ram semen preservation media can improve sperm quality. Kheradmand et al. (2006) reported that the addition of 1 or 2 mg of vitamin E to the egg-yolk/citrate buffer improved the motility and sperm membrane integrity in chilled ram semen. Similarly, Anghel et al. (2010); Azawi and Hussein (2013) and AminiPour et al. (2013) reported that the addition of vitamin E increased the viability and motility of sperm stored at 5 °C for 120 h and after cryopreservation.

Trolox, a water-soluble analog of vitamin E, is a chain-breaking antioxidant that functions as a scavenger of lipid peroxyl radicals (Ross et al., 1995). Its protective effect against lipid peroxidation has been reported for ram semen (Maia et al., 2010; Sicherle et al., 2011; Mata-Campuzano et al., 2014). Mata-Campuzano et al. (2014) reported that the use of Trolox during liquid storage of ram semen had a negative effect on sperm quality. However, Trolox also had a beneficial effect on the post-thawing quality of cryopreserved ram sperm by increasing the integrity of the plasma membrane, as well as motility and viability (Silva et al., 2013). Maia et al. (2007) reported that Trolox at concentrations from 50 to 100 μM increased post-thaw sperm motility, while concentrations that were greater than 100 μM sperm had irreversible effects on the same parameters. Maia et al. (2009), however, observed that addition of Trolox to the freezing extender failed to improve ram sperm motility and viability (intact plasma membrane). The variable outcomes of the addition of Trolox to the preservation media may be due to a dose-dependent effect.

Ascorbic acid, also known as vitamin C, is a water-soluble vitamin that is essential for the normal functioning of the body. It has been associated with fertility but its exact role in reproductive physiology is uncertain (Sönmez et al. 2005). Ascorbic acid may have a role in maintaining the genetic integrity of sperm cells by preventing oxidative damage to sperm DNA (Fraga et al., 1991). Azawi and Hussein (2013) observed an improvement in the viability and motility of Awassi ram sperm extended with Tris-based diluents containing vitamin C at 0.9 mg/mL and stored at 5 °C. In contrast, Sánchez-Partida et al. (1997) reported that the inclusion of ascorbic acid in Tris-based extender at 50 or 100 mM reduced the percentage of motile sperm after thawing compared to the control. Ascorbic acid (AA) may be play a pro-oxidative activity in the presence of transition metals ions (Rietjens et al., 2002; Amid et al., 2016). When ferrous ion is present AA convert $Fe3+$ into $Fe2+$, which reacts with oxygen or hydrogen peroxide resulting in formation of hydroxyl radicals, which then trigger lipid peroxidation (Rietjens et al., 2002). Egg yolk present in the extender contains iron and $Fe3+$ is easily release from ovotransferrin (Ahn, 2014). It is possible that this has caused reduction on sperm motility.

Vitamin B_{12} is another water-soluble vitamin that functions as a coenzyme in a number of biochemical reactions, such as methionine synthesis and the metabolism of branched amino acids (Juanchi, 2000). A modified Tris citrate solution or Tris extender supplemented with vitamin B_{12} at 2 mg/mL improved sperm kinematics in crossbred and Dallagh ram in liquid and frozen forms (Asadpour et al., 2012; Hamedani and Tahmasbi, 2013). In another study, Fu and Youzhang (2003) indicated that vitamin B complex (3% v/v) could improve post-thaw motility and protect the integrity of ram sperm membranes during cryopreservation.

2.3. Amino acids and proteins

Amino acids are important non-enzymatic scavengers with certain antioxidant properties and are present in seminal plasma (SP) at high concentrations. It has been demonstrated that supplementation of extenders using amino acids (e.g. taurine, hypotaurine, proline, glutamine, glycine, histidine, and cysteine) reduced DNA fragmentation and improved the post-thaw motility, viability, membrane integrity and fertility of ram sperm (Sánchez-Partida et al., 1997; Bucak et al., 2009; 2013).

Cysteine is a low molecular weight amino acid containing thiols that participates in glutathione biosynthesis. Cysteine protects sperm from toxic oxygen metabolites induced by lipid peroxidation (Meister and Tate, 1976). Cysteine and glutathione maintain sperm quality and prevent membrane losses and the acrosome integrity of post-thawed ram sperm (Bilodeau et al., 2001). Uysal and Bucak et al., 2007 reported that cysteine at 10 mM protected sperm characteristics after the freezing-thawing process to a greater extent than other concentrations (0, 5, and 20 mM). A Tris-based extender supplemented with cysteine at 1 mM (Coyan et al., 2011) or 5 mM (Bucak et al., 2008) or soybean lecithin extender supplemented with 10 mM of cysteine (Sharafi et al., 2015) resulted in greater post-thaw motility and plasma membrane integrity of post-thawed ram.

Methionine is another amino acid that may be added to ram semen. At 1, 2 and 4 mM methionine improves sperm motility, viability and mitochondrial activity of ram sperm during liquid storage (Coyan et al., 2010; Bucak et al., 2012). Bucak et al. (2009) also reported that a freezing extender supplemented with 5 mM of glutamine improves motility and the results of the hypo-osmotic swelling test (HOST). This finding was strengthened by the study of Roostaei-Ali Mehr and Noori et al. (2013) who observed that sperm motility, viability and functional membrane integrity were improved when 40 to 80 mM of L-Glutamine was added to a conventional freezing medium.

Ergothioneine (2-mercaptohistidine trimethylbetaine) is a scavenger for singlet oxygen hydroxyl radicals and peroxyl radicals (Dahl et al., 1988; Akanmu et al., 1991) and can have beneficial effects on semen quality. When supplementing with ergothioneine at 2 and 4 mM, Coyan et al. (2011) reported an increased subjective sperm motility and progressive motility compared to the controls, following the freeze-thawing process. Also, Najafi et al. (2014a) reported the addition of 6 mM of ergothioneine improved total sperm motility, viability, and membrane functionality and reduced lipid peroxidation during cryopreservation.

Taurine is a sulfonic amino acid that is present in both epididymal and oviduct fluid, and is one of the major non-enzymatic scavengers that has an important role in the protection of sperm against ROS and lipid peroxidation (Sharma and Agarwal, 1996; Saleh and Agarwal, 2002). A beneficial effect of taurine incorporation in extender improves the quality of refrigerated (Rather et al., 2016) as well as post-thaw motility, viability and membrane integrity of ram sperm (Sánchez-Partida et al., 1997; Uysal et al., 2005; Banday et al., 2017). Sangeeta et al. (2015a) have reported that Tris egg yolk supplemented with "L"- glutamine" and "L"- proline" improved total sperm motility compared to the control pre-freezing (equilibrated semen at 4 °C for 2 h) and post-thawing. Inclusion of L-proline and L-glutamine in the diluent also increased the percent of live sperm and total motility and resulted in a greater functional membrane and acrosome integrity than the control group for ram epididymal semen (Sangeeta et al., 2015b).

In terms of proteins, bovine serum albumin (BSA) protects the membrane integrity of sperm cells against heat (Lewis et al., 1997). Uysal and Bucak et al., 2007 reported that extender supplementation with BSA had a positive effect in protecting ram sperm characteristics during the freeze-thawing process. Furthermore, in other studies it has been reported that 10% or 15% of BSA can be used as a substitute for egg yolk in ram semen diluents and enhanced sperm motility and viability following the freeze-thaw process (Matsuoka et al., 2006; Fukui et al., 2007).

2.4. Plant extracts

There have been several sudies where spices and herbs having high antioxidant activities and/or containing phytohemicals such carotenoids, flavonoids and other phenolic compounds have been used for semen processing (Krishnaiah et al., 2011; Embuscado, 2015). The extracts of different kinds of plant materials (*Syzygium aromaticum*, *Rosmarinus officinalis, Camellia sinensis)* have been used to improve ram semen quality (Del Valle et al., 2013; Motlagh et al., 2014; Mehdipour et al., 2016). For example, clove (*Syzygium aromaticum*) is rich in phenolic acids, flavonol glucosides, phenolic volatile oils (eugenol, acetyl eugenol, isoeugenol) and tannins. These compounds provide for the capacity to function as a scavenger for free radicals and as a chelator for metals. Baghshahi et al. (2014) reported that the use of clove buds to supplement Tris egg yolk extender at 35 and 75 μg/mL improved the motility of ram sperm after cooling and freezing–thawing compared to 0 and 115 μg/mL. In the same way, where soybean lecithin-based extender supplemented with 4% and 6% of rosemary aqueous extract improved the percentages of total motility, progressive motility, and plasma membrane functionality of ram frozen semen (Gil et al., 2010; Motlagh et al., 2014). In summary, the beneficial effect of rosemary and clove on sperm parameters is probably due to the antioxidant capacity of these plant materials.

In addition to clove (*Syzygium aromaticum*) and rosemary, green tea (*Camellia sinensis*) contains a variety of biologically active compounds such as polyphenols, catechin, epicatechin, epigallocatechin, epicatechin gallate and apigallocatechin- 3- gallate (Wittayarat et al., 2013), all of which have large amounts of free radical scavenging activity (Nakagawa and Yokozawa, 2002). Mehdipour et al. (2016) reported that the addition of green tea extract to soybean lecithin extender protected ram sperm against damage caused by oxidative stress during cryopreservation.

Soybean lecithin is another plant component that can be used for sperm preservation. It protects the sperm membrane by stabilizing and replacing phospholipids, thus increasing tolerance to the freezing process (Quinn et al., 1980; Watson, 1981; Forouzanfar et al., 2010; de Paz et al., 2010). During the cryopreservation of ram semen, Tris extender supplemented with 1.5% soy lecithin (Forouzanfar et al., 2010; Emamverdi et al., 2013; Najafi et al., 2014b) enhance most semen quality parameters. Nevertheless, during liquid storage at 5 °C the addition of 3.5% soybean lecithin to the extender resulted in decreased motility and mitochondrial activity (Mata-Campuzano et al., 2014). The negative effect of soybean lecithin on mitochondrial activity was also observed in thawed ram semen by Mata-Campuzano et al. (2015b).

Recent studies indicate that the addition of argan oil and cactus seed oil in small amounts to Tris egg yolk/skim milk extenders increased the total sperm motility, progressive motility, viability and membrane integrity, but decreased the spontaneous and induced lipid peroxidation and DNA fragmentation in ram semen at refrigeration temperature (Allai et al., 2015, 2017). Similar effects have been repported when 1% of *Opuntia ficus-indica* extract was added to extenders such as Tris or milk during liquid storage up to 72 h of storage (Allai et al., 2016).

2.5. Other compounds

There are many other compounds that protect semen from the damage caused by liquid or frozen storage deserves mention in this review. Trans-resveratrol and quercetin are non-flavonoid and flavonoid polyphenols, respectively, with antioxidant activity (Stojanović et al. 2001). In ram sperm, the addition of either resveratrol or quercetin (5–20 μg/mL for each compound) to a Tris egg yolk extender decreased the mitochondrial membrane potential, which improved sperm viability (Silva et al., 2012a,b). Furthermore, use of resveratrol inhibited lipid peroxidation of ram sperm most effectively when used at relatively lesser concentrations (15 μg/10^9 sperm), as indicated by the thiobarbituric acid reactive substances (TBARS) test (Sarlos et al., 2002).

Royal jelly (RJ) is mainly composed of water (60%–70%), protein (12%–15%), sugars, lipids, vitamins, salt and free amino acids (10%–16%). It was used at 0.5% or 1% in Tris egg yolk extender to increase the sperm viability, kinetics and plasma membrane functionality of ram sperm during liquid storage (Moradi et al., 2013).

Another compound that has been used to supplement extenders is butylated hydroxytoluene (BHT). The BHT is a synthetic phenolic antioxidant that inhibits the free radical-chain reaction by donating a hydrogen atom (Embuscado, 2015; Yehye et al., 2015). It's use efficiently inhibits lipid peroxidation reactions in biological membranes (Black, 2002). The BHT supplementation of semen extender at a concentration of 0.5–2.0 mM has improved sperm quality and reduced lipid peroxidation in different species (Memon et al., 2012; Khumran et al., 2015; Patel et al., 2015; Trzcińska et al., 2015; Alcay et al., 2016; Seifi-Jamadi et al., 2016). In frozen ram semen, Farshad et al. (2010) reported that Tris/citrate/fructose/yolk supplemented with 2 mM of BHT improved sperm motility, progressive motility, viability and membrane integrity compared to other concentrations.

Use of caffeine (1, 3, 7-trimethyl-2, 6-dioxypurine) has also been reported to have the capacity to stimulate motility and improve ram sperm quality (Anel et al., 1984). For example, Soeparna et al. (2011) reported that Tris egg yolk extender supplemented with 4 mM of caffeine improved the quality of Garut ram sperm. Using the same concentration, Špaleková et al. (2014) reported that caffeine had a positive effect on refrigerated sperm motility and maintained sperm motility for a longer time and reduced apoptosis.

Gelatin also improves the quality of ram semen during liquid storage. Meque et al. (2005) compared different concentrations of gelatin (0.5%, 1%, 2% and 4%) in soya milk extender for ram semen liquid storage and concluded that the most effective concentration was 0.5%. Yániz et al. (2005) indicated that adding gelatin to the extender for the solid storage of ram sperm at 15 °C improved the survival and in vitro penetrating ability of sperm as compared to normal liquid extender. 'The proposed mechanism of action of gelatin is based on the hypothesis that it prevents the sedimentation of live and dead cells at the bottom of the sample, thereby enabling sperm to be more uniformly distributed, and minimizing the detrimental effects of pH and toxic metabolic products (Yániz et al., 2005).

Astaxanthin, a powerful lipid-soluble red carotenoid pigment (McNulty et al., 2007) found in certain marine plants and an animal is another semen extender supplement. Astaxanthin is recognized as being one of the most powerful antioxidants in nature (Lai et al., 2004). Fang et al. (2015) investigated the antioxidant effects of astaxanthin on ram semen during preservation and supplementation improved the viability of stored ram sperm by protecting plasma membrane integrity.

Lycopene is the most plentiful carotenoid in tomatoes and red fruits and is considered to be the most effective antioxidant of all carotenoids (Di Mascio et al., 1989). More precisely, Peker akalin et al. (2016) reported that lycopene added to Tris-based extenders improved ram sperm motility, viability, mitochondrial activity and oxidative stress parameters during liquid storage at 5 °C. In frozen ram semen, Uysal et al. (2007) reported that lycopene had many beneficial effects on cryosurvival by stabilizing the post-thaw reduction in sperm motility, viability and osmotic resistance. Canthaxanthin (β, β-carotene 4, 4′ dione) is another carotenoid found in plants, green algae, bacteria, crustaceans, fish, and birds. There have been some reports on the protective effects of canthaxanthin as an antioxidant in cell cultures in vitro. The addition of 10 and 25 μM of canthaxanthin to a Tris egg yolk extender used for ram semen cryopreservation improved the protection of ram semen from kinetic changes after incubation at 37 °C for 2 h post-thawing (Souza et al., 2017).

3. Seminal plasma

The use of seminal plasma (SP) collected from the same or different species in liquid or frozen extenders has been assessed for its capacity to minimize the negative effects of lower temperatures and cryodamage on mammalian sperm (Baran et al., 2004). The SP contains protein, enzymes and non-enzyme antioxidants that enhance the protection of sperm from oxidative stress and prevent capacitation of ram sperm (van Overveld et al., 2000; Maxwell et al., 2006). It has also been reported that the addition of SP improves characteristics of liquid and frozen-thawed ram sperm such as motility, viability, acrosome integrity, capacitation and mitochondrial respiratory activity (Ashworth et al., 1994; Graham, 1994; Maxwell and Johnson, 1999; López-Pérez et al., 2001; El-Hajj Ghaoui et al., 2007; Ghaoui et al., 2007; Domínguez et al., 2008; Leahy et al., 2010; Bernardini et al., 2011; Rovegno et al., 2013). Mata-Campuzano et al. (2015a) have reported that SP at 20% and 40% concentrations had a protective effect on ram sperm motility after 24 h of liquid storage. Furthermore, Ustuner et al. (2016) reported that with the use of 1% and 10% rainbow trout SP had cryoprotective effects on ram semen during 5 h of incubation.

4. Sugars

Both monosaccharides and disaccharides have been extensively used for storage of ram semen. Sugar has several functions, such as serving as the energy substrate for sperm cells during incubation (Fukuhara and Nishikawa, 1973) and maintaining the osmotic balance of the diluents (Aboagla and Terada, 2003). Previous studies have compared the effects of different concentrations of monosaccharides (glucose, galactose and fructose), disaccharides (sucrose, trehalose) and trisaccharides (raffinose) on sperm parameters in ram semen. Glucose was suggested to be more suitable than fructose, lactose or raffinose in a Tris-based extender for ram semen (Salamon and Visser, 1972).

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside), also known as mycose or tremalose, is a natural alpha-linked disaccharide formed by an α, α-1,1-glucoside bond between two α-glucose units. As a non-reducing sugar, trehalose is a non-permeable disaccharide. It has a protective effect related to both osmotic and specific interactions with membrane phospholipids, resulting in hypertonic dehydration. The use of trehalose can cause cellular osmotic dehydration before freezing and thus reduce cell injury by inhibiting ice crystallization (Schmehl et al., 1986). Trehalose has been used in many experiments to protect ram sperm. Molinia et al. (1994) concluded that trehalose is more suitable for preserving the motility of frozen-thawed ram sperm. Studies have demonstrated that supplementing Tris-based extender with 50 or 100 mM trehalose containing glycerol (5%) and egg yolk (5%) improved the motility (Bucak et al., 2007), viability and membrane integrity (Jafaroghli et al., 2011), and increased frozen-thawed ram sperm

quality (Ahmad et al., 2015). Furthermore, Aisen et al. (2002) reported that 100 mOsm concentration of trehalose had a beneficial effect on sperm motility and acrosome integrity.

Raffinose, a trisaccharide, plays a cryoprotective role by decreasing intracellular ice crystal formation through its interaction with membrane lipids and proteins during cryopreservation (Agca et al., 2002). Nonetheless, Bucak et al. (2013) reported that 10 mM of raffinose in a Tris-based extender maintained motility, viability, mitochondrial activity and acrosome integrity in frozen-thawed ram sperm. Similarly, Jafaroghli et al. (2011) reported an increase in sperm viability and motility with decreased acrosome and total sperm abnormalities in thawed ram sperm frozen with 70 or 100 mM of raffinose in a Tris-based extender.

5. Fatty acids

The supplementation of extenders by fatty acids has mostly been studied in species other than sheep. Hammerstedt et al. (1990) and Hammerstedt (1993) reported that the lipid composition of the sperm plasma membrane is a major determinant of sperm viability. Thus, ram sperm has a greater polyunsaturated/saturated fatty acid ratio than other species (Maxwell and Watson, 1996). This ratio determines the sensitivity of sperm to cold shock (White, 1993). The high content of polyunsaturated fatty acids within the plasma membrane is assumed to impart greater fluidity and a lesser resistance to cold shock due to the presence of many double bonds (Giraud et al., 2000). Unsaturated fatty acids make the sperm susceptible to peroxidation. Previous studies have revealed inconsistent effects of O-3 polyunsaturated fatty acids (PUFA) on sperm quality with use in diets or extenders (Castellano el al., 2010). In contrast, saturated fatty acids are less vulnerable to peroxidation than unsaturated fatty acids (Rael et al., 2004).

Recently, Zadeh Hashem et al. (2017) reported that oleic acid supplementation increased the viability, plasma membrane integrity, total antioxidant capacity and superoxide dismutase and decreased the amounts of malondialdehyde and nitric oxide during liquid storage of ram sperm. Besides oleic acid and linoleic acid, the positive effects of docosahexaenoic acid (DHA) on semen have been shown in feeding trials in sheep (Samadian et al., 2010; Esmaeili et al., 2014; Jafaroghli et al., 2014).

6. Cryoprotectants

Cryoprotectants are substances that are added to the dilution medium to protect the cell against the various injuries inherent in the cryopreservation process. The addition of cryoprotective agents to diluents is important for protecting spermatozoa from freezing damage (Singh et al., 1995), and all freezing media contain permeable and non-permeable cryoprotectants.

Glycerol is the most penetrating cryoprotectant used in diluents for freezing ram semen, whereas egg yolk is a non-penetrating cryoprotectant (Salamon and Maxwell, 2000). The amount of glycerol added to the diluents for cryopreservation of ram semen is limited because of its potential cytotoxicity (Holt, 2000; Watson, 2000). The concentration providing the most desirable post-thaw survival rate is between 4% and 7%. Concentrations of greater than 6% are detrimental to sperm survival because glycerol more readily penetrates the ram sperm (Salamon and Maxwell, 2000). Results of several studies indicated the addition of glycerol at concentrations of 3%–7% in diluents containing 5% to 20% egg yolk provided desirable post-thaw motility recovery (44% to 85%) of ram sperm (Moses et al., 1995; Gil et al., 2000; El-Alamy and Foote, 2001; Anel et al., 2003; Gil et al., 2003). Pelufo et al. (2015) evaluated the effects of the addition of glycerol and/or the disaccharides sucrose and trehalose to hypertonic diluents at 30 or 5 °C in ram semen cryopreservation. It was found that there were no differences in the effect of glycerol at 30 and 5 °C on sperm motility and acrosome integrity, except in the HOST, in which the plasma membrane integrity was preserved to a greater extent if glycerol was added at 5 °C.

According to Salamon and Maxwell (2000) several cryoprotective agents other than glycerol, such as dimethylsulfoxide (DMSO), ethylene glycol, albumin, low molecular weight polyols, polymeric compounds, surfactants, sugars of various types and amino acids have also been examined for cryoprotective action on ram spermatozoa. None of these proved to be more desirable than glycerol as a cryoprotective agent. Nur et al. (2010) investigated the effects of glycerol (6%), propanediol (6%), sucrose (62.5 mM) and trehalose (62.5 mM) in a Tris-based extender with 20% egg yolk on post-thaw sperm parameters in ram semen and found that all cryoprotectants negatively affected sperm motility, sperm morphology and DNA integrity. Silva et al., 2012a,b compared ram semen diluted in a Tris egg yolk extender containing glycerol (5%), ethylene glycol (3% or 5%) or acetamide (3% or 5%), and found that ethylene glycol as well as glycerol were effective at protecting progressive sperm motility, acrosome integrity and oxidative stress during the freezing process. In turn, the greatest protection of plasma membrane integrity resulted from use of 5% glycerol. Similarly, while assessing the effectiveness of dimethylformamide alone or combined with glycerol, as a cryoprotectant for freezing ram semen, Moustacas et al. (2011) found that plasma and acrosomal membrane integrity were preserved to a greater extent in the extender containing 5% glycerol. Furthermore, the use of extenders containing pure dimethylformamide, or more than 2% in combination with glycerol, resulted in sperm motilities close to zero.

Egg yolk protects the sperm against the damaging effects of cold shock, preserves sperm motility, reduces the loss of acrosomal enzymes and maintains the mitochondrial membranes of sperm (Parks and Graham, 1992; Holt, 2000; Salamon and Maxwell, 2000) and its inclusion allows for a reduction of glycerol concentration in the medium. There is a wide variation in the concentrations of egg yolk used in the diluents for the freezing of ram semen. The use of the relatively greater egg yolk concentrations do not necessarily result in increased preservation of sperm motility. Gil et al. (2003) observed that an increase in egg yolk concentration (greater than 5%) in a milk-based extender did not improve post-thaw motility.

Although egg yolk benefits sperm that are cryopreserved, being of animal origin it could represent a potential source of microbiological contaminants that may compromise the quality of sperm (Gil et al., 2003). For this reason, powdered egg yolk could be a safer alternative to fresh egg yolk, as it undergoes a pasteurization process to destroy bacteria (Marco-Jiménez et al., 2004; García

et al., 2017).

To determine whether the source of egg yolk included in a freezing diluent affected ram sperm, Marco-Jiménez et al. (2004) compared fresh egg yolk and powdered egg yolk at 10%, 15% and 20% in diluents for cryopreservation and determined the bacterial contamination in the diluents and the quality of ram sperm after freezing and thawing. From this study, it was reported that the use of media containing powdered egg yolk resulted in greater percentages of total motile cells compared to samples containing fresh egg yolk and that the microbiological contamination was similar in both diluents. Alcay et al. (2015) compared lyophilized egg yolk with fresh egg yolk in extender for the cryopreservation of ram semen using quality assessment such as sperm motility and plasma membrane, acrosome and DNA integrity, and found that lyophilized egg yolk provided similar cryoprotective effects as fresh egg yolk extender and it was concluded that lyophilized egg yolk was effective in ram semen cryopreservation protocols. Gholami et al. (2012) reported a greater post-thaw motility, viability and acrosomal integrity of ram sperm with extender containing turkey egg yolk as compared to chicken egg yolk.

7. Pregnancy rates following use of liquid and frozen storage of semen with extenders supplemented with different compounds

Low pregnancy rates after cervical insemination with fresh and frozen sperm negatively influence the dissemination of the technique. In general, investigators have not performed in vivo assessments of fertility rates because there is no consistency between the results of sperm quality assessment in vitro and field fertility after AI (Sanchez et al., 1999; Paulenz et al., 2003; O'Meara et al., 2005). This highlights the fact that the success of AI is not only dependent on viable semen and successful preservation of semen. For example, it is known that the anatomic structure of the cervical canal of the ewes limits the passage of insemination catheters and thus the fertility success rate when using AI is decreased because of having to get the sperm past this anatomical structure into the uterus. Sperm quality is still an important factor when determining the efficiency of AI in sheep. Different variables of sperm quality have been used to assess those that are the most reliable indicators of the pregnancy rate improvement in sheep and most of these are summarized in Table 1. For example, Tervit and MacMillan (1983) reported that there was marked improvement in fertility if ram semen was frozen in egg yolk-based diluents containing BHT. Aisen et al. (2002) reported that trehalose inclusion in semen extenders enhanced post-thaw fertility. Moreover, Colas et al., 1968 recorded greater fertility (58%) when ram semen was stored for 26 h at 15 °C. The addition of SP has also been reported to improve fertility after artificial insemination (Maxwell et al., 1997; Gunay et al., 2006). Furthermore, the addition of homologous SP (30%–50% v/v) to milk and egg yolk based extenders improved the fertility rates with use of liquid-stored (5 to 15 °C) and frozen-thawed sperm after intracervical AI (Maxwell et al., 1999; Belibasak et al., 2000; Lopez-Perez and Perez-Clariget, 2012). Supplementation with 40% boar SP in cryopreserved sperm increased the pregnancy rate in sheep following intracervical AI (Fang et al., 2018). Fertility was also improved when Tempol (a stable nitroxyl antioxidant) was used for storing ram sperm at 15 °C (Mara et al., 2005). In addition, Bucak et al. (2009) reported that in ram semen supplementation of the extender with glutamine (5 mM) improved the fertility.

Adding 0.30 g of fish oil per 1 g of egg yolk based extender resulted in improved fertility when ewes were AI with semen stored in this extender (Abdi-Benemar et al., 2015). Likewise, the fertility rate obtained after cervical insemination of ewes using frozen-thawed ram sperm was enhanced by supplementation with 100 mM trehalose (Jafaroghli et al., 2011). Also, a preliminary artificial insemination trial indicated that supplementation with GSH in stored liquid ram semen has the potential to improve lambing rates to AI (Mata-Campuzano et al., 2014). Kubovičová et al. (2010) also reported that the addition of glutathione to liquid stored ram semen had a positive effect on the fertilization and pregnancy rate of ewes after cervical insemination.

Table 1
Summary of noteworthy results on fertility of ram semen after insemination.

Supplement	Type of insemination	Conservation Type	Extender	Controls (%)	Improved fertility (%)	Reference
GSH	Cervical	Fresh	EquiPro® (Minitüb)	76	81	Kubovičová et al., 2010
(SOD) and (CAT)	Intra-uterine	Fresh	Tris-glucose-yolk diluents	16	41	Maxwell and Stojanov, 1996
(GSH)	Cervical	Fresh	Tris-citrate-fructose	7	37	Mata-Campuzano et al., 2014
TEMPOL 24 h of storage	Cervical	Fresh	Sodium citrate	19	67	Mara et al., 2005
TEMPOL 72 h of storage	Cervical	Fresh	Sodium citrate	0	52	Mara et al., 2005
Seminal plasma	Cervical	Fresh	Skim milk	5	86	Belibasak et al., 2000
Trehalose	Cervical	Frozen	Tris-fructose-egg yolk	18	47	Aisen et al., 2002
0.30 g fish oil	Intra-cervical	Frozen	Tris-citrate-fructose	18	47	Abdi-Benemar et al., 2015

EquiPro® (Minitüb) is an Equine Semen Extender that is composed of a blend of glucose, sucrose, non-fat dry milk, and antioxidants; SOD, superoxide dismutase; CAT, catalase; GSH, glutathione or reduced glutathione; TEMPOL, (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl or 4-hydroxy TEMPO) is a stable nitroxyl antioxidant that has been used as a protectant in disorders that involve reactive oxygen species (ROS).

8. Concluding remarks and future prospects

In the present review, the effects of the most commonly assessed antioxidants, seminal plasma, sugars and fatty acids when added to extenders on the quality and fertility of ram sperm during liquid or cryopreservation storage were examined. Understanding the biological mechanisms involved by the inclusion of such supplements on semen quality may allow improvement of the existing extenders or development of novel extenders that may enhance the quality of preserved ram sperm and consequently achieve more desirable fertilization rates.

Conflicts of interest

None.

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