2026, Scienceline Publication
World's Veterinary Journal
World Vet J, 16(2): 733-749.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2026.wvj69
PII: S232245682600069-16

Challenges and Perspectives of Cryopreservation in Ram Semen of Indigenous Sheep: A Review

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REVIEW ARTICLE
Received: March 13, 2026
Revised: April 26, 2026
Accepted: May 25, 2026
Published: June 30, 2026

ABSTRACT

Artificial insemination (AI) is a widely used technique to enhance genetic diversity and breeding efficiency in livestock such as cattle, sheep, goats, and pigs. Sheep are important in developing the livelihoods of poor farmers by reducing poverty and promoting sustainable agriculture and food security. Therefore, the present study aimed to explore the challenges and perspectives associated with cryopreserving ram semen to conserve indigenous sheep ecotypes. Indigenous sheep populations are vital for meat production in challenging environmental conditions, where feed is limited and land is scarce. However, concerns have emerged about the decline in indigenous sheep populations, mainly due to the growth of composite breed production. Composite breeds, which are preferred for their faster growth rates and larger body size, reduce the capacity of indigenous sheep to survive in environments affected by climate change. This decline highlighted the importance of assisted reproductive technologies, such as AI and semen cryopreservation, in preserving the genetic resources of indigenous sheep before they become extinct. While AI and semen cryopreservation have been successful in livestock species such as cattle and pigs for genetic preservation, results in sheep have been less successful. Ram semen quality and fertility are often compromised by the freezing and thawing process. Ram spermatozoa produce high levels of reactive oxygen species (ROS) and are sensitive to cold shock, leading to oxidative stress, cellular damage, and conception rates of 30-50%. Adding antioxidants, such as polyunsaturated fatty acids and plant extracts, to semen extenders can reduce ROS production and mitigate oxidative damage, thereby improving sperm quality and functionality during cryopreservation. Despite these advances, optimizing freezing and thawing protocols and addressing knowledge gaps are necessary to enhance semen preservation outcomes.

Keywords: Antioxidant, Artificial insemination, Extender, Nanoparticle, Sheep, Semen cryopreservation

INTRODUCTION

Semen cryopreservation has been shown to improve reproductive efficiency following the use of artificial insemination (AI) by facilitating the exchange of superior genetic material worldwide (Yimer et al., 2016; Kurmi et al., 2018; Maksimovic et al., 2018). Semen cryopreservation is an important technique for enhancing global food security, as it allows the use of superior genetic material to improve livestock performance (Ngcobo et al., 2023). However, the use of semen cryopreservation posed significant challenges at the farm level, especially for rural farmers in underdeveloped and developing nations, due to resource and educational constraints. The use of semen cryopreservation remains in the research phase. In developing countries, although research facilities are available, they are often located far from smallholder farmers, making cryopreservation of semen impractical. Thus, food insecurity is increasing because the indigenous sheep population cannot be preserved, having been diminished by uncontrolled crossbreeding and climate change (Ngcobo et al., 2022). To address the food insecurity crisis in developing and underdeveloped countries, the United Nations has taken action to support farmers, enhance their livelihoods, and protect the global community by implementing the sustainable development goals targets for zero hunger and no poverty. Mitigation strategies included educating farmers on the use of assisted reproductive technologies (ARTs) to improve the reproductive efficiency of their livestock (Sithole et al. 2025). However, there has been limited reporting in developing and underdeveloped countries on advancements in ART applications, particularly in semen cryopreservation. Even in developed countries with adequate resources and education, ram semen cryopreservation continues to pose challenges. Conflicting results have been reported in studies evaluating the use of different semen diluents during ram semen characterization and cryopreservation.

To cite this paper: Patricia MT, Agree NK, Mbali SS, Judas MT, Lottus MM, and Nkululeko NJ (2026). Challenges and Perspectives of Cryopreservation in Ram Semen of Indigenous Sheep: A Review. *World Vet. J.*, 16(2): 733-749. DOI: https://dx.doi.org/10.54203/scil.2026.wvj69

Current studies on ram spermatozoa during and after thawing primarily examine how sperm parameters respond physically across different environments and time points (Yimer et al., 2016; Zhang et al., 2024). To understand sperm responses to cold shock and oxidizing stress induced by reactive oxygen species (ROS), scientists used different semen extenders, antioxidants, and nanoparticles to balance cytoplasmic function and increase the number of natural antioxidants on the sperm plasma membrane during cryopreservation. The addition of some of these diluents and antioxidants has improved semen quality following AI (Yimer et al., 2016; Abbasi et al., 2021; Arruda et al., 2021). However, challenges associated with sperm viability during cryopreservation and thawing affect the success of AI. According to the previous studies, Kurmi and Naqvi (2014) documented low conception rates of 20% and 26.6% following single and double insemination with frozen-thawed semen. Furthermore, Halawa et al. (2025) reported a conception rate of 45.4%, while Najafi et al. (2024) observed a rate of 83.3%. Bernia and Benamora achieved the highest conception rate of 90.47% following the utilization of fresh semen. Gibbon et al. (2019) reported a satisfactory pregnancy rate of 50-70% after using fresh semen. According to Yimer et al. (2016), cryopreservation not only lowers sperm quality, increases DNA damage, decreases mitochondrial function, and increases oxidative stress, but also these damages subsequently elevate the risks of abortion and apoptosis in ewes (Yimer et al., 2016). Consequently, low conception and lambing rates associated with cryopreserved semen have increased the use of liquid preserved semen to achieve optimal pregnancy rates (Najafi et al., 2014). Furthermore, factors including oxidative stress, cryoinjuries, and reduced sperm viability have been identified as major limitations affecting the success of ram semen cryopreservation. Therefore, the current study aimed to address the challenges and perspectives associated with cryopreservation of ram semen to ensure the successful application of AI in conserving indigenous sheep ecotypes.

OVERVIEW OF DIFFERENT SHEEP CONSERVATION METHODS

The ARTs, such as semen cryopreservation and AI, have been used in previous years to conserve sheep biodiversity. However, there are still challenges related to their application. These challenges include limited access to resources and knowledge for rural farmers and limited success with ram semen cryopreservation, which is associated with increased embryonic loss and impacts sheep production (Yimer et al., 2016). According to Ngcobo et al. (2022), the two principal conservation strategies employed were *in situ* (extensive farming) and *ex situ* (intensive farming and the use of ARTs). So far, there has been criticism of the implementation of the *in*-situ approach. Approximately 80% of emerging farmers raised their indigenous breeds under extensive management for communal grazing, with limited monitoring. This situation led to challenges, including uncontrollable crossbreeding and inbreeding, which contributed to the decline in indigenous breed populations. A genetic cross of Dorper in Zulu diversity demonstrated evidence of uncontrolled crossbreeding (Selepe et al., 2018). As Ngcobo et al. (2022) stated, *ex situ* conservation (*in vivo* and *in vitro*) remains a crucial technique for conserving indigenous breeds. To prevent crossbreeding, it is recommended to keep distinct breeds separated in designated areas, such as those at the Agricultural Research Council (ARC), utilizing the *in vivo* conservation method. Additionally, *in vivo* conservation has been successful, as animals were continuously monitored throughout the process. However, challenges arise when applying ARTs *in vitro*.

Semen cryopreservation plays a significant role in breeding and conservation programs, particularly for small ruminants (Kurmi et al., 2018). This method preserves genetic material from superior sires, allowing the widespread transfer of desirable traits to many female sheep and enhancing genetic progress (Kurmi et al., 2018; Selepe et al., 2018). Azizunnesa et al. (2014) observed that semen preservation facilitated the frequent use of AI, the conservation of endangered species, and the international exchange of genetic material. A report highlighting the severe decline in indigenous sheep breeds has caused concern among farmers, since these breeds are vital for wool production and play a crucial role in eradicating poverty and hunger (Ngcobo et al., 2022). Genes associated with high adaptability and disease resistance enable farmers to maintain indigenous breeds at minimal cost. It is important to highlight that around 80% of farmers who depend on indigenous breeds are smallholders (Kunene and Fossey, 2006). However, commercial farmers crossbreed indigenous sheep with exotic breeds to produce a composite breed with improved genetic material (Ngcobo et al., 2019). Therefore, these composite breeds meet market demand, which, in turn, impoverishes rural farmers.

Over 500,000 sheep are artificially inseminated annually in Australia, 300,000 in France, 60,000 in Spain, and 50,000 in Canada, as reported by Gibbons et al. (2019). According to Falch et al. (2022), the production of sheep embryos, *in vivo*-derived and *in vitro*-produced (IVP), increased by 20% between 2018 and 2019. Viana et al. (2022) indicated that although no frozen-thawed embryo transfers were recorded, the study did find a significant increase in fresh IVP embryo transfers. Ngcobo et al. (2022) noted that advanced reproductive biotechnologies, including estrus synchronization, were applied in South African indigenous sheep. Conversely, limited information is available regarding the outcomes of AI using post-thawed semen. Given the decline in indigenous sheep breeds in South Africa, there is an imperative to enhance the application of *ex situ* conservation methods to preserve the remaining genetic resources of these breeds.

However, *ex situ* conservation methods are more effective in developed countries than in underdeveloped or developing nations, as evidenced by the lower number of reports on AI follow-up using frozen semen from these regions. The limited report on AI follow-up studies using frozen-thawed semen in developing countries heightened concerns that ineffective sheep production management could put developing countries such as South Africa at risk of hunger and poverty (Ngcobo et al., 2022). Farming with indigenous sheep is increasingly essential for rural farmers, as these breeds are at risk of extinction and should be conserved (Kunene et al., 2011).

Artificial insemination in sheep has achieved limited success, often depending on chilled semen to improve fertility (Najafi et al., 2014). Laparoscopic AI can help overcome the lower fertility rates usually associated with frozen-thawed semen, which result from the ewe's complex reproductive anatomy, particularly the cervix (Kumar and Naqvi, 2014). Rizkallah et al. (2022) reported a 20-30% pregnancy rate following cervical AI with frozen-thawed semen, making sheep breeders reluctant to use AI due to an inability to achieve positive results, particularly following the use of cryopreserved semen. Laparoscopic AI enables direct semen deposition into the uterus, which enhances the likelihood of fertilization compared to cervical and vaginal AI. Nonetheless, laparoscopic AI applications remain controversial due to factors such as cost, required expertise, and ethical considerations related to surgery and anesthesia (Rizkallah et al. 2022). This approach encourages the use of cervical AI and discourages the use of vaginal AI (Akhter et al. 2023). Vaginal AI is cheaper and simpler than cervical AI. However, imprecise sperm placement during vaginal AI and issues with the cervical barrier may hinder sperm from reaching the site of fertilization, thereby reducing the probability of conception (Masoudi et al., 2017).

Kumar and Naqvi (2014) indicated that controlled internal drug release and equine chorionic gonadotropin are frequently employed exogenous agents at the end of the progesterone therapy during estrus synchronization, with the purpose of promoting cervical relaxation. Several studies have reported that the use of these exogenous substances can effectively modify estrus responses in many mammals, although they do not guarantee optimal fertilization (Najafi et al., 2014; Gibbons et al., 2019; Benia and Benamora, 2023). Fierro et al. (2013) found that the effectiveness of this exogenous substance on the estrus response was influenced by a longer interval between doses, which negatively impacted the fertilization rate. Low fertilization rates in ewes with an optimal body condition score of 3-4 are associated with increased oxidative stress in male germ cells, which is a recognized negative effect of semen cryopreservation (Yimer et al., 2016; Thompson et al., 2024).

The number of services per insemination had a significant effect on lambing rate after trans-cervical AI with frozen semen (Table 1). Double inseminations conducted between 14 and 22 hours following estrus detection yielded higher conception and lambing rates (26.40% and 19.3%) compared to a single insemination performed at 10 hours (20% and 10%; Kumar and Naqvi, 2014). According to Bergstein-Galan et al. (2022), the semen preservation method affected the success of laparoscopic AI in Dorper, white Dorper, and crossbred ewes. A higher conception rate was observed with fresh semen (53.84) than with frozen-thawed semen (30.23). Masoudi et al. (2017) reported the best fertility results with laparoscopic AI compared to the vaginal and trans-cervical AI in Zandi sheep. These results are aligned with the findings reported by Najafi et al. (2014). However, both semen types had lower fertility rates than those reported by Halawa et al. (2025). According to Benia and Benamor (2023), the reproductive performance of ewes utilizing cervical AI was superior in ewes aged three years and above that were inseminated with semen from mature rams, compared to those that were inseminated with semen from younger rams. Regarding the use of vaginal AI, Madrigali et al. (2021) found no substantial difference in fertility results among Assaf, Awassi, and the Assaf × Awassi crossbreed.

Semen cryopreservation

Semen cryopreservation is the long-term storage of animal germ cells at cryogenic temperatures (-150 °C or lower) to support the application of ARTs (Kurmi et al., 2018). This method can be used in countries such as South Africa, where smallholder farms are mostly found in distant areas with no access to research facilities and government services. Despite the advantages of semen cryopreservation, this method has been reported to cause a high level of oxidative stress, which damages spermatozoa and reduces their post-thaw survival (Yimer et al., 2016; Bustani and Baiee, 2021; Sun et al., 2024). Therefore, a decrease of about 40-50% in sperm viability following semen cryopreservation has been documented (Mafolo, 2018). Moreover, poor semen quality has been found to result in poor embryonic development during early gestation and to be associated with higher embryonic losses (Falchi and Yimer et al., 2016). Among the many factors that lead to poor frozen-thawed semen quality, the heightened production of ROS by non-viable spermatozoa and the acidic pH were especially notable (Riesco et al., 2021; Mehdipour et al., 2025). Therefore, enhancing semen extenders with antibiotics and antioxidants is crucial for preserving semen quality, minimizing oxidative stress, and ultimately improving cryopreservation and conception rate (Mphaphathi et al., 2016; Yimer et al., 2016).

Table 1. Effects of different artificial insemination methods on sheep fertility

Breed	AI method	Semen type	Number of females inseminated	Conception rate (%)	Lambing rate (%)	Litter size rate (%)	Twinning rate (%)	References
Bharat merino	Trans-cervical	Frozen	Group 1 100	20	10	-	-	Kumar and Naqvi (2014)
Bharat merino	Trans-cervical	Frozen	Group 2 140	26.4	19.3	-	-	Kumar and Naqvi (2014)
Zandi	Vaginal	Fresh	50	62	62	-	10.0	Masoudi et al. (2017)
Zandi	Laparoscopic	Fresh	50	66	64	-	6.0	Masoudi et al. (2017)
Zandi	Trans-cervical	Fresh	50	64	62	-	6.0	Masoudi et al. (2017)
Ghezel	Laparoscopic	Fresh	30	83.3	76.6	1.30	28.0	Najafi et al. (2024)
Ghezel	Cervical	Fresh	30	60	60	1.28	34.6	Najafi et al. (2014)
Ghezel	Natural mating with PSMG injection	Fresh	30	90	86.6	1.35	18.5	Najafi et al. (2014)
Ghezel	natural mating without PSMG injection	Fresh	30	70	66.7	1.10	10.0	Najafi et al. (2014)
Palestinian Assaf	-	Fresh	37	61.1	-	-	-	Halawa et al. (2025)
Palestinian Assaf	-	Frozen	50	45.4	-	-	-	Halawa et al. (2025)
Palestinian	Natural mating		36	71.9	-	-	-	Halawa et al. (2025)
Bangladesh	Laparoscopic	Frozen	-	72.2	100		-	Kumar and Naqvi (2014)
Rembi	Cervical	Fresh	Group 1 42	61.90	-	-	-	Benia and Benamora (2023)
Rembi	Cervical	Fresh	Group 2 42	78.58	-	-	-	Benia and Benamora (2023)
Rembi	Cervical	Fresh	Group 3 42	90.47	-	-	-	Benia and Benamora (2023)
Awassi	Vaginal	Fresh	94	45.7	56.4	1.23	-	Madrigali et al. (2021)
Assaf	Vaginal	Fresh	228	47.4	67.1	1.47	-	Madrigali et al. (2021)
Crossbreed (Awassi x Assaf)	Vaginal	Fresh	170	46.8	63.9	1.40	-	Madrigali et al. (2021)

Semen extenders

Semen extenders are specialized cryopreservation media designed to protect sperm against damage from freezing, osmotic stress, oxidative damage, and ice crystal formation, thereby preserving sperm viability (Bustani and Baiee, 2021). Notably, semen extenders differ in their chemical and physical properties due to their diverse roles in sperm functionality (Yimer et al., 2016). These roles include protecting sperm cells against cold shock, maintaining optimal pH, supplying essential nutrients, and controlling microbial growth. During semen cryopreservation, sperm survival largely depends on protective semen extenders, such as egg yolk, skim milk, AndroMed®, soybean lectin, and antioxidants, including moringa extracts, ascorbic acid, nanoparticles, and curcumin (Kulaksiz et al., 2010; Mafolo, 2018; Arruda et al., 2021). However, variation in sperm motility and morphometry after dilution during semen cryopreservation is influenced by the extender's chemical composition, concentration, and dilution method.

Since extenders are usually not chemically identical, they can originate from either plants or animals. Materials from the same source can differ in chemical composition. Egg yolk is often used as a semen extender and protein source because of its low levels of low-density lipoproteins, yet its composition can vary between batches (Swelum et al., 2023). However, the content of fatty acids differs among different bird species, which demonstrates significant effects on semen viability (Table 2; Kulaksiz et al., 2016; Swelum et al., 2023).

Table 2. Influence of different semen extenders on ram semen quality

Breeds	Extender	Findings	References
Karayaka	Chucker egg yolk	It demonstrated the most effective cryoprotection, with the highest sperm motility at 54.0%, outperforming the other five avian egg yolks. Frozen sperm had a higher viability rate (59%) than sperm preserved in other species.	Kulaksiz et al. (2010)
Karayaka	Chicken egg yolk	Recorded a lower percentage of sperm viability, motility, membrane integrity, and acrosomal integrity.	Kulaksiz et al. (2010)
INRA 180	Skim milk-based	Sperm quality and seminal plasma composition were superior in low-frequency ejaculation compared with high-frequency ejaculation, and earlier ejaculators exhibited greater quality than later ones. Furthermore, seminal plasma components were correlated with sperm quality after 24 hours of storage.	Benmoula et al. (2022)
Santa Ines	8% Low-Density Lipoprotein -Tris –glucose extender	Improved velocity parameters, motility, and curvilinear velocity	Pereria et al. (2023)
Leccese	Milk lactose egg yolk (Milk- LY)	Milk-LY extender outperforms Tris-Fructose egg yolk in preserving sperm quality at intermediate concentrations (100-500 × 10^6 spermatozoa/mL). However, increasing sperm concentration to 800 × 10^6 spermatozoa/mL negatively impacts sperm characteristics, potentially reducing lambing rates despite higher sperm numbers. For laparoscopic insemination, semen doses of 20-40 × 10^6 spermatozoa per ewe were effective.	D'Alessandro et al. (2021)
Suffolk	Ovipro	Improved sperm motility, viability, and low morphological abnormalities within the short term, from 24 to 48 hours.	Hegedusova et al. (2012)
Suffolk	Triladyl	Demonstrated effective stabilizing properties, yielding consistent results over extended storage periods (24-96 hours).	Hegedusova et al. (2012)
Duolang	Soybean lecithin in Tri's fructose extender	Ram sperm retained their fertilizing ability when preserved at 0°C in a Tris-based extender containing 0.5% soybean lecithin instead of egg yolk, resulting in normal offspring after insemination.	Zhao et al. (2021)
Bangladeshi indigenous	Tris fructose egg yolk	Exhibited less improvement in semen parameters compared to Triladyl.	Rekha et al. (2016)
Bangladeshi indigenous	Triladyl	Triladyl has high sperm motility, viability, and functional integrity	Rekha et al. (2016)
Crossbred- pirot prameka Wurttemberg and lle de France	Soybean lecithin (AndroMed®-based extender)	Improved sperm morphology and motility	Maksimovic et al. (2018)
Dorper	AndroMed®	The percentage of live sperm with intact acrosomes was highest in fresh semen (97.8%) and decreased with refrigeration and freezing.	Quintero-Elisea et al. (2024)
Dorper	OviXcell	Sperm viability rates were 87% in fresh semen, 72% in refrigerated semen, and 55% in post-thawed semen, with a slightly higher rate of 68% observed in a specific post-thawed semen context.	Quintero-Elisea et al. (2024)
Kail	Tris-based extender	Tris-based extender outperformed skim-milk and sodium citrate extenders, yielding higher percentages of motile, progressive motile, rapid progressive, and medium progressive motile sperm	Hameed et al. (2024)
Wrzosówka rams	milk extender containing 5% egg yolk	Replacing egg yolk with soybean lecithin worked well in milk extender but was ineffective in Tris extender.	Gogol et al. (2019)

According to Yimer et al. (2016) and Zhang et al. (2024) excessive dilution can reduce sperm density and effective sperm count, even when the volume of insemination is increased. Given the ewe's complex reproductive anatomy, an increased semen volume with reduced viscosity can worsen reflux of spermatozoa from the cervix, thereby adversely affecting conception and pregnancy rates (D'Alessandro et al., 2021). Conversely, insufficient dilution can threaten sperm survival because it lacks the necessary nutrients, buffers, and antioxidants that maintain stable pH and redox

balance and reduce seminal plasma levels (Zhang et al., 2024). Therefore, choosing the correct semen dilution ratio and method is essential for enhancing and preserving sperm viability and motility (Table 3).

The present results indicated that the milk skim lactose egg yolk extender was more effective than Tris-fructose egg yolk at preserving semen quality when diluted with intermediate sperm concentration (Table 2; 100 to 500 × 10^6 spermatozoa/mL). A rapid increase in the sperm concentration led to a decrease in sperm viability, thus reducing the lambing rate (D'Alessandro et al., 2021). Benmoula et al. (2022) observed positive outcomes with low-frequency ejaculation compared with high-frequency ejaculation when using a skim milk-based extender in INFRA180 rams. The use of Ovipro and Triladyl on Suffolk ram semen improved semen quality, but their capacity was affected by hours during freezing. Ovipro extender improved sperm motility and viability and reduced morphological abnormalities in the short term (24 to 48 hours), whereas Triladyl lasted longer (24 to 96 hours). Consequently, Rekha et al. (2016) reported Triladyl as a superior semen extender compared to tris fructose egg yolk extender. According to Kulaksiz et al. (2010), the use of egg yolk from different avian species indicated that chukar egg yolk may serve as an effective substitute for chicken egg yolk in the cryopreservation of semen. Zhao et al. (2021) reported that soybean lectin in a tris-based extender can be used to achieve successful AI and increase lambing rate following semen cryopreservation. Maksimovic et al. (2018) reported that soybean lectin enhanced semen parameters across different breeds. Quintero-Elisea et al. (2024) compared OviXcell and AndroMed® semen extenders, and the results indicated that both extenders can be used for the cryopreservation of ram semen, as they improved semen quality.

Table 3. Effects of egg yolk from different avian species on rams' semen quality

Avian species	Concentration	Findings	Reference
Japanese quail	15%	The best results were obtained with quail egg yolk compared with chicken egg yolk EY. Thus, quail EY can replace chicken EY.	Swelum et al. (2023)
Chicken, pigeon, duck, Goose and turkey	15%	None of these three avian EYs were suitable substitutes for chicken egg yolk in ram semen extenders, except for quail EY, which resulted in lower post-thaw semen quality.	Swelum et al. (2023)
Pigeon, turkey, Japanese quail, Guinea fowl, and domestic chicken	10%	Sperm motility was best preserved after dilution with Japanese quail and chicken egg yolks, outperforming the other avian egg yolks in terms of cryoprotective effects.	Taha et al. (2020)
Chucker, domestic chicken, goose, turkey, and Japanese quail	15%	Chucker egg yolk could replace chicken egg yolk as a suitable component in semen extenders for cryopreservation.	Kulaksiz et al. (2010)
Chicken	0, 5, 10, 15, and 20%	A 10% egg yolk concentration yielded better membrane integrity and sperm motility in cryopreserved semen, whereas 20% egg yolk concentration resulted in higher acrosome integrity.	Mafolo (2018)
	5, 10, 15, and 20%	Sperm motility, viability, and functional integrity were significantly enhanced during preservation when using 10% egg yolk compared to other concentrations.	Azizunnesa et al. (2014)
Turkey, partridge, ostrich, duck, tortoise, or chicken egg yolk	20%	Semen diluents with partridge egg yolks enhanced cleavage rates and early embryonic development. In contrast, ostrich, turkey, and duck egg yolks yielded fertilization and embryonic development rates comparable to those of traditional diluents containing 20% chicken egg yolk.	Ali et al. (2013)

EY: Egg yolk

Impact of egg yolk from different bird species on ram sperm quality

Different egg yolks from different bird species have been used to preserve ram semen, yielding varying results. A study by Swelum et al. (2023) has explored the relationship between the fatty acid composition of egg yolks from different bird species and the quality of ram semen after thawing. As mentioned previously, egg yolk is the most commonly used semen extender to improve semen quality during storage. The protective effects of egg yolk on sperm during cooling and freezing can be attributed to its rich phospholipid, cholesterol, and low-density lipoprotein composition, which help maintain sperm membrane integrity, prevent cold shock, and improve sperm viability (Azizunnesa et al., 2014). However, several studies have indicated that nutrition, breed, and age of the egg also influence egg yolk fatty acids.

Zhang et al. (2024) reported that as birds grow older, their egg yolks experience notable changes, such as reduced moisture, higher crude fat levels, and increased diversity in fatty acids (Zhang et al., 2024). Since mid-laying eggs have higher fatty acid content than early laying eggs, it is recommended to extract the yolk from eggs collected or hatched during the mid-laying stage. Furthermore, fatty acid profiling has been performed on egg yolk from various avian species, regardless of egg age (Kulaksiz et al., 2010; Swelum et al., 2023). According to Swelum et al. (2023), the egg yolks of turkeys, pigeons, and chickens contain significantly higher percentages of polyunsaturated fatty acids (PUFAs), with levels two to three times higher than those found in duck and goose egg yolks. Consequently, differences in the proportion of PUFAs in avian egg yolk may explain the variation in the results observed when comparing the effectiveness of egg yolk from avian species on ram semen quality (Swelum et al., 2023). Kulaksiz et al. (2010) reported that, when comparing EY across different bird species, chucker EY demonstrated superior semen quality to chicken EY and other species' EY. Furthermore, Taha et al. (2016) disapproved of the use of pigeon, turkey, and guinea fowl EY and suggested replacing chicken EY with Japanese quail EY because of the higher fatty acid level in quail egg yolk compared to chicken egg yolk. Consequently, examining the impact of each EY species on ram semen revealed that each species had a unique EY composition, warranting further investigation. Perumal (2018) noted that differences in EY among species were affected by their habitat. Wild birds, such as quail and chukars, have easy access to omega-3 sources, such as fish, seeds, and insects. In contrast, domestic chickens rely entirely on commercial feed for their omega-3 intake. Thus, omega-3 supplementation in the diet of domestic avian species is critical for improving the nutritional profile and health benefits of egg yolks (Ngcobo et al., 2024; Yaung et al., 2023).

Several studies have indicated that egg yolk levels affect semen preservation, with high concentrations potentially being toxic to semen (Zhang et al., 2024). Since egg yolk comes from animals, it may pose a risk of transmitting diseases through unidentified pathogens. Therefore, understanding the specific impact of each egg yolk and determining the best dilution concentration for semen are essential. According to Azizunnesa et al. (2014), the ideal egg yolk concentration required for semen dilution depends on the preservation method, such as cryopreservation, liquid storage, or freeze-drying. Azizunnesa et al. (2014) found that prolonged preservation time negatively impacted sperm quality, reducing motility, viability, functional integrity, and normal morphology. Reduced sperm quality indicated that, whether egg yolk is used or not, semen freezing reduces motility, damages morphological integrity, and raises embryonic mortality. Fatty acid profiling indicated that the absence of butyric and docosahexaenoic acids in egg yolk reduces its ability to protect sperm from cold shock and maintain the quality of cryopreserved semen (Azizunnesa et al., 2014). Supplementation or advancement of EY fatty acids content is necessary for successful semen cryopreservation. As a result, antioxidants are extensively used in PUFAs to improve EY functionality in preserved semen.

The effects of EY from different bird species are presented in Table 3. According to Swelum et al. (2023), 15% of Japanese quail EY was found to be effective in comparison with other avian EY. Thus, it was recommended to use Japanese quail EY to replace chicken EY on cryopreserved ram semen. These findings were consistent with those of Taha et al. (2016), who observed that the egg yolks of chicken and quail offered superior cryoprotective effects compared to those of other avian species, thereby enhancing sperm motility. Taha et al. (2016) further recommended the inclusion of a 10% concentration of chicken and quail EY. Consequently, Azizunnesa et al. (2014) and Mafolo (2018) found that EY at 10% concentration yielded optimal semen quality compared with 0%, 5%, 15%, and 20% concentrations. Kulaksiz et al. (2010) documented that chucker EY yielded superior results in ram semen compared to chicken EY. In the study conducted by Kulaksiz et al. (2010), a 15% egg yolk concentration was used for all avian species. These results were consistent with those reported by Swelum et al. (2023), who showed that a 15% EY concentration improved ram semen quality. with those of Swelum et al. (2023), who demonstrated that 15% EY enhanced ram semen quality. Furthermore, according to Ali and Floris (2013), using 20% partridge EY yielded higher cleavage rates and enhanced early embryonic development in ewes, compared to EY derived from ostrich, turkey, duck, tortoise, and chicken.

Antioxidants rich in long-chain polyunsaturated fatty acids

There is growing interest in using long-chain polyunsaturated fatty acids (LCPUFAs) as antioxidants to combat oxidative stress and reduce the harmful effects of lipid peroxidation on sperm fertility (Rizkallah et al., 2022). Ram sperm membranes should be altered or improved to enhance ram semen efficiency, since ram sperm membranes have a low phospholipid/cholesterol ratio and a high unsaturated-to-saturated fatty acid ratio (Yimer et al., 2016). As noted by Yimer et al. (2016), antioxidants are classified as either enzymatic, which neutralize excess ROS and prevent cellular damage, or non-enzymatic (dietary supplements), which should be incorporated into ruminant feed owing to the animals' inability to produce specific fatty acids. Additionally, Oppedisano et al. (2020) noted that LCPUFAs, comprising 18, 20, or 22 carbon atoms, can support sperm function and offer therapeutic benefits in different conditions, including cardiovascular disease and hypertension. Omega-3 (alpha-linoleic acid [ALA], docosahexaenoic acid, docosapentaenoic

acid, and eicosatetraenoic acid [EPA]), omega-6 (arachidonic acid, gamma-linoleic acid, and linoleic acid), and omega-9 (oleic acid) are the three categories for LCPUFAs (Ngcobo et al., 2024). When these fatty acids are supplemented in animal diets, mammals convert ALA to EPA, which is essential for the synthesis of reproductive hormones, such as luteinizing hormone (LH), testosterone, and follicle-stimulating hormone (FSH). These reproductive hormones play a vital role in maintaining sperm membrane fluidity, mediating cellular responses, supporting protein synthesis, and sustaining astrocytes and retinal pigment epithelial cells in the testicles (Yaun et al., 2023; Ngcobo et al., 2024). The interactions between antioxidants and sperm function are still poorly understood. However, previous studies have noted that ram sperm membranes have a high ratio of unsaturated to saturated fatty acids and low lipoprotein levels, along with the absence of a robust antioxidant system, unlike those in other ruminants (Azizunnesa et al., 2014; Yimer et al., 2016; Akther et al., 2023).

According to Yimer et al. (2016), freezing and dilution of sperm decreased the levels of natural antioxidants, thereby increasing ROS and impairing sperm quality and fertilization potential. As a result, several studies indicated that using PUFAs can help resolve sperm quality abnormalities (Table 4; Zarei et al., 2018; Ngcobo et al., 2024; Abdollahzadeh et al., 2025). Flaxseed oil, a plant-based source of omega-3, can be supplemented to the diet to improve sperm's low phospholipid concentration and lower malondialdehyde (MDA) levels during lipid peroxidation (Ngcobo et al., 2024). Additionally, vitamin E can enhance reproductive processes without reducing sperm efficiency (Saini et al., 2021; Yuan et al., 2023). Moreover, Masoudi and Dadashpour-Davachi (2021) reported that fish oil improved semen quality and ewe fertility parameters. An optimal hydrophobic tail of fatty acids was reported to help preserve sperm cell membrane integrity and improve fluidity, thereby supporting sperm motility and function (Yimer et al., 2016). Additionally, fatty acids can integrate into the lipid bilayer of the sperm membrane, protect sperm from oxidative damage, and reduce ROS. The addition of these hydrophobic antioxidants can help balance lipid, antioxidant, and ROS levels in the sperm cytoplasm. Therefore, it is crucial to sustain a balanced intake of LPUFAS to replace the utilization of hydrophilic antioxidants that are not absorbed by ROS during semen cryopreservation.

Table 4. Effects of different semen antioxidants on rams' semen quality

Breed	Type of antioxidant	Level used/concentration	Findings	References
Hu	Alpha-lipoic acid (ALA)	0, 0.025, 0.05, 0.1, 0.5, 1 mM	Alpha-lipoic acid (ALA) supplementation at 0.1 mM effectively mitigates oxidative stress in sperm and improves semen quality during preservation at 4°C.	Sun et al. (2024)
Zandi	Fish oil	-	Fish oil (FO) supplementation significantly improved all aspects of sperm quality and quantity compared to the control diet.	Alizadeh et al. (2014)
Afshari	Flaxseed oil	5%	Improved semen during liquid storage	Zarei et al. (2018)
Bapedi, Zulu, Damara, and Namaqua	Flaxseed oil	FLO - 5%	A combination of flaxseed oil and ascorbic acid (FLO and ASA) improved semen quality, in vitro fertilization, and cleavage rate.	Ngcobo et al. (2024)
Bapedi, Zulu, Damara, and Namaqua	ascorbic oil	4%	Adding ascorbic acid improved semen quality compared to the negative and positive controls	Ngcobo et al. (2024)
	Docosahexaenoic (DHA)	0.15, 0.30, or 0.45 g	Adding Docosahexaenoic acid to ram semen extenders enhances semen quality and fertility after thawing.	Abdi-Benemar et al. (2015)
Moghani	Persia fat	-	Adding fish oil or Persia fat to the diet enhanced the resilience of sperm to freezing and thawing, resulting in better quality Adding fish oil or Persia fat to the diet enhanced the resilience of sperm to freeze and thawing, resulting in better quality.	Hedayat-Evrigh et al. (2019)
Shall	Omega-6 to omega-3 fatty acids	Low ratio 4:1	Dietary supplementation of a low ration improved semen parameters and reduced DNA damage compared to a high ration	Abdollahzadeh et al. (2025)
Iranian Zandi	Fish oil		Fish oil supplementation enhanced semen parameters and reproductive performance, leading to better pregnancy and lambing rates	Masoudi et al. (2021)
Moghani	Sunflower oil + vitamin C	diet without SFO and VC	Rams fed with SFO and VC supplements showed improved semen quality and higher fertility rates	Ezazi et al. (2019)

Antioxidants play different vital roles in ram semen quality during cryopreservation. For instance, Abdi-Benemar et al. (2013) and Sun et al. (2024) used ALA and DHA (precursors of LCPUFAs) and found that 0.1 mM at 40°C noticeably reduced sperm damage from oxidative stress and improved semen quality. Moreover, dietary supplementation of fish oil has been demonstrated to improve sperm quality and quantity (Alizadeh et al., 2014; Hedayat-Evrigh et al., 2019; Abdollahi et al., 2021). Given that the sperm dilution method did not harm sperm function, it instead led to an increase in pregnancy and lambing rates in ewes supplemented with fish oil compared with palm oil. However, Hedayat-Evrigh et al. (2019) observed no significant difference in the quality of ram semen when comparing Persian oil to fish oil after feeding. *In vitro* fertilization, cleavage rate, and semen quality shelf life were all improved in sheep by dietary supplementation with 5% flaxseed oil and ascorbic acid (Zarei et al., 2018; Ngcobo et al., 2024). Additionally, Alizadeh et al. (2014) found that adding fish oil to a vitamin E diet resulted in beneficial effects compared with a control diet lacking fish oil. Recent findings by Abdollahzadeh et al. (2025) revealed that semen samples with a lower ratio (4:1) significantly improved semen quality parameters and exhibited reduced DNA damage. Finally, Ezazi et al. (2019) found that the quality of semen has increased, maintained sperm health and fertility with a synergetic effect following the use of sunflower oil and vitamin C.

Nanotechnology and cryopreservation

Nanoparticles (NPs) are tiny particles measuring 1-100 nm (Khan et al., 2017; Abbasi et al., 2021; Arruda et al., 2021). According to Khan et al. (2017), NPs are divided into four groups, namely, metal, ceramic, fullerene, and polymeric. Because of their large surface area and nanoscale size, NPs offer diverse applications, including transgenesis, targeted delivery to sperm cells, and antimicrobial and antioxidant effects (Khan et al., 2017; Khalil et al., 2024a). Several studies have investigated the potential of NPs to improve the physical and chemical properties of cryopreserved semen. According to Arruda et al. (2021), there was an improvement in semen quality in bulls, boars, camels, mice, rams, bucks, and other species following the use of NPs. Khalil et al. (2024a) reported that adding 100 µg/mL of zinc oxide NPs, selenium NPs, silver NPs, and copper NPs to semen extender improved the cryotolerance and reproductive potential of buffalo bull sperm. The small particle sizes of NPs can penetrate the sperm membrane and sperm nucleus without causing toxicity to the whole sperm cell. However, an excessive increase in the NPs concentration on the sperm during semen storage was reported to cause toxicity and reduce sperm viability. The toxicity was discovered following the use of 1000 µg/mL of zinc oxide on cryopreserved semen. In contrast, Arruda et al. (2021) observed that zinc oxide nanoparticles at 10-100 µg/mL had no cytotoxic effects on ram sperm motility; rather, motility was enhanced, attributed to zinc's involvement in ATP production and its regulatory function in phospholipid energy metabolism. Abbasi et al. (2021) reported that magnetic oxide NPs improved the vitrification of immature mouse oocytes and altered the expression of pluripotent genes. According to Arruda et al. (2021) and Khalil et al. (2024), Fe_3O_4 NPs minimized recrystallisation and devitrification during warming, protected immature oocytes from cryodamage, improved vitrification results, and increased the pluripotency of resultant embryos. Kanwar et al. (2023) evaluated the antibacterial properties of silver NPs and reported notable improvements in both pre- and post-freezing motility, along with increased antibacterial activity. The microbial load in frozen-thawed semen from the control and supplemented groups remained within acceptable limits. Furthermore, compared with the control group, Khalil et al. (2024) observed a decline in the overall numbers of bacteria, fungi, and yeast in the semen of the group that received zinc oxide NPs. The effects of adding iron oxide and silver NPs to ram semen on bacterial growth control and other sperm quality and function parameters were examined by Tsakmakidis et al. (2021). The findings of Tsakmakidis et al. (2021) demonstrated that while silver NPs offered more antibacterial qualities as well as cytotoxicity, iron NPs had stronger antibacterial impacts. Therefore, these findings suggested that NPs have antibacterial properties on semen.

The results obtained in Table 5 by Moradi et al. (2022) when comparing semen treated with magnetic NPs and untreated semen (control group). There were notable differences in several sperm characteristics, including viability, membrane function, abnormalities, lipid peroxidation (LPO) levels, and DNA integrity among magnetic NPs treated semen and control groups. Moreover, Arruda et al. (2021) indicated that application of zinc oxide NPs increased the mitochondrial membrane potential without affecting the ram spermatozoa's kinetics following the freezing-thawing procedure. Improved sperm motility post semen dilution with zinc oxide was reported to be associated with zinc chemical properties. The use of selenium NPs was reported to improve motility, viability index, and membrane integrity and reduce acrosome deficiencies (Hozyen et al., 2019). In addition, Hozyen et al. (2019) reported that the addition of selenium NPs to extenders resulted in reduced DNA damage. Murawski et al. (2014) reported that deionized water can be used on semen extenders to improve the fertilizing ability of frozen-thawed semen and further improve the lambing rate following AI.

Table 5. Effects of different nanoparticles on rams' semen quality

Breed	Type of nanoparticles	Level used/concentration	Findings	References
Unknown	Magnetic nanoparticles	50 µg/ml MNPs	Adding magnetic nanoparticles (MNPs) to ram semen extenders didn't significantly impact key semen quality parameters, including viability, membrane function, abnormality, oxidative stress, or DNA integrity, compared with the control group.	Moradi et al. (2021)
Santa Inês	Zinc oxide nanoparticles)	0, 10, 50, 100, or 200 µg/mL	The addition of all concentrations improved semen quality without altering kinetics and protecting spermatozoa membranes during freezing and thawing.	Arruda et al. (2021)
Lacunae	Iron oxide nanoparticles	3.072 mg Fe3O4/mL	Iron oxide possesses antibacterial effects.	Tsakmakidis et al. (2021)
Lacunae	Silver (Ag) nanoparticles	2.048 mg Ag-Fe/mL	Silver nanoparticles exhibited strong antibacterial effects but also showed toxic effects on cells.	Tsakmakidis et al. (2021)
Barki	Selenium nanoparticles	1 µg/ml	Improved semen parameters while also reducing DNA deterioration.	Hozyen et al. (2019)
synthetic breed: Berrichon, Charolaise, and PON	Non-water (Deionized water)	1 µg/ml	Improved semen quality and ewe fertility following artificial insemination.	Murawski (2014)

Plant extracts to preserve sheep semen

Choosing semen diluents, whether plant- or animal-derived, is essential for successful sperm cryopreservation. However, the use of animal-based sources was poorly investigated. According to Ngcobo et al. (2024), the use of animal-derived diluents was unsustainable due to increasing competition for food between humans and animals. Consequently, the widespread use of animal products worsened the living conditions of poor people. However, given the rising demand for resources to sustain a growing human population, the use of animal-derived diluents is unsustainable (Ngcobo et al., 2024). Therefore, this limitation recommended the use of plant-based extracts as a viable alternative to animal-origin sources for semen cryopreservation, reducing reliance on animal products and mitigating competition for food resources. Plant extracts possess natural antioxidant, antimicrobial, and anti-inflammatory qualities, making them a promising, low-cost, and natural additive for preserving and enhancing sperm function during storage (Ros-Santaella et al., 2021). The application of plant extract to preserve sheep semen quality is a promising field of study (Berean et al., 2024). According to Ros-Santaella et al. (2021), plant extracts have emerged as promising, cost-effective, and natural additives for preserving and enhancing sperm function during semen storage. In addition, plant extracts presented an environmentally sustainable, non-animal-based method of preserving animal genetics, thereby reducing the potential transfer of hormonal and antibiotic-resistant genes to humans via the food chain (Ngcobo et al., 2024). The inclusion of plant extracts in ram and other male semen depends on their ability to minimize oxidative damage and scavenge ROS. Moreover, according to Mphaphathi et al. (2024), plant extracts are an integral natural antioxidant source with lower cytotoxicity than synthetic therapeutic antioxidants. Their antimicrobial and antioxidant properties enabled them to reduce oxidative stress and to scavenge ROS.

Published findings indicate that the method of preparing and extracting plant extracts, together with the concentration of the semen extender, influences both their interaction with semen extenders and the resulting semen quality. The ability of plant extracts to interact with sperm suggested that plant extracts can be used to treat semen as an antioxidant. Adding 20 µmol/L of curcumin to refrigerated semen increased the levels of multi-antioxidant enzymes and notably reduced ROS production during freezing (Table 6; Ji et al., 2024). Rosemary aqueous extract (Motlagh et al., 2018), wild marjoram (Alenezy et al., 2019), and quinoa seed extract (Khalil et al., 2024) have been reported to enhance the antioxidant capacity of ram semen during cryopreservation. Vahedi et al. (2018) observed improvements in sperm parameters following thymus V supplementation after freezing and thawing. According to Shorky et al. (2021), the use of *Moringa oleifera* leaf extract improved post-thaw ram semen quality and fertility by boosting antioxidant enzyme activity, reducing DNA fragmentation, and minimizing lipid peroxidation. In addition, Alenezy et al. (2019) observed improvements in ram sperm parameters after treatment with *Origanum vulgare* (oregano) extract in a capacitation medium. El-Harairy et al. (2018) indicated that there was no negative impact on semen after using soybean lectin, and soybean lectin was recommended to replace the use of EY in preserved semen. Furthermore, Allai et al. (2016) noted that ram semen quality can be improved by supplementing skim milk and egg yolks with 1% acetone extract ACTEX.

Table 6. Effects of different plant extracts on preserving ram semen

Breed	Plant extract	Concentration	Findings	References
Hu ram	Curcumin	20 µmol/L.	Increased level of multi-antioxidant enzymes and significantly reduced production of ROS during freezing.	Ji et al. (2024)
Rahmani ram	Quinoa seed extracts	0, 250, 500, 750, and 1000	Promoted sperm antioxidants-related genes and reduced apoptosis-related genes.	Khalil et al. (2024)
Moghani ram	Thymus vulgaris	2, 4, 8, 12, and 16Ml/Dl	Improved sperm parameters were observed after supplementation with thymus V following freezing and thawing. However, only the addition of 16Ml/dl extract was criticized.	Vahedi et al. (2018)
Barki ram	*Moringa oleifera* leaves extract (MOLE)	Control-0%, group 2 (MOLE) - 300mg/mL, group 3 (MOLE)- 600mg/mL, group 4 (vit and selenium)- 2.5mg/mL, and group 5 (Vit and selenium) 5mg/mL	Improved ram semen and reduced spermatozoa DNA fragmentation and MDA.	Shorky et al. (2021)
Santa Inês ram	Aqueous extract of noni	0, 24, 72, and 120 µg/mL	The addition of tris-egg-yolk-glycerol-based did not improve semen parameters. Only the aqueous extract of noni improved and maintained sperm functioning and membrane integrity.	Nascimento et al. (2018)
Rahmani ram	Propolis ethanolic extract	0, 0.1, 0.5, and 1.0 mg/ml	No detrimental effect on semen was reported following the use of soybean lectin. Thus, concluded that soybean lectin can replace EY on preserved semen.	El-Harairy et al. (2018)
Chal-ram	Rosemary aqueous extract	0, 2, 4, 6, and 8%	Supplementation of SL extender with rosemary aqueous extract influences post-thawed ram sperm quality in a dose-dependent manner.	Motlagh et al. (2014)
	Wild Marjoram (Origanum vulgare)	0.3, 0.6, 1.2 µg/ml and 25.0, 50.0, 100.0 µg/ml	Origanum vulgare extract had a positive impact on ram sperm quality when added to the capacitation medium.	Alenezy et al. (2019)
Boujaâd rams	*Opuntia ficus*-indica extract (ACTEX)	0%, 1%, 2%, 4%, and 8%	The addition of 1% of ACTEX on skim milk and Tris-based EY improved semen parameters.	Allai et al. (2016)

CHALLENGES IN CRYOPRESERVING

Cryoprotectant toxicity and different dilution rates

Although semen storage techniques have improved, finding an ideal extender that maintains sperm characteristics while minimizing damage remains a challenge (Kameni et al., 2021). The diluent is crucial for sperm viability, providing essential nutrients, maintaining a stable environment, and extending survival during storage (Zhang et al., 2023). Different cryoprotectants can affect sperm quality differently, depending on factors such as concentration, composition, storage time, and temperature. Zhang et al. (2024) evaluated different dilutions (1:1, 1:2, 1:3, and 1:4) of diluent I (Tris-based with egg yolk) on Hu ram semen, while maintaining diluent II (diluent I with glycerol) at a fixed 1:1 ratio. The findings of Zhang et al. (2024) indicated that a two-step dilution (1:3, 1:2) was the most suitable method and ratio for diluting Hu ram semen after cryopreservation. The 1:1 and 1:4 ratios compromised the semen quality. Therefore, a specific concentration is necessary for improving semen quality. However, sperm viability may be compromised by improper diluent addition. The quality of the semen diluent and its capacity to preserve semen quality during lipid peroxidation are linked to semen toxicity. Anel-Lopez et al. (2021) raised concerns about the use of gentamicin, lincomycin, and spectinomycin in semen, indicating their harmful effects and the risk of developing antibiotic-resistant bacteria. These medications had no antibacterial activity and were ineffective on semen quality. Therefore, one of the main problems leading to the failure of semen cryopreservation in many species is the lack of standardized dilution and concentration ratios.

Unimproved protocols for semen cryopreservation

Kameni et al. (2021) observed that prolonged storage of semen consistently led to a decline in quality, reflected in reduced intracellular enzymatic activity, viability, and motility, independent of diluent composition, storage temperature, collection method, or dilution factor. Ari et al. (2011) reported that collecting semen daily (with 1 day of rest) improved post-thaw quality, motility, viability, and membrane integrity, and reduced abnormalities and acrosomal damage, compared with collecting semen every 4 days. D'Alessandro et al. (2021) evaluated sperm motility and function at different preservation times and found improvements in semen quality six hours post-storage. D'Alessandro et al. (2021) observed that the extender's initial impact on motility and function from 3 to 6 hours was reversible. Since these early changes may result from sub-lethal damage, it is recommended that ram sperm adapt to the extender for three to six hours before conducting the quality assessment. Preliminary details, such as storage temperature, duration, dilution rate,

and storage conditions, are crucial and should align with optimal-quality diluents. Da Silva Passarelli et al. (2020) found that a 2-hour equilibration time provided superior cryotolerance than a 4-hour period at 5°C, suggesting that a simplified freezing protocol enhanced the success of semen cryopreservation. Therefore, it is essential to perform proper dilution steps, allowing spermatozoa enough time to osmotically adapt to their changing environment and prevent osmotic stress during freezing.

Ejaculation frequency

The frequency of semen collection, whether through electroejaculation (EE) or artificial vagina (AV), affects semen quality. Bearden and Fuquay (1980) found that EE and AV produced similar total sperm counts and fertility rates, though sperm collected via AV exhibited greater resistance to cold shock. Regular collection intervals are essential for preserving semen quality, as Mahmuda (2014) emphasized that consistent collection promoted sperm maturity during spermatogenesis. Nevertheless, repeated ejaculations may decrease semen volume, concentration, and motility (Jennings and Mcweeney, 1976).

Effects of an imbalanced temperature level during semen freezing

Sheep semen is frequently preserved at low temperatures (around 4°C), and determining an appropriate diluent is essential for successful preservation (Zhang et al., 2018). Compared to frozen or chilled storage, ram spermatozoa kept in liquid at 23°C did not have a standardized concentration (Rizkallah et al., 2022). Ram semen is extremely vulnerable to freezing and cold shock, which can reduce sperm fertility and viability (Kanwar et al., 2018). Extreme ROS production from cryopreservation damages sperm functioning (Kameni et al., 2021). Post-thaw semen quality can be compromised when equilibration conditions are changed (Benmoula et al., 2019; Rizkallah et al., 2022). Compromised semen results could be justified by increased bacterial proliferation, such as *Staphylococcus* species and *Escherichia coli* (*E. coli*). Consequently, semen extenders were supplemented with antibiotics and NPs to reduce oxidative stress, provide protection against cold shock, and inhibit microbial growth (Larbi Allai et al., 2018). However, the use of some antibiotics and antioxidants has been challenged by bacterial resistance.

Bacterial contamination

As noted by Ahmen et al. (2018), semen collection is not a sterile procedure, which can lead to bacterial contamination. *Escherichia coli*, *Staphylococcus* species, and *Bacillus* species are the most common bacteria found in ram semen and are transmitted from the surface of the penis and prepuce, collection area, equipment, and people during semen handling. Cross-contamination extensively affects females' reproductive tracts during AI. However, a higher bacterial load in sperm count is associated with decreased sperm viability. Ahmen et al. (2018) found that antibiotics in semen extenders inhibited bacterial growth and helped prevent bacterial transmission during AI. Yániz et al. (2010) found that enterobacterial contamination affected ram semen quality during storage at 15°C, while antibiotics such as gentamicin and ceftiofur demonstrated strong antimicrobial effects. Yániz et al. (2010) reported bacterial resistance to a wide range of antibiotics, recording resistance rates of 20% for ampicillin, 53% for penicillin, 15% for streptomycin, 4% for spectinomycin, 47% for erythromycin, 33% for oxytetracycline, 13% for polymyxin B, and 15% for co-trimoxazole. The bacterial resistance rates for these antibiotics exhibited that some of the antibiotics were ineffective against microbial growth, leading to resistance among the bacteria. Moreover, Anel-Lopez et al. (2021) expressed concerns regarding the use of gentamycin, lincomycin, and spectinomycin due to their toxic effects on sperm quality.

PROSPECTS TO IMPROVE THE PRESERVABILITY OF RAM SEMEN

Standardization of semen freezing protocols

Developing established protocols is essential for semen storage, offering specific guidelines on storage duration, temperature, collection procedures aligned with the method, dilution rates, and storage conditions. These protocols help maintain sperm quality during freezing, ensuring consistency and optimal sperm health (Zhang et al., 2024). Unimproved protocols and imbalanced temperatures negatively impacted semen quality. Improving these protocols is expected to help farmers follow reliable guidelines, thereby enhancing the reproductive efficiency of their sheep and other breeds (Kameni et al., 2021).

Optimal concentration and standardization of plant extracts

Optimal levels of plant extracts and antioxidants are essential for semen preservation, as they protect sperm cells and help maintain their viability and function (Yimer et al., 2016). This process begins by analyzing the chemical composition of the selected plants, enabling scientists to establish safe and effective concentrations that do not

compromise semen quality. Plant extracts differ from synthetic extracts, because plant extracts are natural substances whose nutritional profiles depend on climate and extraction methods. The optimal concentrations for improving ram semen quality were 20 µmol/L of curcumin and 1% *Opuntia ficus-indica* extract (Allai et al., 2016; Ji et al., 2024). Additionally, studying the bioactive compounds in plant extracts can further enhance semen preservation and increase the success of AI procedures (Zhang et al., 2024).

The development of established protocols for the extraction and processing of plant-derived additives used in semen extenders is crucial (Alenezy et al., 2019). To effectively use plant extracts in semen cryopreservation, it is essential to maintain the integrity of bioactive compounds and preserve the quality of the extracted materials (Mphaphathi et al., 2025). A comprehensive analysis of the chemical compositions of plant extracts, including fatty acid profiles, is necessary to understand their impact on semen quality and identify potential sperm shortages (Ngcobo et al., 2024). Additionally, toxicological evaluations are essential to determine the safety of plant extracts for use in semen cryopreservation. Plant extracts should demonstrate sufficient antioxidant activity to protect spermatozoa from oxidative stress during cryopreservation (Alenezy et al., 2019; Shorky et al., 2021). The antioxidant properties of plant extracts play a vital role in preserving sperm cells from oxidative damage. Phenolic-rich extracts and flavonoid-rich extracts, known for their high antioxidant activity, might be beneficial in protecting sperm cells (Allai et al., 2016). Advanced technologies, such as automated semen analyzers and nanomechanical sensors, can aid in assessing sperm parameters and evaluating the effects of plant extracts on sperm function. By optimizing extraction methods, standardizing plant extracts, and conducting thorough toxicological evaluations, scientists can develop effective and safe solutions for semen cryopreservation. The integration of advanced technologies and a thorough understanding of the effects of plant extracts on semen quality can ultimately improve the success rates of cryopreservation protocols (El-Harairy et al., 2018).

In vivo studies for validating the efficacy of plant extracts

Although plant extracts have demonstrated potential to enhance the quality of cryopreserved semen, additional in *vivo* studies are needed to verify their role in improving ovine reproductive efficiency. These studies should investigate the impact of plant extracts on semen quality parameters, such as motility, viability, and fertility, and their potential to optimize AI protocols. Additionally, although efforts have been made to enhance semen quality, the impact of plant extracts on ewe reproductive efficiency remains insufficiently understood. Direct dietary supplementation of plant extracts to ewes may improve reproductive performance, and further studies are needed to explore the potential benefits of different plant extracts on both male and female fertility.

CONCLUSION

Studies have underscored the importance of conserving indigenous sheep ecotypes. The limited success of AI highlighted the urgent need to improve semen cryopreservation methods to protect the genetic diversity of indigenous sheep ecotypes before they become extinct. Despite considerable advances in developed countries in oxidative damage and other factors influencing semen cryopreservation, the inability to enhance the effectiveness of frozen semen for AI could worsen food insecurity and deepen poverty in underdeveloped and developing countries. To improve the success of sheep semen cryopreservation, further studies are required to establish a standardized freezing protocol for ram semen that effectively reduces freezing-related stressors. This freezing protocol should thoroughly assess the effects of plant extracts on semen quality, fertility, and reproductive performance in ewes. Furthermore, conducting *in vivo* studies and exploring the advantages of directly incorporating plant extracts into diets can provide cost-effective, evidence-based methods to improve sheep fertility.

DECLARATIONS

Acknowledgements

The authors would like to acknowledge Tshwane University of Technology SEEDFUND and the Agricultural Research Council, Irene, for their financial support in conducting this study.

Author's contributions

Thandiwe Patricia Mlambo, Jabulani Nkululeko Ngcobo, and Sindisiwe Mbali Sithole conceptualized the article and contributed to writing the manuscript. Thandiwe Patricia Mlambo, Jabulani Nkululeko Ngcobo, Sindisiwe Mbali Sithole, Masindi Lottus Mphaphathi, Takalani Judas Mpofu, and Khathutshedzo Agree Nephawe reviewed the manuscript. All authors considered and agreed on the final edition of the manuscript for publication in the present journal**.**

Availability of data and materials

The data used and analyzed in the present study were obtained from publicly accessible publications cited in this manuscript.

Competing interests

The authors declared no conflict of interest.

Ethical considerations

All authors have reviewed ethical considerations, including plagiarism, consent to publish, misconduct, data fabrication and falsification, duplicate publication and submission, and redundancy. Furthermore, the authors did not utilize any artificial intelligence tools in the data preparation, writing, or revision of the manuscript.

Funding

This research was funded by Tshwane University of Technology (TUT-SEED FUND) and the Agricultural Research Council under contract number API012403000057 for the Agricultural Research Council.

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