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Update on semen cryopreservation in sheep and goats: A review

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Abstract Despite the long history of artificial insemination, its widespread use in many species, including the poultry, sheep, and goat, has been faced with many challenges especially when frozen semen is used. The freezing-thawing process results in physical and chemical insults on the sperm which subsequently decrease the fertility of the sperm. The decrease in fertility is much greater in most species compared with the species such as cattle. Many factors affect the fertility of the frozen-thawed sperm, and different procedures, including the use of various extenders, have been investigated to improve the quality of frozen-thawed sperm. Glycerol and egg yolk are traditionally used as the protective components in many extenders. Despite the positive effects of the egg yolk (mainly as a result of its low-density lipoproteins), there have been concerns with the use of animal products in semen extenders. Therefore, attempts have been made to substitute the egg yolk with other sources such as lecithin. There are reports that several additives such as disaccharides, antioxidants, and essential oils may have beneficial effects on the fertility of frozen-thawed sperm. Variable success rates were reported, and a small number of publications contained fertility data. In this paper, I shall review the most recent research findings on frozen-thawed sperm in sheep and goats, supplemented with data on other mammals when appropriate.

Keywords: artificial insemination, semen freezing, sperm cryostorage, ram, buck, mammals

Introduction

Artificial insemination (AI) is the most effective reproductive technology for the genetic improvement of livestock species. Despite the widespread application of AI in cattle, its use in genetic improvement of sheep and goats, especially native breeds, has been very limited due to specific challenges facing the use of reproductive technologies in most countries (Holt, 2000; Leboeuf et al., 2000). In most developing countries, including Iran, AI is mostly used in upgrading programs aimed at increasing the productivity of local breeds through insemination of imported semen or semen prepared locally from exotic animals having the desired characteristics. With the exception of several countries, sheep AI has not become a common practice in most sheep producing regions (Gordon, 1997).This is due to problems relating to the management of flocks, the cost involved in AI, and the handling procedures necessary for ram semen. The fact that most sheep AI, as currently practiced in Russia, is still based on fresh undiluted semen shows that methods of dilution and frozen storage of semen need much more refining for use in this species (Gordon, 1997), as well as in goats.

A single ejaculate contains many more sperm cells than needed to fertilize a single female, and for more extensive use of a proven sire, the ejaculate needs to be extended or diluted. Besides, the availability of procedures for long-term preservation of extended semen would increase the flexibility of AI. At prevailing ambient temperatures, the fertilizing capacity of un-extended semen decreases rapidly due to increased metabolic activity of the sperm cells. Decreasing the semen storage temperature, and provision of suitable energy sources would, to some extent, increase the fertile of sperm cells. On the other hand, cooling or freezing procedures, and thawing of frozen semen, will damage the cells, resulting in decreased sperm longevity and fertility. Freezing is the preferred method of semen preservation, and while the spermatozoa of some species are more tolerant of the cooling insults, sheep and goat spermatozoa are more prone to the detrimental impacts of cooling and freezing (Evan and Maxwell, 1987).

Effects of low-temperature preservation on sperm structure and function

Low temperature, especially sub-zero one, imparts physical and chemical changes in the cell membrane resulting in damages in sperm functionality or even death. These damages are due to oxidative stress, and cold and osmotic shocks imparted on the sperm during cooling, freezing, and thawing procedures (Aitkin, 2020). As a result of these changes, sperm motility and fertility will be reduced (Salamon and Maxwell, 2000; Aisen et al., 2005; Aitkin, 2020). Despite many years of research, the quality of cold or frozen spermatozoa has not improved as desired. During cold storage, reasonable sperm quality can be obtained only for few days, and cryopreservation in liquid nitrogen or on dry-ice induces substantial damage and death of spermatozoa in many animals, including the ovine and caprine spermatozoa. Many endogenous and exogenous factors impact on the quality of cryopreserved spermatozoa, including the species, breed, individual variations, seasonal variations in the quality of sperm, sensitivity of the acrosome to temperature, and intracellular ice crystal formation (La Falci et al., 2002). Detrimental effects of the reactive oxidative stress on spermatozoa have been reviewed over the past decades, and very recently by Professor R. J. Aitkin, a well-known authority in this field (Aitkin, 2020).

Early research from 1930 to 1993 on the storage of sheep semen at reduced temperature (0-15°C) and at ambient temperature was reviewed by Maxwell and Salamon (1993). A rapid decline in fertility occurs when ram semen is stored for more than 24 h and used in cervical insemination. It is noteworthy that in New Zealand, Upreti et al. (1995) developed a chemically defined ram semen diluent which maintained sperm motility in diluted semen incubated at 38°C for about 24 h, whereas the conventional milk-based diluent supported motility for less than 6 h at that temperature.

Semen extenders must provide a suitable pH, buffering capacity, and osmolality, and should be able to protect the spermatozoa from cryogenic injury (Salamon and Maxwell, 2000). The extender for cryopreservation of spermatozoa usually contains a non- penetrating cryoprotectant (e.g., milk or egg yolk), a penetrating cryoprotectant (e.g., glycerol, or dimethyl sulfoxide), a buffer (e.g., Tris), sugars (e.g., glucose, raffinose, or trehalose), a salt of citric acid, and some antibiotics; frequently, dried skimmed milk or Tris–glucose based hypertonic diluents are used (Evans and Maxwell 1987; Fischer et al., 1987; Medeiros et al., 2002; Purdy, 2006).

Penetrating cryoprotectants increase membrane fluidity, cause greater dehydration at low temperatures, and decrease the formation of intracellular ice crystals which can damage the plasma membrane. On the other hand, non- penetrating cryoprotectants decrease extracellular ice formation by lowering the freezing temperature of the medium (Amman, 1999; Aisen et al., 2002; Holt, 2000).

Sperm membrane non-diffusible carbohydrates such as sucrose, lactose, raffinose, or trehalose are used to induce cell dehydration and decrease intracellular ice formation (Salamon and Maxwell, 2000; Aisen et al. 2002). In Tris extenders, monosaccharides provide a greater cryoprotective effect than do disaccharides (Molinia et al., 1994). It has been reported that high trehalose, by increasing the membrane fluidity, provides the greatest protection in terms of sperm motility preservation, recovery rate, and acrosomal integrity (Aboagla and Terada 2003).

Plasma and outer acrosomal membranes are the most cryosensitive structures.

Biochemical changes resulting from freezing and thawing include: the release of glutamic-oxaloacetic transaminase (GOT); loss of lipoproteins, amino acids, and prostaglandins; decreased activity of phosphatase and acrosomal proteases; decrease in loosely bound cholesterol protein; increase in sodium and decrease in potassium contents; inactivation of hyaluronidase and acrosin; decreased synthesis of ATP and ADP (Salamon and Maxwell, 1995).

Increased membrane permeability and restructuring, and calcium uptake to the sperm cause capacitation-like changes and fusion between plasma and acrosomal membranes, a phenomenon referred to as cryocapacitation (Purdy, 2006).

The shape, and size of the head seem to determine the sensitivity of sperm to cryoinjury. It has been shown that there is a negative correlation between the size of the head and the cryostability of the sperm (Gao et al., 1997). Differences in sperm cryosensitivity can also be attributed to the species (Purdy, 2006; Gangwar et al., 2016), breeds, individuals within breeds, inter-ejaculate variations (Hiemstra et al., 2005; Medrano et al., 2010; Ramon et al., 2013), and composition of the sperm plasma membrane (Bailey et al., 2000; Medeiros et al., 2002). Boar sperm are highly sensitive to cold shock. Bull, ram, and stallion sperm are very sensitive. Dog and cat sperm are somewhat sensitive while rabbit, human, and rooster sperm are the least sensitive to low temperatures (Parks, 1997).

Several mechanisms have been proposed by which oxidative stress can be created in spermatozoa (Aitkin, 2020). The spermatozoa of several species are known to possess L-amino oxidases (Upreti et al., 1998) that are a significant source of damaging ROS in domestic animals (bull, stallion, and ram) exposed to high concentrations of aromatic amino acids in egg yolk-based cryopreservation media. Lipoxygenase is another potential source of ROS, the presence of which may reflect the retention of excess residual cytoplasm as a consequence of defective spermiogenesis and the presence of excess free unesterified free fatty acids (Walters et al., 2018). The mitochondria are a major source of ROS in spermatozoa and are heavily involved in the induction of senescence and apoptosis (Koppers et al., 2011). Many species, not mouse, also possess an NADPH oxidase (NOX5) that is capable of generating ROS in a calcium-dependent manner. Overexpression of *NOX5* has been linked to the loss of sperm motility (Vatannejad et al., 2019). Excessive production of hydrogen peroxide (H_2O_2), the final product of oxidative stress, results in lipid peroxidation of unsaturated fatty acids in the plasma and mitochondrial membranes leading to the production of electrophilic aldehydes. The latter then bind to nucleophilic centers within DNA and proteins, and severely damage the sperm morphology and DNA.

Many strategies have been used to increase the quality of frozen semen such as increasing the concentration of cryoprotectants (Bucak et al., 2007), inclusion of various additives in the extenders (Leahy et al., 2010; Valente et al., 2010), and different methods of thawing and packaging the diluted semen (Chandler et al., 1994). Glycerol is mostly used as an intracellular cryoprotectant in the extender; however, the final concentration of a penetrating cryoprotectant is determined by its toxicity and beneficial effect on the spermatozoa. Glycerol can also induce osmotic damage to spermatozoa (Holt, 2000; Watson, 2000), but the extent of the damage varies according to the species. Goat spermatozoa are reasonably tolerant to these osmotic conditions and can withstand a rapid exposure to glycerol (Purdy, 2006).

According to Büyükleblebici et al. (2014), ethylene glycol or dimethylsulfoxide offered no advantage when replaced glycerol in a Tris-egg yolk extender for freezing Angora goat spermatozoa. Bezerra et al. (2011) demonstrated that dimethylformamide could be used as an alternative cryoprotectant in Tris egg yolk extender for goat semen freezing but concluded that no benefits were derived by using dimethylformamide at an equal 6% concentration.

As a substitute for glycerol, trehalose has been tested in extenders for several species (Molinia et al., 1994; Sanchez-Partida et al., 1998). Trehalose promotes cell dehydration, which reduces the negative effects of water flow through the sperm membrane during freezing (Yildiz et al., 2000) and the formation of ice crystals (Aisen et al., 2002, 2005). Trehalose also interacts with the membrane phospholipids and proteins, providing the membrane more flexibility against cryo-injuries (Aisen et al., 2002; Bucak et al., 2007).

In 1940, Phillips and Lardy showed that egg yolk inclusion in the extender was beneficial to bull spermatozoa stored at 5°C. Since then, egg yolk has become a common extracellular cryoprotectant for spermatozoa of many species, despite contains substances that may interfere with the cell metabolism, and reduce sperm motility (Moussa et al., 2002). Egg yolk gives protection against cold shock during the freezing stage (Tonieto et al., 2010); however, it is less effective for the ram compared with the bull spermatozoa. Egg yolk has been used in extenders for its effect on maintaining the sperm motility and longevity, as well as the integrity of the acrosomal and mitochondrial membranes during physical and chemical stresses (Salamon and Maxwell, 2000). The optimum concentration of the egg yolk in the extender is affected by many factors, including the species, and the type and level of other constituents (Salamon and Maxwell, 2000; Purdy, 2006).

Traditionally, chicken egg yolk has been used as an additive for the freeze preservation of spermatozoa be-

cause of its wide availability. However, the chemical composition of the egg yolks of different avian species varies, particularly in terms of the cholesterol, fatty acid and phospholipid contents (Burris and Webb, 2009), which may influence their effectiveness during cooling, freezing, and thawing steps (Bathgate et al., 2006). It has been shown that egg yolk from avian species, other than the chicken, might be more beneficial for cryopreservation of sperm from stallion (Clulow et al., 2007; Burris and Webb, 2009), jackass (Trimeche et al., 1997), bull (Su et al., 2008), and ram (Kulaksiz et al., 2010; Gholami et al., 2012).

However, the use of egg yolk in the goat semen extenders was met with complications, including sperm coagulation and death (Leboeuf et al., 2000). The harmful interaction between the buck seminal plasma and egg yolk was later reported for milk-based extenders, and was shown to be due to phospholipase A and/or a glycoprotein lipase (named SBUIII, and later BUSgp60) secreted from the bulbourethral (Cowper's) glands. The release of toxic substances as a consequence of the interaction between these secretions and sperm membrane would cause sperm coagulation and death; however, the precise mechanism (s) of action of these secretions has not been verified [see Amoah and Gelaye, 1997; Leboeuf et al., 2000 for more comprehensive coverage of the subject]. The procedures for handling this problem were comprehensively reviewed by Purdy (2006).

Dorado et al. (2007) compared the post-thaw quality of Florida buck spermatozoa extended in two diluents. Tris extender provided more effective preservation of total motility, velocity parameters, and amplitude of lateral head displacement after freezing. The percentage of acrosome intact spermatozoa was significantly higher in samples diluted with milk extender. In the insemination doses, mean values of velocity parameters and lateral head displacement were higher in doses processed in Tris. Although Tris extender resulted in better *in vitro* performance compared to milk extender, these improvements were not reflected in fertility results. Mohammed et al. (2012) studied the effects of breed, semen diluents and freezing method on sperm quality in Aradi and Damascus buck spermatozoa, and showed that in terms of post-thaw semen viability, fertility, kidding, fecundity and prolificacy, milk-based extender was superior to Tris and Na-citrate based diluents. Black Bengal buck semen held good motility and proportion of normal spermatozoa at 4 to 7°C for up to 2 days in the glucose-citrate-egg yolk (GCEY) or Tris-fructose-egg yolk (TFEY) but not in skim milk extenders (Shamsuddin et al., 2000).

Sarıozkan et al. (2010) did not recommend centrifugation and washing of Angora buck semen prior to cryopreservation in Tris egg yolk extender, but suggested that use of Bioxcell with or without centrifugation/washing of semen may be recommended to facilitate semen cryopreservation system. They concluded that differences in fertility rates among extenders (47 to 76%) were not statistically important. Bajuk et al. (2018) suggested that dialysis of Saanen buck semen, using 300-kDa cut-off semi-permeable cellulose tubing, was an alternative method for reducing phospholipase A2 in semen before cryopreservation.

Concerns about the use of egg yolk in semen extenders

Several concerns have been raised in relation to the use of egg yolk in semen extenders, including: 1) concentration of lipoproteins in the yolk varies and is affected by the genetics of the bird, number of days in production, and egg storage condition, therefore, preparation of standard concentrations of yolk in the extender is almost impossible (Moussa et al., 2002); 2) the yolk also contains high-density lipoproteins which to some extent may counteract the efficiency of LDL (Tonieto et al., 2010); 3) the yolk contains progesterone which may advance sperm capacitation (Bencharif et al., 2008); 4) if yolk is contaminated, insemination of the extended semen would increase the chance of female contamination (Gil et al., 2000); 5) some of the yolk constituent increase the extender viscosity, and by preventing cellular respiration decrease the motility and longevity of the spermatozoa (Pace and Graham, 1974; Moussa et al., 2002; Aires et al., 2003); 6) Certain components of the yolk interfere with sperm evaluation, especially with CASA, in the diluted semen (Vera-Munoz et al., 2009); 7) high concentration of yolk in the extender is associated with a lower percentage of intact acrosome (Fukui et al., 2007); 8) dilution of goat semen with extenders containing egg yolk can be detrimental to the sperm cells as described previously.

The beneficial effects of the egg yolk have been attributed to its low-density lipoprotein (LDL) contents; however, the function (s) of the yolk proteins and lipids, if any, in this process has not been elucidated. The egg low-density lipoproteins (LDL) have increased sperm resistance against cold shock in rams (Tonieto et al., 2010), bulls (Moussa et al., 2002), boars (Jiang et al., 2007), and

dogs (Varela et al., 2009). Low-density lipoproteins, by binding to seminal plasma proteins, prevent the outflow of phospholipids and cholesterol from the sperm membrane. The lipids present in the LDL also form a physical barrier that protects the membrane against cold shock (Manjunath and Thérien, 2002; Bergeron et al., 2004; Bergeron and Manjunath, 2006). The phosphatidyl choline and phosphatidyl serine, present in the LDL, replace the membrane phospholipids that are continuously lost during the freezing-thawing process (Graham and Foote, 1987).

Serum albumin, LDL, and lecithin as the extender constituents

Several alternatives were proposed to overcome these limitations of the egg yolk, including the use of serum albumin, LDL, and soybean lecithin.

Bovine serum albumin

Bovine serum albumin has been used in lieu of egg yolk in extenders for the ram (Fukui et al., 2007), turkey (Bakst and Cecil, 1992) and rainbow trout (Cabrita et al., 2001) semen. According to Fukui et al. (2007), there were no significant differences in the pregnancy and lambing rates when ram semen was extended in diluents containing 10% bovine serum albumin or 20% egg yolk. Besides, albumin, being an animal product, could be a source of microbial contamination as is the egg yolk.

Low-density lipoproteins (LDL)

The published data indicated that LDL was at least equal or superior to egg yolk when used in semen extenders for the ram (Tonieto et al., 2010), goat (Ali Al-Ahmad et al., 2008), bull (Moussa et al., 2002; Amirat et al., 2004; Amirat-Briand et al., 2009; Hu et al., 2011), boar (Jiang et al.,2007; Hu et al., 2008), buffalo bull (Akhter et al., 2011), and dog (Bencharif et al., 2008). However, LDL extraction and purification procedures are complex and time-consuming, and LDL preparations have limited storing capacity, requiring continuous extraction.

Soybean lecithin

Lecithin forms about 10% of the yolk phospholipids, and because of its structural similarities with the egg yolk and soya been phospholipids, soybean was suggested as a suitable substitute for the egg yolk in semen extenders, thus reducing the chance of contamination from the animal products (Fukui et al., 2008).

There is a general belief that soybean lecithin-based extenders better preserve the sperm structures than do the egg yolk- based extenders (Hinsch and Hinsch, 1997). However, soy lecithin resulted in decreased sperm motility when it replaced the egg yolk in the human freezing extenders (Jeyendran et al., 2008; Reed et al., 2009), although it did not affect the sperm morphology and DNA integrity (Reed et al., 2009). Suitability of soy lecithin as a replacement for egg yolk in cold-storage of the dog spermatozoa at 5°C for up to 8 days (Beccaglia et al. 2009; Kmenta et al., 2011), and for semen freezing in the horses (Papa et al., 2011), cattle (Gil et al., 2000; Aires et al., 2003), goats (Vidal et al., 2013) and sheep (Sharafi et al., 2009; Forouzanfar et al., 2010; Emamverdi et al., 2013) was reported almost a decade earlier. In most studies, at least some of the sperm attributes showed better improvement in the presence of soy lecithin compared with the egg yolk, where the concentration of soy lecithin ranged from as low as 0.4% to 1%).

Soy lecithin at 1.5% concentration could successfully replace egg yolk in Tris-fructose-citrate extender for cryopreservation of Mahabadi goat sperm (Salmani et al., 2104). Supplementation of soybean lecithin at 3.5% in Tris extender could better preserve the ram and buck sperm characteristics after the equilibration period and thawing (Khalifa and Abdel-Hafez, 2013).

In Toggenburg bucks, removal of seminal plasma improved the quality of chilled semen that was cooled in a soybean lecithin-based extender, especially when using 2% soybean lecithin (Silva et al., 2019).

Chelucci et al. (2015) showed that 1% lecithin in Tris extender for freezing Sarda buck semen was the most effective level in terms of viability, percentages of progressive motile, and rapid spermatozoa, and DNA integrity after thawing. The heterologous *in vitro* fertilization test showed that Tris- lecithin better preserved the sperm functionality, as demonstrated by the higher fertilization rates compared with a commercial extender, and the extender containing egg yolk. Tris based extenders supplemented with 20% egg yolk or 2.0% soybean lecithin had similar effects on cryopreserved spermatozoa of Chongming White goats (Sun et al., 2020).

Sugars as constituents of the semen extenders

As a sugar, raffinose was first used in the bull semen extender by Nagase et al. (1968) who also showed that the sugars with higher molecular weight more effective than the low molecular weight sugars. In addition to

monosaccharides, di- and oligosaccharides have been evaluated for their effects on preserving the cryostored spermatozoa.

In 1980, Marinov et al. found that sucrose was able to better preserve the ram sperm acrosomal integrity as compared with glucose, fructose, or lactose; after which sucrose became an important constituent in semen extenders. Ram semen is low in antioxidant capacity; therefore, synthetic antioxidants were included in the extenders that contained sucrose (Marinov et al., 1980). There are reports that inclusion of more complex saccharides in extenders greatly improved the quality of frozen-thawed ram (Bucak et al., 2007), bull (De Leeuw et al., 1993), goat (Farshad and Akhondzadeh, 2008; Naing et al., 2010; Tuncer et al., 2010) and boar (Gomez-Fernandez et al., 2012) spermatozoa. It is noteworthy that the inclusion of several sugars in the extenders was found to be more effective than single ones (Nauk, 1991; Gomez-Fernandez et al., 2012).

According to Jafaroghli et al. (2011), sucrose, trehalose or raffinose significantly decreased the percentages of abnormal sperm and acrosome in frozen ram spermatozoa, while the fertility rate was highest for the spermatozoa extended in trehalose-containing diluent and inseminated intracervically. In both Markhoz (Khalili et al., 2009) and Mahabadi (Mohammadian, 2014) goats, a combination of trehalose and sucrose could better maintain the quality of frozen spermatozoa compared with either trehalose or sucrose alone.

Synthetic and natural antioxidants as constituents of the semen extenders

Optimal cellular functions require a proper balance between the oxidants and antioxidants. Under various stressful conditions, the production of oxidants damages the cells both structurally and functionally (Aitken, 2020). Compared to other cells, sperm cells posses much lower antioxidant capacity which makes them more prone to oxidative stress (Sreejith et al., 2006). Seminal plasma and spermatozoa contain various antioxidants, including enzymes (superoxide dismutase, catalase, and glutathione peroxidase), low-molecular weight antioxidants (ascorbate, alpha-tocopherol, and beta-carotene), transferrin, lactoferrin, and ceruloplasmin (Sanocka and Kurpisz, 2004; Aitkin, 2020). A large proportion of cytoplasm and associated contents, including the antioxidants, are lost during spermatogenesis, making spermatozoa very sensitive to peroxidation reactions. Spermatozoa of several species also contain a high concentration of polyunsaturated fatty acids that are easily oxidized, thus lowering the sperm fertility rate. It has been shown that supplementation of the semen extender with antioxidants provides a constant level of the reactive species, and decreases the reactivity of free radicals, which could help maintain the quality and functionality of the cryopreserved spermatozoa (Kreider et al., 1984; Bailey et al., 2000; Moretti et al., 2012).

Pyruvate was reported to be an effective antioxidant for the preservation of the stallion (Bruemmer et al., 2002), ram and bull (Upreti et al., 1998) spermatozoa, and butylated hydroxytoluene (BHT) resulted in higher post-thaw motility of the bull sperm (Killian et al., 1989; Shoae and Zamiri, 2008). Several studies also indicated at least some beneficial effects of vitamin E (Kheradmand et al., 2006), vitamin B_{12} (Hamedani et al., 2013), BHT (Watson and Anderson, 1983), cysteine, and glycine (Khalili et al., 2010) on frozen ram spermatozoa. The improved sperm quality of frozen-thawed sperm was also reported when several antioxidants were included in the ram semen extenders (Bucak et al., 2007). Higher sperm motility and lower malondialdehyde production were recorded when a soy lecithin-based medium supplemented with 4 and 6% rosemary (*Rosmarinus officinalis* L.) extract was used for cryopreservation of ram spermatozoa (Khodaei Motlagh et al., 2014). The protective effect of silymarin against the toxicity of bisphenol A (BPA) on boar sperm quality was reported by Jang et al. (2011), and Purdy et al. (2004) showed that the flavonoids, silibinin and catechin, improved the motility of extended cooled caprine sperm. Silymarin also improved the post-thaw quality of ram sperm (Delfanipour, 2015). The beneficial effect of quercetin on post-thaw sperm characteristics was reported in several species, including the human (Moretti et al., 2102), rat (Ben Abdallah et al., 2011), horse (Gibb et al., 2013), and sheep (Silva et al., 2012; Delfanipour, 2015). However, supplementation of the cryopreservation medium with both silymarin and quercetin resulted in substantial deterioration of ram sperm quality, including the viability, acrosomal integrity, malondialdehyde level, and CASA parameters (Delfanipour, 2015).

An ethanolic extract of fennel (*Foeniculum vulgare*) seeds effectively improved most of the quality parameters of the ram semen processed in Tris-base extenders, including several CASA attributes (Mohammadian, 2017). Malo et al. (2012) reported that fennel essences provided antioxidant protection for boar semen cryopreserved in yolk-based extender; improving the sperm motility and viability but not the hypoosmotic swelling test and acrosomal integrity.

There is evidence that catalase supplementation of the extenders might be beneficial for sperm preservation. Shannon and Curson (1982) reported that in cattle, the non-return rate was not affected when semen was stored at 5°C in 20% egg yolk diluents with catalase but was significantly increased with semen stored at 15-23°C. Asadpour et al. (2011) found no significant differences in bull sperm viability and motility following the addition of catalase (100 IU or 200 IU mL^{-1}) to citrate-egg yolk extender; however, the highest sperm viability was recorded by addition of catalase to Tris-egg yolk extender compared with the control group. Malondialdehyde levels did not change with the addition of catalase in either extender. Combinations of vitamin C and catalase (100 IU mL^{-1}) in Tris extender for cryopreservation of bull sperm resulted in increased sperm motility, viability, plasma membrane integrity, and acrosomal integrity (Eidan, 2016).

According to Guerra (2011), the addition of CAT (100 and 200 U mL^{-1}) reduced the deleterious effects of cooling on total motility in ram sperm maintained at 5 °C for 24 h, but it did not affect the functionality of the sperm membranes. The same levels of catalase imparted great benefits to the post-thaw quality when ram spermatozoa were cryopreserved in egg yolk extender (Yazdinejad, 2018). Irrespective of the concentration used, catalase and superoxide dismutase, alone or in combination, significantly improved post-thaw boar sperm survival, in terms of total sperm motility (assessed with CASA) and viability, and reduced post-thaw ROS generation, without any influence on MDA production (Roca et al., 2005). In human, Li et al. (2010) concluded that appropriate catalase or ascorbate supplementation of cryopreservation medium restrained the level of the reactive oxygen species, and the resultant sperm characteristics, including the viability, motility, mitochondrial membrane potential, apoptosis, and DNA integrity.

There were no beneficial effects of taurine, trehalose, or cysteine in Solomon's Tris extender on post-thaw motility, acrosomal integrity, sperm membrane integrity, and malondialdehyde levels in Angora goats (Atessahin et al., 2008). The Solomon's diluent supplemented with 2.5 and 5mM glutamine led to higher post-thaw motility and HOST rates in Angora bucks. Sperm membrane integrity was also improved by 500 µL/mL hyaluronan. There was no effect of glutamine or hyaluronan on acrosomal and total sperm abnormality (Bucak et al., 2009a).

Salmani et al. (2013) showed that for cryopreservation of Mahabadi goat semen, soybean lecithin-based extender could be a suitable replacement for Tris egg yolk, and addition of glutathione to the former did not significantly impact on most of the post-thaw sperm quality parameters.

Small improvements (2-5 percentage points) in motility, membrane integrity, morphology, acrosomal integrity and viability in post-thaw Boer goat spermatozoa at 2mM concentration of butylated hydroxytoluene (BHT) in Tris egg yolk extender (Memon et al., 2011). In Beetal bucks, no effect of BHT in Tris egg yolk extender was recorded on motility, livability, and sperm membrane integrity, but acrosomal integrity showed a small improvement (Iqbal et al., 2015).

The antioxidants, hypotaurine and cysteamine, improved sperm motility, morphology, and functional membrane integrity, without influencing ROS formation and elevating antioxidant capacity, after freeze–thawing of Angora goat semen (Bucak et al., 2009b). Butylated hydroxyanisole in the presence of 5% glycerol or dimethyl sulfoxide resulted in better post-thaw sperm quality in Mahabadi goats during the non-breeding season (Rahmatzadeh et al., 2017). Pyridoxine alone or in specific combinations with vitamin E, vitamin C, or melatonin improved the viability and reduced oxidative stress parameters of cryopreserved sperm in West African Dwarf goats (Daramola et al., 2017).

The addition of rosemary aqueous extract at 4% level in Tris extender improved the post-thaw sperm quality in Kurdi bucks (Zanganeh et al. 2013). Avocado seed extract at 20% concentration in the Tris-citric acid extender improved sperm (motility, livability, membrane and acrosomal integrity) and oxidative stress indices in West African Dwarf goats (Olamitibo et al., 2016). Supplementation of egg yolk- skim milk extender with 3% extract of water clover resulted in improvement in post-thaw motility, viability, and plasma membrane integrity (8-15% points) with no effect on sperm abnormality in bucks (of unknown breed) (Wahjuningsih et al., 2019). A crude extract of fig fruit (*Ficus carica L*) added to Tris egg yolk extender at 6% concentration mitigated the decline in quality of buck semen stored at 5°C (Zaenuri et al., 2014). Daramola et al. (2016) studied the effects orange (*Citrus sinensis*), cucumber (*Cucumis sativus*) and pineapple (*Ananas comosus*) juices, or their combinations, in Tris-egg yolk extenders on frozen West African Dwarf goat spermatozoa. Their findings revealed that extenders supplemented with orange and pineapple at 10% consistently improved post-thaw motility, acrosomal integrity and membrane integrity, and reduced sperm abnormality compared to the control extender while no particular combination of these fruit-juices was consistently

superior in all the parameters. Grape seed procyanidin extract was able to mitigate the decrease in goat sperm quality stored for up to 120 h at 4°C, at an optimum concentration of 30 mg/L; however, there were practically no differences in the pregnancy rate (72.5 vs. 68.7%) and litter size (1.50 vs. 1.47) due to insemination of the semen stored for 72 h (Wen et al., 2019).

Supplementation of Tris extender with 10 mM quercetin in combination with dimethylacetamide preserved the motion kinetics, and it was concluded that dimethylacetamide could be an alternative to glycerol as a cryoprotectant (Seifi-Jamadi et al., 2017).

Addition of bull seminal plasma crude protein to egg yolk-milk diluent (2.5 mg/mL) improved buck sperm motility and viability and decreased cytochrome C expression during and after thawing (Vahyu Suprayogiand and Susilowati, 2018). The rainbow trout seminal plasma at 8% concentration in a soybean lecithin-based extender, used for freezing of Saanen buck spermatozoa, preserved sperm motility, acrosomal integrity, plasma membrane functional integrity and mitochondrial function better than the control group (Alcay et al., 2020). Inclusion of resveratrol in the Optidyl, a commercial extender for bull semen, at 10 or 50 µM concentrations resulted in higher total motility, progressive motility, livability, membrane and acrosomal integrity, and mitochondrial activity in post-thaw goat spermatozoa; ROS production was lower but there was no significant effect on abnormal morphology (Lv et al., 2019).

Conclusions

Since the introduction of the most widely-used extenders for cold- preservation of the ovine and caprine spermatozoa, many variants have been prepared by substituting the common constituents or including novel compound(s) in the extender. A number of published articles contained insufficient data, wrong interpretation of the finding, or incorrect method of statistical analysis; therefore, these were not included in this review. Whilst accrediting the significance of the un-cited works, for the sake of brevity only a selected number of published papers were covered in the present review.

Variable success rates have been reported in terms of the sperm quality measures, whilst field fertility data were only reported in a small number of the publications searched for preparation of this review. Based on the information in the publications covered in the present review, it seems unlikely that the findings presented in the literature could replace the widely-used extenders, and field application in the ovine and caprine artificial insemination programs. In most studied reporting the field fertility data due to artificial insemination of the extended semen, the number of inseminated females was not large enough for suggesting the use of those extender(s) in large flocks or herds. Future experimental works for modifying the semen extenders need to provide field fertility data on a larger number of females that are artificially inseminated with the semen diluted in each modified extender.

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