Agronomía Mesoamericana

UNIVERSIDAD DE COSTA RICA

Literature review
Volumen 36: Artículo 59591, 2025
e-ISSN 2215-3608, https://doi.org/10.15517/am.2024.59591
https://revistas.ucr.ac.cr/index.php/agromeso/index

An overview on extenders used in ram sperm cryopreservation*

Panorama de los medios de dilución espermática para la criopreservación en carnero

Francisco Sevilla[1,4], Gerald Muça[2], Luigj Turmalaj[2], Miguel A. Silvestre[3], Ignacio Araya-Zúñiga[4], Anthony Valverde[4]

* Reception: April 22th, 2024. Acceptance: August 13th, 2024. This work was part of the research project VIE-2151-083 "Optimización de la conservación y búsqueda de parámetros de la fertilidad en espermatozoides de animales de interés productivo" from the Vicerrectoría de Investigación y Extensión (VIE), Instituto Tecnológico de Costa Rica.

1 Instituto Tecnológico de Costa Rica – Universidad Nacional – Universidad Estatal a Distancia, Doctorado en Ciencias Naturales para el Desarrollo (DOCINADE). Alajuela, Costa Rica. f.sevilla@tec.ac.cr (https://orcid.org/0000-0003-1480-4141).

2 Universiteti Bujqësor i Tiranës, Fakulteti i Mjekësisë Veterinare, Departamenti i Lëndëve Klinike. Rruga Paisi Vodica 1025, Tiranë, Shqipëri/Albania. gmuca@ubt.edu.al (https://orcid.org/0000-0002-7209-9885), lturmalaj@ubt.edu.al (https://orcid.org/0009-0003-3154-8554).

3 Universitat de València, Departament de Biologia Cel·lular, Biologia Funcional i Antropologia Física, Campus Burjassot, C/Dr. Moliner 50, 46100 Burjassot, Spain. miguel.silvestre@uv.es (https://orcid.org/0000-0001-9260-5792).

4 Instituto Tecnológico de Costa Rica, Escuela de Agronomía, Laboratorio de Reproducción Animal, San Carlos Campus, 223-21002, Alajuela, Costa Rica. igaraya@estudiantec.cr (https://orcid.org/0000-0002-4292-2287); anvalverde@tec.ac.cr (Author for correspondence, https://orcid.org/0000-0002-3191-6965).

Abstract

Introduction. One of the most critical methods for improving domestic animal reproduction is sperm cryopreservation. To achieve the best results, this complex process requires the meticulous balance of numerous variables. Sperm cryopreservation is an essential technique in programs that improve breeding and conservation for many species, especially small ruminants. In this case, genetic material from a limited number of exceptional sires can be used to breed a large number of female sheep. **Objective.** To analyze the current advances in extenders used in ram sperm cryopreservation. **Development.** In rams, spermatozoa have a lower intramembrane cholesterol-to-phospholipid ratio than other species, making them more susceptible to cryopreservation damage than other livestock species. Adequate freezing and thawing can prevent metabolic and structural damage to ram sperm cells, a goal that has not yet been achieved. The success of the sperm freezing process depends on several factors, such as freezing/thawing rate, equilibration time, spermatozoa, and the composition of medium surrounding spermatozoa, among others. In this sense, sperm extender is one of the most critical factors in the cryopreservation process. **Conclusions.** The vulnerabilities of ram sperm to cryogenic stress poses challenges to maintaining viability after thawing and achieving consistently high fertility rates. Further research to refine supplementation strategies, optimize freezing protocols, and explore novel cryoprotectants is essential to overcome these challenges and enhance the efficacy of ram semen cryopreservation to improve reproductive outcomes in ovine breeding programs.

Keywords: Freezing, semen extenders, sperm quality, animal reproduction, cryobiology.

Resumen

Introducción. Uno de los métodos más utilizados para mejorar la reproducción de los animales domésticos es la criopreservación del esperma. Para lograr los mejores resultados, este proceso complejo requiere el control meticuloso de numerosas variables. La criopreservación del esperma es una técnica utilizada en programas de mejora genética y conservación de muchas especies, especialmente los pequeños rumiantes. En este caso, el material genético de un número limitado de machos seleccionados puede utilizarse para inseminar un gran número de ovejas. **Objetivo.** Analizar los avances actuales en diluyentes utilizados en la criopreservación de esperma de carnero. **Desarrollo.** En los carneros, los espermatozoides tienen una proporción intra-membrana colesterol: fosfolípidos más baja que otras especies, lo que los hace más susceptibles a los daños asociados a la criopreservación que otros animales utilizados en la producción ganadera. La congelación y descongelación adecuada pueden evitar daños metabólicos y estructurales de los espermatozoides en la especie ovina, algo que no ha sido posible hasta el momento. El éxito del proceso de congelación de espermatozoides depende de varios factores como la velocidad de congelación/descongelación, el tiempo de equilibrio, los propios gametos o la composición del medio que rodea a los espermatozoides, entre otros. Los medios de dilución del semen podrían afectar los procesos de criopreservación. **Conclusión.** La vulnerabilidad del esperma de carnero al estrés criogénico plantea retos para mantener la viabilidad después de la descongelación y lograr tasas de fertilidad altas. Es necesario realizar más investigaciones centradas en refinar las estrategias de suplementación, optimizar los protocolos de congelación y explorar nuevos crioprotectores para superar estos desafíos y hacer más eficaz la criopreservación del semen de carnero para mejorar los resultados reproductivos en los programas de mejora genética ovina.

Palabras clave: congelación, diluyentes de semen, calidad espermática, reproducción, criobiología.

Introduction

One of the many benefits of using frozen-thawed ram semen is the preservation of biodiversity, which helps to protect both endangered species and livestock species. On the other hand, sperm cryopreservation together with artificial insemination (AI) makes it possible to inseminate many sheep at different farms far away from the collection center. Also, cryopreservation may have applications in improving the precision in agriculture and biotechnology (Sharafi et al., 2022). This fact facilitates the testing of breeding males and the dissemination of high-quality genetic material (Cseh et al., 2012). Finally, as semen doses of rams could be used for longer periods of time or throughout different seasons of the year, research into artificial spermatozoa storage was encouraged (Salamon & Maxwell, 2000). When spermatozoa are cryopreserved and stored for a long time, they undergo biochemical and functional changes that lower their fertility. Sudden temperature changes, such as cold shock, ice formation, and melting during the freezing and thawing processes, affect the acrosome, nucleus, mitochondria, axoneme, and plasma membrane (Alcay et al., 2015).

Sperm cryopreservation makes it possible to preserve germplasm and maximize sperm doses during artificial insemination and *in vitro* fertilization (Valente et al., 2010). Because of the poor ability of ram sperm to withstand the freezing-thawing procedure and the consequent low efficiency, this strategy is only seldom used in ovine breeding projects, and fresh semen is normally used (Cseh et al., 2012). Frozen ram semen is rarely used in artificial insemination due to poor pregnancy rates and the cervix's anatomy interfering with the spermatozoa's ability to transit and reach the egg during the freezing-thawing procedure (Zalazar et al., 2020). Because of their low cholesterol content in the spermatozoa plasma membrane, high unsaturated:saturated fatty acid ratio (large quantity of polyunsaturated fatty acids), and inadequate antioxidant ability, ram sperm are more susceptible to

structural damage (El-Seadawy et al., 2022; Ledesma et al., 2019; Valente et al., 2010), when compared to sperm from bulls, rabbits, or men.

Cryopreservation has several detrimental effects, including decreased motility, increased ion permeability, membrane polyunsaturated fatty acid peroxidation, reactive oxygen species production, decreased DNA stability, and decreased mitochondrial activity (Gosálvez et al., 2011; Zalazar et al., 2020). While optimal levels of reactive oxygen species (ROS) are integrated into the physiological capacitation reaction and fertilizing potential of spermatozoa, higher levels of ROS produced during the cryopreservation process lead to oxidative stress, which in turn causes lipid peroxidation and protein denaturation in the sperm membrane, thus disrupting the spermatozoa fluidity, intactness, and ability to fertilize (El-Seadawy et al., 2022; Sarıözkan et al., 2009). The early induction of the sperm capacitation, known as cryo-capacitation, is a significant harmful effect induced by cryopreservation (Benko et al., 2022). The hallmarks of cryo-capacitation include a restructuring of the membrane with a loss of cholesterol and polyunsaturated fatty acids, as well as the establishment of molecular hallmarks such as changes in intracellular ion concentrations and protein phosphorylation, hyperpolarization, actin polymerization, an increase in energy metabolism, and activation of cAMP-dependent protein (Ledesma et al., 2019; Peris-Frau et al., 2020).

Several experiments have been conducted to modify semen diluents to test their effectiveness in preserving sperm during the freeze-thaw process (Ramírez-Vasquez, Cesari, et al., 2019). Post-thaw sperm quality is reduced as a result of osmotic stress and cold shock that arise during the freeze-thawing process (Saha et al., 2022). Most of this damage can be prevented by using cryoprotectant additives and appropriate extenders (Emamverdi et al., 2013). In the realm of scientific and technological advances, the viability of ram sperm after thawing still encounters several obstacles (Salamon & Maxwell, 2000). The decrease in viability during the freeze-thaw procedure is mainly due to changes in medium osmolarity and sperm shape, which negatively affect post-breeding fertility rates (Batissaco et al., 2020).

The survival of the preserved sperm may be affected by several factors, including storage temperature, cryoprotectant concentration, cooling rate, thawing protocol, extender composition, sperm dilution, free radical contents, seminal plasma contents, and antiseptic components (Kumar Jha et al., 2019; Sobeh et al., 2020). To enhance sperm quality, two types of supplementation methods related to freezing and subsequent thawing are provided. The first ones are affected by interactions with non-defined freezing extenders and are essentially inconsistent. Supplementation of extenders or thawing medium with other substances, such as carbohydrates, lipids, antioxidants, or other cryoprotectants, has been the subject of numerous investigations (Zalazar et al., 2020). This review aims to analyze the current advances in extenders used in ram sperm cryopreservation.

Semen extenders

Similar to other species, ram semen extenders must have adequate pH, osmolality, and buffering capacity in order to preserve spermatozoa against cryogenic damage (Zhang et al., 2024). This section's diluents have been explained based on their intended uses, with notes made regarding their effectiveness in relation to fertility and the newly discovered supplements and extenders.

Tris-based diluents

The formula for tris, also known as tris (hydroxymethyl) aminomethane, tris $(HOCH_2)_3CNH_2$, functions as a chemical buffer in semen extenders (Yániz et al., 2012). Ram spermatozoa's motility and metabolism have been shown to be unaffected by tris concentrations ranging from 10 to 50 mM. Higher tris concentrations were subsequently found to be beneficial in diluents for refrigerated storage (Salamon & Maxwell, 2000). Tris-based

extenders contain citric acid and phospholipids (egg yolk), carbohydrates (glucose, fructose, trehalose), glycerol and glycine (Moradi et al., 2022; Saha et al., 2022; Toker et al., 2016; Valente et al., 2010). Tris-based extenders can also be combined with soybean lecithin (Asadzadeh et al., 2021; Emamverdi et al., 2013; Sobeh et al., 2020), ethylene glycol, dimethylsulfoxide (Najafi et al., 2017), cholesterol-loaded cyclodextrin (Batissaco et al., 2020), followed by antioxidants and antibiotics (Barbas et al., 2023; Liu et al., 2020).

Citrate sugar-based diluents

Various sugars are added to the citrate medium in this type of extender. Some of the sugars employed in this diluent medium are arabinose, fructose, or glucose, with post-thawing effectiveness serving as the criterion (Rostami et al., 2020). Ram semen handled the above range in diluent tonicity with good tolerance, with a mean osmotic pressure of 7.7 atm. In general, hypertonic citrate-glucose-yolk or citrate-fructose-yolk diluents with an osmotic pressure of 8-12 atm were utilized since the glycerol in the extender induced a drop in osmotic pressure. Because monosaccharides can penetrate sperm cells and level the osmotic gradient, ram spermatozoa can also withstand glucose or fructose concentrations twice as high as isotonicity (Salamon & Maxwell, 2000).

Milk-based diluents

One of the earliest diluent combinations frequently utilized in the sperm cryopreservation of ram was milk-based diluent (Plante et al., 2015). Like egg yolk, milk-based diluents are categorized as non-penetrant cryoprotectants (Saha et al., 2022; Salamon & Maxwell, 2000). There are hygienic concerns, just like with egg yolks; for this reason, skimmed milk powder is used (Alcay et al., 2015; Gil et al., 2003). Casein micelles, which interact with phospholipid binding proteins to minimize membrane lipid loss while preserving sperm motility and viability, are hypothesized to be the mechanism behind skimmed milk's capacity to protect spermatozoa from cold shock (Allai et al., 2016; Bergeron & Manjunath, 2006; Saha et al., 2022). Skimmed milk is generally used in a concentration from 5 % when combined with other cryoprotectants, and up to 11 % when the extender is composed mainly of skimmed milk (Allai et al., 2023; Emamverdi et al., 2013; Zalazar et al., 2020).

It has been possible to freeze ram semen using milk, primarily in reconstituted form, together with egg yolk, fructose, or arabinose (Allai et al., 2023). A number of French workers employed the milk-citrate medium that was developed by National Institute for Agricultural Research (INRA); it was added to the partially diluted, cooled lactose-yolk as a glycerolated phase of the two-step dilution procedure. In both cases, the two-step dilution and the freezing, skim milk extender was utilized (Saha et al., 2022). Following cervical insemination using milk-frozen semen, the fertility outcomes have ranged from 23 to 45 % (Salamon & Maxwell, 2000).

Cryoprotective compounds

To date, there are different types of cryoprotective agents utilized in cell cryopreservation penetrant compounds, including diols (i. e., glycerol, ethylene glycol) and sulfoxide (i. e., dimethyl sulfoxide) groups (Marcantonini et al., 2022).

Permeant cryoprotectant agents

Among penetrant agents, glycerol is the most commonly used as a cryoprotectant for mammal sperm cryopreservation at a concentration between 4-8 % (Saha et al., 2022; Salamon & Maxwell, 2000). Cell dehydration is the result of spermatozoa cells being penetrated by glycerol, which then displaces intracellular water. Additionally,

it inhibits the development of intracellular ice by binding to intracellular water, and it is hypothesized that glycerol decreases cell damage by increasing the fluidity of the spermatozoa membrane (Luna-Orozco et al., 2019; Paul et al., 2021). The harmful effects of glycerol on spermatozoa have been taken into consideration and include osmotic stress, changes in membrane lipid organization, fluidity, and permeability (Watson, 1995). Glycerol toxicity varies depending on how it is added, how quickly it cools down and freezes, and what kind of extender it is composed of (Salamon & Maxwell, 2000). It is advised to utilize a two-step progressive addition of glycerol at a temperature of 2.5-5 °C for optimal cryoprotection and to reduce the toxicity, as opposed to adding it at a temperature near 0 °C (Colas, 1975; Emamverdi et al., 2013; Kumar Jha et al., 2019). It was observed that 6 % glycerol and 0.25 % carboxymethyl cellulose together functioned better and produced superior cryopreservation outcomes (Paul et al., 2021).

Due to concerns about glycerol toxicity, numerous investigations have been conducted to find better alternatives. Other constituents, including ethylene glycol, polymeric compounds, different kinds of sugars, albumin, and surfactants, could be used instead of glycerol. It was believed that none of these compounds could match the effectiveness of glycerol until the last 20 years (Salamon & Maxwell, 2000). Later on, nevertheless, it was revealed that ethylene glycol enhanced sperm viability, acrosome integrity, plasma membrane integrity, and conception rate while also decreasing abnormalities (Keskin et al., 2020). A suitable substitute for glycerol could be ethylene glycol at a concentration of 3-5 %. It was shown that this quantity of ethylene glycol produces the same cryopreservation effects as glycerol in soybean lecithin-based extenders (Najafi et al., 2017). In a different study, a combination of 1.5-5 % ethylene glycol and +100 mM trehalose in a tris-based extender offered better protection than combinations of glycerol 3-5 % and trehalose groups (Keskin et al., 2020). It was also shown that ethylene glycol is a good replacement for glycerol in egg yolk-based extenders; even this latter one provided superior protection for the plasma membrane (Valcácia Silva et al., 2013).

Non-permeable cryoprotectant agents

Also, in literature, non-penetrant compounds or non-permeable cryoprotectants, including proteins and saccharides, among others, have been found (Marcantonini et al., 2022). As previously mentioned, during the cryopreservation process, sperm undergo important osmotic stress. Osmotic tolerance limit is species-specific, and supplementation of freezing media with egg yolk, milk or sugars extended this limit (Benson et al., 2012).

Proteins

Typically, tris or citrate-based media for sperm are supplemented with egg yolk, milk or soy lecithin, providing protein among other substances (Allai et al., 2017; Üstüner et al., 2014). During cooling storage, skimmed milk has a protective effect analogous to egg yolk (Bergeron et al., 2007; Del Valle et al., 2013). The beneficial effect of milk and egg yolk appears to be based on the sequestration of binder of sperm proteins (BSP) from seminal plasma, which prevents BSP from link to the sperm membrane during cooling, thus avoiding the cholesterol loss of the membrane (Bergeron et al., 2007); in milk (casein micelles) and egg yolk, it would be lipoproteins (mainly low-density lipoproteins; LDL) (Bergeron et al., 2007; Plante et al., 2015).

Egg yolk is applied at concentrations ranging from 5 % to 20 % and, after freezing, it preserves the viability, motility, and integrity of spermatozoid membranes (Kumar Jha et al., 2019; Saha et al., 2022; Salamon & Maxwell, 2000). Low-density lipoproteins found in egg yolks shield the phospholipid membrane during cryopreservation against degenerative alterations (Ramírez-Vasquez, Cano, et al., 2019). There are a few issues with egg yolk utilization; as a product made from chicken, the composition varies between batches (Najafi et al., 2014), and its potential hazard to human health and microbial contamination that produces toxic byproducts are causes for

concern (Asadzadeh et al., 2021; Toker et al., 2016). Recent research has shown that egg yolks enhance semen pre-capacitation and agglutination, which lowers fertility and semen quality (Pini et al., 2018; Ramírez-Vasquez, Cano, et al., 2019). Moreover, it has been observed that egg yolk causes antigenic reactions in the reproductive system and systemic circulation (Toker et al., 2016; Üstüner et al., 2014).

Egg yolk is currently being replaced with an alternate cryoprotectant to minimize these disadvantages and, most importantly, microbiological contamination (Layek et al., 2016). Soybean lecithin is one of the alternative animal protein-free extenders to conserve ram sperm (Toker et al., 2016). Soybean comes from plants, which eliminates variations in egg yolk batches and is safer in terms of hygiene (Emamverdi et al., 2013). Likewise, lecithin reproduces the cryopreservation-related protective effect of a low-density lipoprotein fraction derived from egg yolk. It causes cryoprotection by stabilizing and replacing the phospholipids in spermatozoa membranes (Allai et al., 2018; Forouzanfar et al., 2010). Lecithin at 1 % and glycerol at 7 % leaded or 1.5 % soybean lecithin in tris-based extender give optimal cryopreservation results (Emamverdi et al., 2013; Forouzanfar et al., 2010). When a lecithin nanoliposome was utilized in place of soybean lecithin, this effect was slightly greater (Mehdipour et al., 2017) (Table 1).

Saccharides

Adding non-permeable cryoprotectants to the freezing media increases medium osmolarity and enables cell dehydration. Ram spermatozoa tolerate hypertonic freezing media supplemented with trehalose (Aisen et al., 2005). Some of the most commonly used sugars for cryopreservation are saccharose and trehalose (Aisen et al., 2002; Freitas Bittencourt et al., 2018; Najafi et al., 2013). Authors have shown that supplementation media with sugars (trehalose, sucrose, raffinose) and antioxidants improves the quality parameters of ram spermatozoa after cryopreservation (Rostami et al., 2020). It has been shown that different combinations of 3-5 % glycerol and 100 mOsm trehalose improved semen cryopreservation and had a synergistic effect (Aisen et al., 2002; Najafi et al., 2013).

Use of new substances in semen extenders

Antioxidants

As discussed above, oxidative stress is one of the factors that deteriorate sperm quality during the cryopreservation process. To avoid the deleterious effect of excessive ROS production, adding antioxidants to the extender has been proposed (Amidi et al., 2016). One strategy to prevent oxidative damage to sperm inherent in the conservation process, both refrigeration and cryopreservation, is the supplementation of antioxidants in extenders (Silvestre et al., 2021). Polyphenols such as resveratrol have been used as antioxidants in cryopreserved ram semen (Zhu et al., 2023). Very recently, authors showed that the addition of resveratrol increased both sperm motility and several reduced proteins after cryopreservation (Chen et al., 2024). Moreover, given its function in the seasonal cycles in ovine reproduction, melatonin has been used in the cryopreservation of ovine spermatozoa for its antioxidant effects (Ofosu et al., 2021). It was observed that melatonin supplementation in the extender increased motility and decreased reduced mitochondrial superoxide in frozen/thawed ram spermatozoa (Pool et al., 2021).

Vitamin E supplementation also has beneficial effects on sperm quality parameters (Valcácia Silva et al., 2013). Moreover, in a *in vitro* sperm test, Riesco et al. (2021) tested several antioxidant compounds like crocin,

Table 1. Protocols in ram sperm cryopreservation.

Cuadro 1. Protocolos en la criopreservación de semen de carnero.

Diluents	Equilibration time	Freezing steps	Freezing rate	Concentration	Reference
Tris/glucose/glycerol 6 %/egg yolk 20 %	5 °C for 2 h	Two	5 cm above nitrogen liquid/10 min	200 × 10^6 cells/mL	Zalazar et al. (2020)
Tris/glucose/glycerol 7 %/egg yolk 10 %	5 °C for 2 h	Two	5 cm above nitrogen liquid/10 min	100 × 10^6 cells/mL	Ledesma et al. (2019)
Tris/glucose/glycerol 7 %/egg yolk 20 %, soybean lecithin 1 %	4 °C for 90 minutes	Two	Above nitrogen liquid (–70 °C) for 10 min	4 × 10^9 cells/mL	Masoudi et al. (2019)
Tris/glucose/glycerol 5 %/egg yolk 20 %	5 °C for 2 h	Three	+5 °C to –80 °C at –11.33 °C/min followed by 2 L LN (4 cm deep) and left for 1.5 min to decrease the temperature to –120 °C at –26.66 °C/min, and finally 2 L LN (6 cm deep) left for 1.5 min to decrease the temperature to –140 °C	10 × 10^6 cells/mL	Kumar Jha et al. (2019)
Tris/fructose/egg yolk 15 %/ ethylene glycol 5 %/ ethylene glycol 1.5 %	5° C for 3 h	Two	4 cm above nitrogen liquid/15 min	4 × 10^8 cells/mL	Keskin et al. (2020)
Tris/Soybean lecithin/glycerol 5 %	5° C for 4 h	Two	Above nitrogen liquid/10 min	220 × 10^6 cells/mL	Sobeh et al. (2020)
Tris/glucose/egg yolk 20 %/glycerol 6 %/ dimethylacetamide 3 %	5 °C for 2 h	Two	Frozen in liquid nitrogen vapor	80 × 10^6 cells/mL	Freitas Bittencourt et al. (2018)
Tris/glucose/egg yolk/glycerol	5 °C for 2 h	Two	6.5 cm above nitrogen liquid/15 min	2.7 × 10^9 cells/mL	El-Seadawy et al. (2022)
Tris/egg yolk 10 %/glycerol 3 %/trehalose 100 mOsm	5 °C for 2 h	Two	above nitrogen liquid/–100°C	1 × 10^9 cells/mL	Aisen et al. (2002)
Tris/lyophilized egg yolk 20 %/glycerol 6 %	5 °C for 2 h	Three	3 °C/min from +5 to –8 °C 15 °C/min from –8 to –120 °C	Not mentioned	Alcay et al. (2015)
Tris/egg yolk 20 %/glycerol 7 %/soybean lecithin 1.5 %	4 °C for 2 h	Two	5 cm above nitrogen liquid/12 min	35 × 10^7 cells/mL	Emamverdi et al. (2013)
Tris/egg yolk 15/glycerol 5 %	5 °C for 4 h	Three	5 °C/min from 4 to –10 °C 40 °C/min from –10 to –100 °C	10 × 10^9 cells/mL	Liu et al. (2020)
Tris/soybean lecithin 1.5 %/ ethylene glycol 3-5 %	4 °C for 2 h	Two	4 cm above nitrogen liquid/ 7 min	4 × 10^8 cells/mL	Najafi et al. (2017)

GSH and Trolox (vitamin E), and found that Trolox supplementation improved fertility rates in high fertility males and increased multiple lambing frequency in low fertility males (Riesco et al., 2021). Ascorbic acid and vitamin E are also present, both of which have antioxidant qualities (Najafi et al., 2017; Vieira de Souza et al., 2019). The quality of post-thawed semen in rams is improved by the new mitochondria-targeted antioxidant (Mito-TEMPO) at concentrations of 5 and 50 μM (Asadzadeh et al., 2021). The addition of antioxidants such as cysteine at 10 mM in soybean lecithin extenders improved even more the post-thawed quality of ram semen (Najafi et al., 2014).

Plant extracts

Evidence has shown that a combination of plant-based extenders, such as 10 mg of prickly pear extract, and milk-based extenders (Jihad Neamah, 2022), *Spirulina platensis* and *Salvia verbenaca* extracts (Ben Moula et al., 2023), acetone extract from *Opuntia ficus* (Allai et al., 2016), and cactus seed oil (Allai et al., 2017) enhance the characteristics of the semen in rams during liquid preservation. When plant extracts like rosemary are added to soybean lecithin extenders at 4 and 6 %, a comparable result is observed (Motlagh et al., 2014). An extract of 375 μg/mL from *Entada abyssinica* (Splinter bean) also improved semen cryoprotection and reduced peroxidation (Sobeh et al., 2020). Ram sperm post-thawing quality was enhanced by the addition of green tea extract (10 mg/L) to soybean lecithin-based extenders (Mehdipour et al., 2016). Pomegranate extract, at 7.5 mg/kg in soybean lecithin extender, is another supplement that enhances the quality of post-thaw semen (Mehdipour et al., 2017).

Other supplementations

Nowadays, there are several novel materials or ingredients added to semen extenders that have been demonstrated to enhance semen cryopreservation (Abdelnour et al., 2020). Many of them have antioxidant qualities; for example, egg yolk-based extenders and soybean lecithin include 2 μM of Coenzyme Q10 (CoQ10), which increases semen viability, offers cryopreservation, and lowers peroxidation (Masoudi et al., 2019). It has been demonstrated that adding bioactive peptides to a Tris-based extender at a dose of 60 μg/mL enhances the cryoprotection of ram semen (Liu et al., 2020). Adding nano-water to the semen extender improves the ability of frozen-thawed ram semen (Murawski et al., 2015). Ram semen cryopreservation can be further improved by adding royal jelly to semen extenders (0.1-3 %) (Abdelnour et al., 2020).

Steps of adding of cryoprotectant agents

Greater results were achieved with a two-step dilution and a three-step freezing approach using a manual freezing process with a Tris-based extender, adding 20 % egg yolk and 5 % glycerol. Glycerol was present in the second portion of the diluent but not in the first. The samples were cooled at a rate of –0.25 °C/min for 120 minutes until they reached 5 °C. Two-step: at –11.33 °C/min from +5 °C to –80 °C and –30 °C/min from –80 °C to –140 °C; and three-step: at –11.33 °C/min from +5 °C to –80 °C, at –26.66 °C/min from –80 °C to –120 °C, and at –13.33 °C/min from –120 °C to –140 °C (Kumar Jha et al., 2019). When cooling at 5 °C at 0.47 °C/min and allowing it to equilibrate for two hours, a Tris-based extender containing 20 % egg yolk was observed to provide strong cryoprotective characteristics. It was then frozen in liquid nitrogen vapor (Freitas Bittencourt et al., 2018).

In a Tris-based extender with 20 % lyophilized egg yolk and 6 % glycerol, a freezing protocol was followed at 3 °C/min from +5 to –8 °C and at 15 °C/min from –8 to –120 °C (Alcay et al., 2015). In a Tris-based extender adding soybean lecithin as a cryoprotectant, an equilibration time of 4 hours at 5 °C was used (Sobeh et al., 2020). In a Tris-based extender, an equilibration time of 3 hours was used until the straw reached 5 °C, and then it was

frozen 4 cm above liquid nitrogen vapor (Keskin et al., 2020). After being chilled to 4 °C for 90 minutes, the Tris-based extender with glycerol was exposed to liquid nitrogen vapor for 7 minutes, and then liquid nitrogen was plugged in (Masoudi et al., 2019).

The semen extender, which was made up of 10-20 % egg yolk and 6-7 % glycerol, was chilled for two hours until it reached 5 °C. It was then kept at that temperature for two more hours before freezing (Ledesma et al., 2019). Ram semen was extracted by electroporation, and as an extender, two-step dilutions of glycerol (6 %) and egg yolk (20 %) were performed. After completing one phase without glycerol and a second step in which glycerol was gradually added until the temperature reached between 30 and 50 °C, equilibrated straws were frozen five centimeters above liquid nitrogen vapor and then submerged in liquid nitrogen (Zalazar et al., 2020).

Conclusions

The use of frozen and thawed ram semen offers promising perspectives for biodiversity conservation and breeding programs. The inherent vulnerabilities of ram sperm to cryogenic stress pose challenges in maintaining post-thaw viability. Advances in extender formulations and the addition of cryoprotective compounds, including Tris-based and citrate sugar-based diluents, along with the incorporation of cryoprotective agents such as glycerol and ethylene glycol, maintaining post-thaw sperm viability, remain a complex issue, and achieving consistently high fertility rates is still elusive. Further research focusing on refining supplementation strategies, optimizing freezing protocols, and exploring novel cryoprotectants is essential to overcome these challenges and enhance the efficacy of ram semen cryopreservation for improved reproductive outcomes in ovine breeding programs.

Funding

This research was funded by the Instituto Tecnológico de Costa Rica (Vicerrectoría de Investigación y Extensión), Project VIE-2151-083. It was also supported by the AGROALNEXT program (AGROALNEXT/2022/063) and the Valencian Government (CIAICO/2022/174). The funders had no role in the study design, data collection, analysis, decision to publish, or manuscript preparation.

Acknowledgement

The authors would like to thank the Instituto Tecnológico de Costa Rica (Vicerrectoría de Investigación y Extensión) for financing this study. They are also grateful to the AGROALNEXT program (AGROALNEXT/2022/063) for their support. F.S., e I.A.Z., thank the Postgraduate Office of the Costa Rica Institute of Technology (ITCR).

Interests conflict

The authors declare that there is no conflict of interest related to this study.

References

Abdelnour, S. A., Abd El-Hack, M. E., Alagawany, M., Taha, A. E., Elnesr, S. S., Abd Elmonem, O. M., & Swelum, A. A. (2020). Useful impacts of royal jelly on reproductive sides, fertility rate and sperm traits of animals. *Journal of Animal Physiology and Animal Nutrition, 104*(6), 1798–1808. https://doi.org/10.1111/jpn.13303

Aisen, E. G., Medina, V. H., & Venturino, A. (2002). Cryopreservation and post-thawed fertility of ram semen frozen in different trehalose concentrations. *Theriogenology, 57*(7), 1801–1808. https://doi.org/10.1016/S0093-691X(02)00653-2

Aisen, E., Quintana, M., Medina, V., Morello, H., & Venturino, A. (2005). Ultramicroscopic and biochemical changes in ram spermatozoa cryopreserved with trehalose-based hypertonic extenders. *Cryobiology, 50*(3), 239–249. https://doi.org/10.1016/j.cryobiol.2005.02.002

Alcay, S., Berk Toker, M., Gokce, E., Ustuner, B., Tekin Onder, N., Sagirkaya, H., Nur, Z., & Kemal Soylu, M. (2015). Successful ram semen cryopreservation with lyophilized egg yolk-based extender. *Cryobiology, 71*(2), 329–333. https://doi.org/10.1016/j.cryobiol.2015.08.008

Allai, L., Benmoula, A., Marciane da Silva, M., Nasser, B., & El Amiri, B. (2018). Supplementation of ram semen extender to improve seminal quality and fertility rate. *Animal Reproduction Science, 192*, 6–17. https://doi.org/10.1016/j.anireprosci.2018.03.019

Allai, L., Druart, X., Louanjli, N., Contell, J., Nasser, B., & El Amiri, B. (2017). Improvements of ram semen quality using cactus seed oil during liquid preservation in Tris egg yolk and skim milk based extenders. *Small Ruminant Research, 151*, 16–21. https://doi.org/10.1016/j.smallrumres.2017.02.001

Allai, L., Druart, X., Öztürk, M., BenMoula, A., Nasser, B., & El Amiri, B. (2016). Protective effects of *Opuntia ficus-indica* extract on ram sperm quality, lipid peroxidation and DNA fragmentation during liquid storage. *Animal Reproduction Science, 175*, 1–9. https://doi.org/10.1016/j.anireprosci.2016.09.013

Allai, L., Druart, X., Terzioğlu, P., Louanjli, N., Nasser, B., Ozturk, M., & El Amiri, B. (2023). The addition of *Opuntia ficus-indica* ethanolic extract to a skimmed milk-based extender impacts ram sperm quality. *Veterinary Medicine International, 2023*(1), Article 6248890. https://doi.org/10.1155/2023/6248890

Amidi, F., Pazhohan, A., Nashtaei, M. S., Khodarahmian, M., & Nekoonam, S. (2016). The role of antioxidants in sperm freezing: a review. *Cell and Tissue Banking, 17*, 745–756. https://doi.org/10.1007/S10561-016-9566-5

Asadzadeh, N., Abdollahi, Z., Esmaeilkhanian, S., & Masoudi, R. (2021). Fertility and flow cytometry evaluations of ram frozen semen in plant-based extender supplemented with Mito-TEMPO. *Animal Reproduction Science, 233*, Article 106836. https://doi.org/10.1016/j.anireprosci.2021.106836

Barbas, J. P., Pimenta, J., Baptista, M. C., Marques, C. C., Pereira, R. M. L. N., Carolino, N., & Simões, J. (2023). Ram semen cryopreservation for Portuguese native breeds: Season and breed effects on semen quality variation. *Animals, 13*(4), Article 579. https://doi.org/10.3390/ani13040579

Batissaco, L., Paes de Arruda, R., Rodrigues Alves, M. B., Andrade Torres, M., Menegon Lemes, K., Romano Prado-Filho, R., Guibu de Almeida, T., De Andrade, A. F. C., & Carvalho Celeghini, E. C. (2020). Cholesterol-loaded cyclodextrin is efficient in preserving sperm quality of cryopreserved ram semen with low freezability. *Reproductive Biology, 20*(1), 14–24. https://doi.org/10.1016/j.repbio.2020.01.002

Ben Moula, A., Rahim, A., Chentouf, M., Raes, M., Kirschvink, N., & El Amiri, B. (2023). Inclusion of *Spirulina platensis* and *Salvia verbenaca* extracts to boost semen quality and fertilization ability in sheep. *Reproduction in Domestic Animals, 58*(5), 637–645. https://doi.org/10.1111/rda.14332

Benko, F., Mohammadi-Sangcheshmeh, A., Ďuračka, M., Lukáč, N., & Tvrdá, E. (2022). *In vitro* versus cryo-induced capacitation of bovine spermatozoa, part 1: structural, functional, and oxidative similarities and differences. *PLoS ONE, 17*(10), Article e0276683. https://doi.org/10.1371/journal.pone.0276683

Benson, J. D., Woods, E. J., Walters, E. M., & Critser, J. K. (2012). The cryobiology of spermatozoa. *Theriogenology, 78*(8), 1682–1699. https://doi.org/10.1016/j.theriogenology.2012.06.007

Bergeron, A., Brindle, Y., Blondin, P., & Manjunath, P. (2007). Milk caseins decrease the binding of the major bovine seminal plasma proteins to sperm and prevent lipid loss from the sperm membrane during sperm storage. *Biology of Reproduction, 77*(1), 120–126. https://doi.org/10.1095/biolreprod.106.058248

Bergeron, A., & Manjunath, P. (2006). New insights towards understanding the mechanisms of sperm protection by egg yolk and milk. *Molecular Reproduction and Development, 73*(10), 1338–1344. https://doi.org/10.1002/mrd.20565

Chen, Y., Meng, F., Liu, Y., Zhu, C., Ling, Y., Liu, C., Li, L., Liu, Y., He, X., Cao, J., & Zhang, Y. (2024). Effects of resveratrol on DLD and NDUFB9 decrease in frozen semen of Mongolian sheep. *Cryobiology, 114*, Article 104791. https://doi.org/10.1016/j.cryobiol.2023.104791

Colas, G. (1975). Effect of initial freezing temperature, addition of glycerol and dilution on the survival and fertilizing ability of deep-frozen ram semen. *Reproduction, 42*(2), 277–285. https://doi.org/10.1530/jrf.0.0420277

Cseh, S., Faigl, V., & Amiridis, G. S. (2012). Semen processing and artificial insemination in health management of small ruminants. *Animal Reproduction Science, 130*(3-4), 187–192. https://doi.org/10.1016/j.anireprosci.2012.01.014

Del Valle, I., Souter, A., Maxwell, W. M. C., Muiño-Blanco, T., & Cebrián-Pérez, J. A. (2013). Function of ram spermatozoa frozen in diluents supplemented with casein and vegetable oils. *Animal Reproduction Science, 138*(3-4), 213–219. https://doi.org/10.1016/j.anireprosci.2013.02.022

El-Seadawy, I. E., Kotp, M. S., El-Maaty, A. M. A., Fadl, A. M., El-Sherbiny, H. R., & Abdelnaby, E. A. (2022). The impact of varying doses of moringa leaf methanolic extract supplementation in the cryopreservation media on sperm quality, oxidants, and antioxidant capacity of frozen-thawed ram sperm. *Tropical Animal Health and Production, 54*(6), Article 344. https://doi.org/10.1007/s11250-022-03344-y

Emamverdi, M., Zhandi, M., Zare Shahneh, A., Sharafi, M., & Akbari-Sharif, A. (2013). Optimization of ram semen cryopreservation using a chemically defined soybean lecithin-based extender. *Reproduction in Domestic Animals, 48*(6), 899–904. https://doi.org/10.1111/rda.12183

Forouzanfar, M., Sharafi, M., Hosseini, S. M., Ostadhosseini, S., Hajian, M., Hosseini, L., Abedi, P., Nili, N., Rahmani, H. R., & Nasr-Esfahani, M. H. (2010). *In vitro* comparison of egg yolk–based and soybean lecithin–based extenders for cryopreservation of ram semen. *Theriogenology, 73*(4), 480–487. https://doi.org/10.1016/j.theriogenology.2009.10.005

Freitas Bittencourt, R., Oba, E., De Almeida Biscarde, C. E., Costa Azevedo, H., Vasconcelos Bittencourt, M., Oliveira de Menezes, G. F., Da Silva Lima, A., Da Mata Fuchs, K., & De Lisboa Ribeiro Filho, A. (2018). Dimethylacetamide and trehalose for ram semen cryopreservation. *Cryobiology, 85*, 1–6. https://doi.org/10.1016/j.cryobiol.2018.10.266

Gil, J., Lundeheim, N., Söderquist, L., & Rodrı´guez-Martı´nez, H. (2003). Influence of extender, temperature, and addition of glycerol on post-thaw sperm parameters in ram semen. *Theriogenology, 59*(5-6), 1241–1255. https://doi.org/10.1016/S0093-691X(02)01177-9

Gosálvez, J., López-Fernández, C., Fernández, J. L., Gouraud, A., & Holt, W. V. (2011). Relationships between the dynamics of iatrogenic DNA damage and genomic design in mammalian spermatozoa from eleven species. *Molecular Reproduction and Development, 78*(12), 951–961. https://doi.org/10.1002/MRD.21394

Jihad Neamah, H. (2022). Evaluation of adding prickly pear extracts to the diluted ram's semen at preservation. *Journal of Animal Health and Production, 11*(1), 94–98. https://doi.org/10.17582/journal.jahp/2023/11.1.94.98

Keskin, N., Erdogan, C., Bucak, M. N., Ozturk, A. E., Bodu, M., Ili, P., Baspinar, N., & Dursun, S. (2020). Cryopreservation effects on ram sperm ultrastructure. *Biopreservation and Biobanking, 18*(5), 441–448. https://doi.org/10.1089/bio.2020.0056

Kumar Jha, P., Shahi Alam, M. G., Mansur, A. A., Naher, N., Islam, T., Uddin Bhuiyan, M., & Bari, F. Y. (2019). Cryopreservation of Bangladeshi ram semen using different diluents and manual freezing techniques. *Cryobiology, 89*, 35–41. https://doi.org/10.1016/j.cryobiol.2019.06.001

Layek, S. S., Mohanty, T. K., Kumaresan, A., & Parks, J. E. (2016). Cryopreservation of bull semen: Evolution from egg yolk based to soybean based extenders. *Animal Reproduction Science, 172*, 1–9. https://doi.org/10.1016/j.anireprosci.2016.04.013

Ledesma, A., Zalazar, L., Buchelly Imbachi, F., Pastore, J. I., Brown, P., Eddy, E. M., Hozbor, F., & Cesari, A. (2019). Recombinant peptide reverses cryo-capacitation in ram sperm and improves *in vitro* fertilization. *Animal Reproduction Science, 207*, 61–72. https://doi.org/10.1016/j.anireprosci.2019.05.016

Liu, G., Pan, B., Li, S., Ren, J., Wang, B., Wang, C., Su, X., & Dai, Y. (2020). Effect of bioactive peptide on ram semen cryopreservation. *Cryobiology, 97*, 153–158. https://doi.org/10.1016/j.cryobiol.2020.08.007

Luna-Orozco, J. R., González-Ramos, M. A., Calderón-Leyva, G., Gaytán-Alemán, L. R., Arellano-Rodríguez, F., Ángel-García, O., & Véliz-Deras, F. G. (2019). Comparison of different diluents based on liposomes and egg yolk for ram semen cooling and cryopreservation. *Iranian Journal of Veterinary Research, 20*(2), 126–130. https://doi.org/10.22099/ijvr.2019.5262

Marcantonini, G., Bartolini, D., Zatini, L., Costa, S., Passerini, M., Rende, M., Luca, G., Basta, G., Murdolo, G., Calafiore, R., & Galli, F. (2022). Natural cryoprotective and cytoprotective agents in cryopreservation: a focus on melatonin. *Molecules, 27*(10), Article 3254. https://doi.org/10.3390/molecules27103254

Masoudi, R., Sharafi, M., & Shahneh, A. Z. (2019). Effects of CoQ10 on the quality of ram sperm during cryopreservation in plant and animal based extenders. *Animal Reproduction Science, 208*, Article 106103. https://doi.org/10.1016/j.anireprosci.2019.06.015

Mehdipour, M., Daghigh Kia, H., Najafi, A., Vaseghi Dodaran, H., & García-Álvarez, O. (2016). Effect of green tea (Camellia sinensis) extract and pre-freezing equilibration time on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender. *Cryobiology, 73*(3), 297–303. https://doi.org/10.1016/j.cryobiol.2016.10.008

Mehdipour, M., Daghigh Kia, H., Nazari, M., & Najafi, A. (2017). Effect of lecithin nanoliposome or soybean lecithin supplemented by pomegranate extract on post-thaw flow cytometric, microscopic and oxidative parameters in ram semen. *Cryobiology, 78*, 34–40. https://doi.org/10.1016/j.cryobiol.2017.07.005

Moradi, M., Hajarian, H., Karamishabankareh, H., Soltani, L., & Soleymani, B. (2022). Pre-treatment of ram semen extender with magnetic nanoparticles on freeze-thawed spermatozoa. *Veterinary Medicine and Science, 8*(2), 792–798. https://doi.org/10.1002/vms3.689

Motlagh, M. K., Sharafi, M., Zhandi, M., Mohammadi-Sangcheshmeh, A., Shakeri, M., Soleimani, M., & Zeinoaldini, S. (2014). Antioxidant effect of rosemary (*Rosmarinus officinalis* L.) extract in soybean lecithin-based semen extender following freeze–thawing process of ram sperm. *Cryobiology, 69*(2), 217–222. https://doi.org/10.1016/j.cryobiol.2014.07.007

Murawski, M., Schwarz, T., Grygier, J., Patkowski, K., Oszczęda, Z., Jelkin, I., Kosiek, A., Gruszecki, T. M., Szymanowska, A., Skrzypek, T., Zieba, D. A., & Bartlewski, P. M. (2015). The utility of nanowater for ram semen cryopreservation. *Experimental Biology and Medicine, 240*(5), 611–617. https://doi.org/10.1177/1535370214557219

Najafi, A., Daghigh-Kia, H., Dodaran, H. V., Mehdipour, M., & Alvarez-Rodriguez, M. (2017). Ethylene glycol, but not DMSO, could replace glycerol inclusion in soybean lecithin-based extenders in ram sperm cryopreservation. *Animal Reproduction Science, 177*, 35–41. https://doi.org/10.1016/j.anireprosci.2016.12.004

Najafi, A., Najafi, M., Zanganeh, Z., Sharafi, M., Martinez-Pastor, F., & Adeldust, H. (2014). Cryopreservation of ram semen in extenders containing soybean lecithin as cryoprotectant and hyaluronic acid as antioxidant. *Reproduction in Domestic Animals, 49*(6), 934–940. https://doi.org/10.1111/rda.12405

Najafi, A., Zhandi, M., Towhidi, A., Sharafi, M., Akbari Sharif, A., Khodaei Motlagh, M., & Martinez-Pastor, F. (2013). Trehalose and glycerol have a dose-dependent synergistic effect on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender. *Cryobiology, 66*(3), 275–282. https://doi.org/10.1016/j.cryobiol.2013.03.002

Ofosu, J., Qazi, I. H., Fang, Y., & Zhou, G. (2021). Use of melatonin in sperm cryopreservation of farm animals: a brief review. A*nimal Reproduction Science, 233*, Article 106850. https://doi.org/10.1016/j.anireprosci.2021.106850

Paul, R. K., Kumar, D., & Singh, R. (2021). Carboxymethyl cellulose and glycerol act synergistically as cryoprotectant during cryopreservation of ram semen. *Cryobiology, 101*, 61–66. https://doi.org/10.1016/j.cryobiol.2021.06.001

Peris-Frau, P., Martín-Maestro, A., Iniesta-Cuerda, M., Sánchez-Ajofrín, I., Cesari, A., Garde, J. J., Villar, M., & Soler, A. J. (2020). Cryopreservation of ram sperm alters the dynamic changes associated with *in vitro* capacitation. *Theriogenology, 145*, 100–108. https://doi.org/10.1016/j.theriogenology.2020.01.046

Pini, T., Rickard, J. P., Leahy, T., Crossett, B., Druart, X., & De Graaf, S. P. (2018). Cryopreservation and egg yolk medium alter the proteome of ram spermatozoa. *Journal of Proteomics, 181*, 73–82. https://doi.org/10.1016/j.jprot.2018.04.001

Plante, G., Lusignan, M.-F., Lafleur, M., & Manjunath, P. (2015). Interaction of milk proteins and binder of sperm (BSP) proteins from boar, stallion and ram semen. *Reproductive Biology and Endocrinology, 13*(1), Article 92. https://doi.org/10.1186/s12958-015-0093-1

Pool, K. R., Rickard, J. P., & De Graaf, S. P. (2021). Melatonin improves the motility and DNA integrity of frozen-thawed ram spermatozoa likely via suppression of mitochondrial superoxide production. *Domestic Animal Endocrinology, 74*, Article 106516. https://doi.org/10.1016/j.domaniend.2020.106516

Ramírez-Vasquez, R. R. A., Cano, A., Hozbor, F. A., & Cesari, A. (2019). Cryopreservation and egg yolk extender components modify the interaction between seminal plasma proteins and the sperm surface. *Theriogenology, 140*, 153–163. https://doi.org/10.1016/j.theriogenology.2019.08.025

Ramírez-Vasquez, R., Cesari, A., Greco, M. B., Cano, A., & Hozbor, F. (2019). Extenders modify the seminal plasma ability to minimize freeze-thaw damage on ram sperm. *Reproduction in Domestic Animals, 54*(12), 1621–1629. https://doi.org/10.1111/rda.13571

Riesco, M. F., Alvarez, M., Anel-Lopez, L., Neila-Montero, M., Palacin-Martinez, C., Montes-Garrido, R., Boixo, J. C., De Paz, P., & Anel, L. (2021). Multiparametric study of antioxidant effect on ram sperm cryopreservation—from field trials to research bench. *Animals, 11*(2), Article 283. https://doi.org/10.3390/ani11020283

Rostami, B., Ebrahimi, D., Sadeghipanah, H., Masoumi, R., & Shahir, M. H. (2020). Effects of supplementation of tris-egg yolk extender with different sugars and antioxidants on freezability of ram semen. *Cryobiology, 92*, 62–66. https://doi.org/10.1016/j.cryobiol.2019.10.198

Saha, A., Asaduzzaman, M., & Bari, F. Y. (2022). Cryopreservation techniques for ram sperm. *Veterinary Medicine International, 2022*(1), Article 7378379. https://doi.org/10.1155/2022/7378379

Salamon, S., & Maxwell, W. M. C. (2000). Storage of ram semen. *Animal Reproduction Science, 62*(1–3), 77–111. https://doi.org/10.1016/S0378-4320(00)00155-X

Sarıözkan, S., Bucak, M. N., Tuncer, P. B., Ulutaş, P. A., & Bilgen, A. (2009). The influence of cysteine and taurine on microscopic–oxidative stress parameters and fertilizing ability of bull semen following cryopreservation. *Cryobiology, 58*(2), 134–138. https://doi.org/10.1016/j.cryobiol.2008.11.006

Sharafi, M., Borghei-Rad, S. M., Hezavehei, M., Shahverdi, A., & Benson, J. D. (2022). Cryopreservation of semen in domestic animals: a review of current challenges, applications, and prospective strategies. *Animals, 12*(23), Article 3271. https://doi.org/10.3390/ani12233271

Silvestre, M. A., Yániz, J. L., Peña, F. J., Santolaria, P., & Castelló-Ruiz, M. (2021). Role of antioxidants in cooled liquid storage of mammal spermatozoa. *Antioxidants, 10*(7), Article 1096. https://doi.org/10.3390/antiox10071096

Sobeh, M., Hassan, S. A., Hassan, M. A. E., Khalil, W. A., Abdelfattah, M. A. O., Wink, M., & Yasri, A. (2020). A polyphenol-rich extract from *Entada abyssinica* reduces oxidative damage in cryopreserved ram semen. *Frontiers in Veterinary Science, 7*, Article 604477. https://doi.org/10.3389/fvets.2020.604477

Toker, M. B., Alcay, S., Gokce, E., & Ustuner, B. (2016). Cryopreservation of ram semen with antioxidant supplemented soybean lecithin-based extenders and impacts on incubation resilience. *Cryobiology, 72*(3), 205–209. https://doi.org/10.1016/j.cryobiol.2016.05.001

Üstüner, B., Alçay, S., Nur, Z., Sağirkaya, H., & Soylu, M. K. (2014). Effect of egg yolk and soybean lecithin on Tris-based extender in post-thaw ram semen quality and in vitro fertility. *Kafkas Universitesi Veteriner Fakultesi Dergisi, 20*(3), 393-398. https://scispace.com/pdf/effect-of-egg-yolk-and-soybean-lecithin-on-tris-based-1vo9a8dhsy.pdf

Valcácia Silva, S., Trindade Soares, A., Batista, A. M., Costa Almeida, F., Ferreira Nunes, J., Alves Peixoto, C., & Pessoa Guerra, M. M. (2013). Vitamin E (Trolox) addition to Tris-egg yolk extender preserves ram spermatozoon structure and kinematics after cryopreservation. *Animal Reproduction Science, 137*(1–2), 37–44. https://doi.org/10.1016/j.anireprosci.2012.12.002

Valente, S. S., Pereira, R. M., Baptista, M. C., Marques, C. C., Vasques, M. I., Silva Pereira, M. V. C., Horta, A. E. M., & Barbas, J. P. (2010). *In vitro* and *in vivo* fertility of ram semen cryopreserved in different extenders. *Animal Reproduction Science, 117*(1–2), 74–77. https://doi.org/10.1016/j.anireprosci.2009.04.007

Vieira de Souza, C., Zandonadi Brandão, F., Dantas Rodrigues Santos, J., Angélico Pereira Alfradique, V., Moreira Barbosa dos Santos, V., Da Cruz Morais, M. C., Cerqueira Rangel, P. S., Amaral da Silva, A., & Gonçalves Souza-Fabjan, J. M. (2019). Effect of different concentrations of l-carnitine in extender for semen cryopreservation in sheep. *Cryobiology, 89*, 104–108. https://doi.org/10.1016/j.cryobiol.2019.05.009

Watson, P. F. (1995). Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reproduction, Fertility and Development, 7*(4), 871–891. https://doi.org/10.1071/RD9950871

Yániz, J. L., Mateos, J. A., & Santolaria, P. (2012). Tris buffer improves fluorescence yield of ram spermatozoa when evaluating membrane integrity. *Microscopy Research and Technique, 75*(4), 520–523. https://doi.org/10.1002/jemt.21086

Zalazar, L., Iniesta-Cuerda, M., Sánchez-Ajofrín, I., Garde, J. J., Soler Valls, A. J., & Cesari, A. (2020). Recombinant SPINK3 improves ram sperm quality and in vitro fertility after cryopreservation. *Theriogenology, 144*, 45–55. https://doi.org/10.1016/j.theriogenology.2019.12.019

Zhang, L., Wang, X., Jiang, C., Sohail, T., Sun, Y., Sun, X., Wang, J., & Li, Y. (2024). Effects of different diluents and freezing methods on cryopreservation of Hu ram semen. *Veterinary Sciences, 11*(6), Article 251. https://doi.org/10.3390/vetsci11060251

Zhu, Z., Zhao, H., Cui, H., Adetunji, A. O., & Min, L. (2023). Resveratrol improves the frozen-thawed ram sperm quality. *Animals, 13*(24), Article 3887. https://doi.org/10.3390/ani13243887