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Review Article

Goat semen cryopreservation: A review of practical aspects and prospective strategies

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Abstract

Cryopreservation, a widely used technique in animal reproductive biotechnology, plays a crucial role in preserving genetic material for future use. However, the process of freezing and thawing inflicts both physical and chemical damage on sperm, leading to a reduction in fertility. Glycerol and egg yolk have been conventionally utilised as protective elements in various extenders. However, in goats, interaction between seminal plasma and egg yolk has a negative impact on sperm viability. Therefore, attempts have been made to substitute the egg yolk with lecithin, skim milk and commercially available extenders. Several reports are available regarding addition of different disaccharides, additives, antioxidants having beneficial effect on frozen-thawed goat semen. Recently, proteome profiling of goat semen showed association of specific proteins with fertility in goat semen. Hence, acquiring an extensive knowledge of goat sperm freezing techniques would enhance the viability of cryopreservation and the reproductive potential of the sperm. It encompasses impact of goat seminal plasma on the process of cryopreservation, the manipulation of sperm dilution and concentration, the methodology employed in freezing and thawing, the constituents of cryopreservation diluents, as well as conventional and newly explored cryoprotectants. It also provides an updated analysis of the cryopreservation techniques employed for goat semen and variation in conception rates after employing different insemination techniques, extenders and additives.

Keywords: Cryopreservation, Diluents, Goat, Seminal plasma, Spermatozoa

Highlights

- Seminal plasma plays a crucial role in the regulation of sperm motility and fertilisation
- Egg yolk coagulating enzyme originating from bulbourethral gland is detrimental to goat sperm
- Goat seminal plasma protein profiling can differentiate high fertile from low fertile semen samples
- Soyabean-lecithin based extender provides better conception rate than egg-yolk based extender in goats
- Supplementation of semen additives to extender enhance post-thaw motility and conception rate in goats

INTRODUCTION

The cryopreservation of goat semen is a multifaceted procedure that necessitates the careful management of various parameters in order to attain optimal outcomes. In order to achieve even modest levels of success, it is imperative to consider not only the appropriate diluent, sperm dilution rate, cooling rate, and thawing pace, but also possess a comprehensive understanding of the physiological characteristics of the semen (Post thaw motility= 40-50% according to Department of Animal Husbandry and Dairying [DAHD], 2019). This information is crucial for maximising the post-thaw recovery of sperm and, subsequently, enhancing fertility. Goat sperm serves as an excellent example in this context due to its resemblance to sperm from other domestic species. The cryopreservation process for goat sperm involves the use of comparable cryopreservation media used in other domestic species, cryoprotectants, and freezing and thawing rates. However, it is crucial to provide distinct care for goat sperm to optimise its viability after thawing. For instance, the existence of a deleterious interaction between egg yolk and bulbourethral gland secretions has been observed specifically in goat semen, whereas similar interactions are not observed in other species such as the bull, boar, or ram (Roy, 1957; Iritani & Nishikawa, 1963; Iritani et al., 1964). Furthermore, only 50-60% sperm population survive after cryopreservation. In the event that the total number of spermatozoa in a dose of cryopreserved semen contains less completely functional spermatozoa than is required, then the

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fertility of the individual would be diminished (Watson, 2000). A study conducted by Bispo et al. (2012) in Brazil reported a conception rate ranging from 7% to 79% when utilising artificial insemination (AI) with frozen-thawed Alpino and Saanen goat semen. They had employed both cooled storage (5°C) and frozen storage (-196°C) methods for cryopreservation.

In light of the aforementioned variations observed in the sperm of domestic species, the present review aims to examine the impact of seminal plasma on caprine species, as well as the influence of semen cryopreservation media, cryoprotectants, semen dilution, cooling rate, and thawing rate on caprine sperm. Previous review articles have extensively explored various facets of goat semen cryopreservation (Leboeuf et al., 2000; Purdy, 2006; Gangwar et al., 2016).

Goat semen, like that of other mammals, is composed of sperm cells and seminal plasma, which contains a range of biochemical and physiological constituents. These constituents play a pivotal role in sperm motility, viability, and overall semen quality. Among the physicochemical constituents, pH, electrolytes, sugars are important. Whereas, motility, morphology, viability are crucial parameters of goat semen.

Seminal plasma

The prevailing diluent utilised for cryopreservation of goat semen often includes either egg yolk or dried skimmed milk. Although, it is worth noting that both components have the potential to adversely affect the viability of goat sperm cells. The initial documentation of the detrimental interaction occurred when Roy (1957) observed certain biochemical factors during seminal plasma interactions with egg yolk. Nunes et al. (1982) further expanded on this observation by documenting a similar interaction between these factors and milk. According to existing reports, it has been observed that goat spermatozoa exhibit vulnerability to a range of biochemical and physiological challenges when subjected to freezing and thawing procedures. In addition, it is worth noting that seminal plasma contains certain biochemical factors that play a crucial role in mitigating the detrimental effects induced by cryogenic procedures such as cholesterol, glucose, proteins, antioxidant, mineral elements, and enzymes. Prior researches have demonstrated that seminal plasma plays a crucial role in the regulation of sperm motility and fertilisation (Bezerra et al., 2019; Gonzalez-Cadavid et al., 2014; Intasqui et al., 2016). Jia et al. (2021) conducted proteome profiling of Yunshang black goat semen and showed that 175 proteins were upregulated in the semen sample having high motility. Among the differentially expressed proteins, beta-galactosidase, ATP-citrate synthase, 3-phosphoinositide-dependent protein kinase1-like, trafficking protein particle compound subunit 13 isoform X2, kinesin-like protein, a protein phosphatase inhibitor 2 were important. Dias et al. (2017) observed seasonal changes among the constituents of seminal plasma in Alpine brown goats. They carried out proteome profiling of goat semen by polyacrylamide gel electrophoresis to observe that the abundance of proteins with molecular weight of 7.5 and 34.3-34.5 kDa were higher during reproductive season (March-August). They also observed seasonality among the mineral component of seminal plasma. The concentration of calcium was higher during reproductive season, whereas phosphorus was higher during anestrus season (September-February). According to Roy's study conducted in 1957, it was observed that when seminal plasma was removed, sperm cells were able to maintain their viability in egg yolk diluents. This phenomenon was attributed to the presence of an enzyme called egg yolk-coagulating enzyme (EYCE), which originates from the bulbourethral gland. Pellicer-Rubio et al. (1997) characterised SBUIII as a glycoprotein lipase with a molecular weight of 55-60 kDa, derived from the bulbourethral gland of goats. The optimal method for freezing goat semen involves washing it before diluting in Tris diluents that contain 6% glycerol as a cryoprotectant, as determined by Chauhan and Anand (1991). Various alternative diluents have been suggested as potential strategies to reduce sperm and seminal plasma interactions. These include the utilisation of lipid-free cow milk, a diluent devoid of triglycerides that incorporates milk protein casein, the exploration of milk from non-dairy cow species, and the incorporation of BUSgp60 lipase inhibitors (Pellicer-Rubio & Combarnous, 1998; Liang et al., 2023). Based on the aforementioned data, it is imperative to establish biochemically defined diluents and cryogenic processes for goat semen cryopreservation. This development has the potential to enhance the survivability and fertility of goat spermatozoa after the process of freezing and thawing to ensure production of goats as major food animals in the global scale.

Semen extender

One essential characteristic of an effective extender for cryopreservation is its ability to provide sperm

cells with adequate energy sources, safeguard them from biochemical and physical harm, and provide a favourable environment that enables the survival of spermatozoa during the cryopreservation process (Purdy, 2006). Generally, the extender used for cryopreservation of goat sperm consists of a non-penetrating cryoprotectant, such as milk or egg yolk, which helps protect the sperm during the freezing process. Additionally, a penetrating cryoprotectant, such as glycerol, propylene glycol, or dimethyl sulfoxide, is included to further enhance the sperm's ability to survive freezing and thawing. A buffer, specifically Tris, is added to maintain the pH of the diluent and provide a stable environment for the sperm. Furthermore, one or more sugars, such as fructose, lactose, glucose or trehalose, are included to provide energy for the sperm during the cryopreservation process. Sodium citrate, an organic acid, is added to help maintain the proper osmotic balance of the extender. Lastly, antibiotics like penicillin and streptomycin are included to prevent bacterial contamination during the cryopreservation process (Evans & Maxwell, 1987). The extender most frequently employed for cryopreservation of goat semen are non-fat dry skim milk extender, as documented by Corteel in 1974, and a Tris-glucose extender, as described by Salamon and Ritar (1982). Konyak et al. (2018) found that a 1% concentration of soybean-lecithin improved in-vitro sperm characteristics. In a similar study, soyabean-lecithin based extender has given better results in terms of fertilization rate (66.2% vs 38.7%) compared to egg yolk-based extender (Chelucci et al., 2015). Based on these studies, researchers have concluded that, soybean-lecithin at a 1% concentration can be used as a replacement for egg yolk in the extender. Roof et al. (2012) conducted a separate investigation wherein they noticed that Bioxcell® (IMV Technologies, France), a commercially available soy-based extender, demonstrated enhanced efficacy in retaining the motility of cryopreserved goat sperm as compared to Irvine TYB (TEST- yolk buffer), an egg yolk-based extender. The researchers employed a two-step method in their investigation. The findings from several studies (Dorado et al., 2007; Khalil & Al-Saef, 2012; Manivannan et al., 2017) indicate that Tris-citric acid has been identified as a potentially effective buffering system and a superior extender for goat spermatozoa. Several extenders used for goat semen cryopreservation along with conception rate have been shown in Table 1.

Osmolality

The phenomenon of osmotic stress, which arises from variations in the relative permeability of cryoprotectants, has been identified as a significant contributing factor in the process of cryopreservation (Lovelock, 1953). The cryopreservation of goat spermatozoa has been observed to result in their sustained viability and fertility when stored in a diluent containing a diverse array of components. Due to the relatively lower permeability of most cryoprotectants compared to water, a phenomenon occurs where water enters the cells, leading to an observable increase in cell volume. The preference for a hyperosmotic media for cryopreservation of goat spermatozoa arises due to the potential variation in osmolality of the diluent within certain limitations. Various sugars, salts, and buffers can be incorporated into the cryopreservation diluent at different molar concentrations, ensuring the spermatozoa remain unharmed during the process. In a study conducted by Salamon and Ritar in 1982, it was observed that the optimal tonicity for a Tris-glucose-citric acid buffer

Table 1. Conception rates with frozen-thawed goat semen using different semen extenders

Extenders	Conception rate (%)	References
Tris egg yolk citrate (TEYC)	42.86-70	Dorado et al., 2007; Khalil and Al-Saef, 2012; Manivannan et al., 2017
Milk	52.38-57.76	Dorado et al., 2007; Khalil and Al-Saef, 2012
Triladyl	38	Rimi et al., 2023
Andromed	35	Rimi et al., 2023
Cholesterol added to Tris	60	Kaewkesa et al., 2016
Albumin added to Tris	30	Kaewkesa et al., 2016
Na-citrate	34.40	Khalil and Al-Saef, 2012
Tris with 3% soyabean-lecithin	55.56	Fathi et al., 2019
OptiXcell (Plant-based)	61.54	Fathi et al., 2019
BullXcell (Egg yolk-based)	57.50	Fathi et al., 2019
Bioxcell	76.5	Sariözkan et al., 2010
Skim milk	66.7	Mara et al., 2007

was determined to be 948 kPa (540 mOsm). In addition, it was observed by Bowen et al. (1988) that the cryopreservation of goat sperm in buffers with an osmolality of 425 and 525 mOsm resulted in reduced damage, as compared to diluents with an osmolality of either less than 325 mOsm or greater than 625 mOsm. Therefore, it can be inferred that goat sperm necessitates a higher level of osmolality in the diluent for the process of cryopreservation.

Buffering systems

The occurrence of notable alterations in the pH of semen has the potential to induce detrimental effects on the cellular and sub-cellular structures of spermatozoa. To ensure the long-term survival and fertilising capacity of sperm, it is imperative to regulate pH changes in cryopreservation solutions, hence maintaining an optimal environment. Hence, the process of diluting semen in an appropriate buffer solution is a critical determinant that impacts the viability of sperm cells during the cryopreservation procedure. According to Graham et al. (1972), an optimal buffer should possess several key characteristics viz. neutral pH, high water solubility, least reactivity with salts, least vulnerability with temperature change, high ionic strength and chemical stability. The investigation of oxygen uptake in goat sperm and its correlation with sperm cell motility has revealed that the highest levels of both parameters are observed at a pH of 7.2. Jaiswal and Majumder (1998), investigated the impact of pH fluctuation on sperm sensitivity. They found that when the pH of themediumwas increased from 7 to 8, approximately 50% of the sperm from the cauda epididymis exhibited motility. The researchers made the observation that manipulating the pH of the external environment leads to a corresponding increase in the pH within the cells, hence triggering the onset of progressive movement in sperm cells. The activation of protein kinase A, which serves as an indicator of capacitation in spermatozoa, is also observed in response to elevated intracellular pH (Visconti & Kopf, 1998). Salamon and Ritar (1982), observed higher post-thaw motility while using Tris buffer. Additionally, the researchers examined the relationship between the concentration of Tris and the sugars found in the medium. Semen samples cryopreserved in the Tris-citric acid buffer exhibited a notably higher progressive motility (44%) and viability (49%) in comparison to samples cryopreserved in Bes, Tes, and Test buffers (zwitterionic buffers). Molinia et al. (1994) observed that monosaccharides combined with Tris exhibited more pronounced cryoprotective effects compared to disaccharides combined with Tris. Deka and Rao (1987) compared the effectiveness of Tris buffer with that of skim milk, Tes yolk, and egg yolk citrate extenders. Based on the aforementioned studies, it has been proposed that zwitterion buffers, specifically Tris-citric acid buffers with a pH closer to the pKa value, could potentially serve as an optimal buffering system for enhancing the fertility of goat spermatozoa.

Sugars

Sugars are known to have significant implications in the process of sperm respiration, as well as in maintaining osmotic equilibrium and providing cryoprotection in diluent solutions. The inclusion of sugars in a diluent is a reasonable practice due to the presence of sugars in seminal plasma. Among the various sugars, fructose exhibits the highest molar concentration in fresh goat semen, as reported by Aboagla and Terada (2003). The inclusion of sugar in a diluent may be determined by the chemical's functioning. In the case of goat seminal plasma, fructose serves as the principal substrate for glycolysis. Therefore, it is reasonable to incorporate this sugar into the extender (Pellicer-Rubio et al., 1997). Similarly, glucose can serve as a very suitable substrate in the metabolic processes of goat sperm. It plays a crucial role in supplying the necessary energy for the proper functioning of sperm cells in a physiological way, as evidenced by studies conducted by Corteel (1974).

The addition of non-permeating sugars, such as lactose, raffinose, dextrans, or trehalose, to the diluents results in an increase in osmotic pressure. This increase in osmotic pressure leads to cell dehydration, which subsequently reduces the occurrence of intracellular ice formation. The sugars in the extender also engage in interactions with the phospholipids present in the sperm plasma membrane, resulting in a reorganisation of the plasma membrane that is more conducive to the cryopreservation process necessary for survival (Molinia et al., 1994). In contrast to monosaccharides such as fructose and glucose, disaccharides predominantly function as cryoprotectants. According to Salamon and Ritar (1982), the inclusion of lactose (29%) or raffinose (17%) led to reduced proportions of motile cells in comparison to fructose. The study revealed that goat sperm exhibited a preference for fructose or glucose when mixed with Tris, and it was found that the concentration of these sugars needed for effective cryopreservation was minimal, ranging from 0 to 62.4 millimolar. In contrast, Gangwar et al. (2020)

found that the extender containing fructose resulted in a significant enhancement in sperm motility, viability, and acrosomal integrity compared to the extender containing glucose. Moreover, the use of glucose extender resulted in a significant rise in sperm abnormalities and lipid peroxidation when compared to the fructose extender. Aboagla and Terada (2003) used trehalose along with Tris-citric acid-glucose (TCG) extender. They observed that the presence of trehalose can lead to an elevation in membrane fluidity as a result of reorganisation of proteins and phospholipids. The precise mechanism through which trehalose and other non-penetrating sugars exert their effects on the sperm plasma membrane remains unclear. However, these sugars are capable of penetrating the sperm plasma membrane and establishing hydrogen bonds with the polar head groups of phospholipids (Roy et al., 2016). Additionally, trehalose can mitigate the detrimental effects of membrane lipid phase transition. In contrast, Karunakaran et al. (2019) did not observe any significant enhancements in the cryopreservability of Black Bengal goat semen following the supplementation of trehalose.

Dilution rate

In order to optimise fertility rates while minimising the number of inseminations and the quantity of sperm used per insemination, it is crucial to ensure proper dilution of semen samples. This ensures an adequate concentration of sperm cells per insemination dose. The site at which semen is deposited is a critical determinant in the quantity of sperm necessary to achieve satisfactory fertility outcomes. In addition to these factors, the quantity of spermatozoa in an inseminating dosage is influenced by other variables, including the type of artificial insemination process, the time of year (breeding or non-breeding season), the type of estrus (spontaneous or induced), the category of the animal (pluriparous or nulliparous), and the physiological state of the animal (nursing or dry). The impact of a higher concentration of spermatozoa, i.e., above 400 million per dosage, during the freezing process has been observed to have implications on the post-thaw quality and fertility of ram semen (Alvarez et al., 2012). In the past, semen samples in farm animals were diluted using precise quantities of diluent. However, contemporary practices involve diluting semen based on the sperm concentration present in the semen (Karatzas et al., 1997). Successful utilisation of different dilution rates, ranging from 1:10 to 1:23 (volume/volume; semen to diluent), has been documented in previous studies (Ritar et al., 1990). Successful cryopreservation of sperm with satisfactory fertility has been achieved using samples containing a range of 80 to 500×10^6 sperm cells/mL (Fathi et al., 2019; Rimi et al., 2023). Nordstoga et al. (2010) carried out a study in Norway, observed non-return rates (NRR) and kidding rates in Norwegian dairy goats following single insemination with frozen-thawed spermatozoa ($400X10^6$) were 64.3% and 58.3%, respectively. Additionally, double inseminations resulted in an NRR of 62% and a kidding rate of 57%.

Cryoprotectants

Cryoprotectants are substances that are utilised to mitigate the adverse effects caused by the cooling, freezing, and thawing processes on spermatozoa. These compounds are employed to alleviate both the physical and chemical stresses that arise during these procedures. Cryoprotectants can be classified into two categories: penetrating and non-penetrating. Glycerol is commonly employed as a penetrating cryoprotectant due to its ability to permeate the cell membrane. Moreover, it has been observed to exhibit non-toxic properties towards cells when incorporated in lower concentrations. According to the findings of Kulaksiz et al. (2013), the concentration of glycerol plays a significant role in enhancing the post-thaw quality of various goat breeds (Angora, Kilis, and Saanen breeds). Penetrating cryoprotectants possess the ability to permeate cellular membranes and exert their effects both within the intracellular and extracellular environments. Penetrating cryoprotectants refer to solutes that induce dehydration of spermatozoa by the osmotically induced movement of water. The extent of this dehydration varies depending on the specific chemical properties of the cryoprotectant. The reduction in intracellular water content of the sperm cell is accompanied by a corresponding decrease in its freezing point. Consequently, this alteration in freezing point leads to a diminished occurrence of intracellular ice formation, thereby conferring advantageous effects. The presence of ice within the cells leads to cellular demise, resulting in a subsequent decline in semen fertility. The introduction of penetrating cryoprotectants induces changes in membrane lipids and proteins, leading to enhanced membrane fluidity, heightened dehydration at lower temperatures, and consequently improved capacity for withstanding cryopreservation (Holt, 2000). In addition, penetrating cryoprotectants refer to solvents that possess the capability to dissolve sugars and salts present in the extender during cryopreservation.

Several studies have investigated the efficacy of various membrane-permeable cryoprotectants, such as ethylene glycol, glycerol, propylene glycol, and dimethyl sulfoxide, either individually or in combination, in preserving the quality of goat semen (Singh et al., 1995; Kundu et al., 2000; Leboeuf et al., 2000; Sikarwar & Ramachandran, 2020). However, it is worth noting that glycerol is the most widely used penetrating cryoprotectant. The incorporation of glycerol can be carried out using a methodology that involves one, two, or three steps, as described by Leboeuf et al. (2000). The process can be conducted at either 37°C or 5°C. The implementation of a stepwise glycerolization protocol at a temperature of 37°C yielded comparable outcomes in terms of the percentage of progressively motile and viable sperm when compared to a single-step glycerolization process conducted at the same temperature. Additionally, the stepwise dilution technique performed at a temperature of 5°C also produced similar results. In contrast, the stepwise approach of glycerolization at a temperature of 37°C exhibited a notably lower release of glutamic oxaloacetic transaminase (GOT) after thawing, with a measurement of 99 units/mL, as compared to the other procedures, which yielded measurements of 105 and 108 units/mL, respectively. Based on the findings obtained, it can be inferred that the addition of glycerol to sperm can be effectively accomplished in a single step.

The non-penetrating cryoprotectants most frequently employed in various studies include egg yolk, with concentrations ranging from 2% to 20% (Ranjan et al., 2015), and non-fat skim milk at a concentration of 10% (w/v) (Leboeuf et al., 1998). The existing literature has extensively documented that the primary factor responsible for safeguarding sperm during cryopreservation is the presence of low-density lipoproteins (LDL) found in egg yolk (Watson, 1976). According to previous research conducted by Graham and Foote (1987), and Quinn et al. (1980), it has been proposed that low-density lipoprotein (LDL) has the ability to bind to the sperm membrane and offer protection by enhancing its stability. Priyadharsini et al. (2011) observed that the use of egg yolk at a concentration of 10% yielded superior results in terms of maintaining functional membrane integrity and intactness of the acrosome in Jakhrana goat semen, compared to egg yolk at 20%. Kundu et al. (2001), observed that amino acids, specifically l-proline, l-alanine, glycine, or glutamine at concentrations of 100-150 mM, exhibited cryoprotective properties during the cryopreservation of goat cauda epididymal sperm. The inclusion of these amino acids resulted in improved post-thaw outcomes, with 8-14% forward motility and 11-19% total motility recovery. In contrast, the control group, which lacked amino acids, showed no motile cells (0% motility). In addition, the amino acids demonstrated a heightened level of protection when utilised in conjunction with glycerol (0.87 M) and DMSO (0.76 M), leading to a post-thaw progressive motility rate of 50-55%. Bispo et al. (2012) recommended to utilise a glucose-EDTA extender with a low concentration of egg yolk (2.5%) in order to achieve enhanced reproductive outcomes in goats. The utilisation of these dextrans resulted in post-thaw total and progressive motility rates of 23% and 25%, respectively.

Other additives

In recent years, there has been a significant emphasis on investigating the diverse range of additives present in the diluent solution with the objective of achieving enhanced post-thaw motility and conception rates. The application of glutamine at a concentration of 2.5 to 5 millimolar (mM) and hyaluronan at a concentration of 500 microliters per millilitre (μL/mL) resulted in a reduction in the activity of superoxide dismutase (SOD) in semen obtained from Angora goats (Bucak et al., 2009). Addition of methionine (2.5 mM), carnitine (7.5 mM), and inositol (7.5 mM) prior to the cryopreservation procedure effectively preserved the integrity of DNA by mitigating the detrimental effects of cryodamage (Bucak et al., 2010). The addition of vitamin E at a concentration of 5 mM resulted in a considerable enhancement of post-thaw motility and preservation of DNA integrity in semen samples from individuals with normal sperm count and reduced sperm motility, as demonstrated in study conducted by Saraswat et al. (2014). Wang et al. (2014) found that Butylated hydroxy toluene (BHT), a lipid soluble antioxidant, had a positive effect on many seminal attributes such as viability, membrane integrity, motility, and acrosomal integrity in Boer goat sperm during both the pre-freeze and post-thaw stages. According to a study conducted by Saraswat et al. (2012), the addition of reduced glutathione at a concentration of 7 mM to Tris-egg yolk extender resulted in enhanced motility, viability, acrosome integrity, functional membrane integrity, and DNA integrity of goat spermatozoa after freezing and thawing. Padilha et al. (2012) observed that the addition of IGF-I (Insulin-like Growth Factor-I) to Tris extender resulted in enhanced sperm motility and improved structural integrity of the plasma

Table 2. Various additives used for goat semen cryopreservation

Antioxidants	References
Quercetin	Seifi-Jamadi et al., 2017; Mao et al., 2018; Diniz et al., 2021; Kumar, Prasad, et al., 2024
Trolox (A synthetic Vit E analogue)	Soares et al., 2015
Vit C	Wang et al., 2014; Gangwar et al., 2015; Sarangi et al., 2017
BHT	Iqbal et al., 2015; El-Khawagah et al., 2020; Tudu, Mandal et al., 2023
Fullerenol	Murugan et al., 2002
L- arginine	Maidin et al., 2014; Singh et al., 2021; Fahlevi et al., 2022; Ahmad and Hamood, 2022
GSH	Hazarika et al., 2016; Rawash et al., 2018; Das et al., 2021
L-cysteine	Metwaly et al., 2016; Fayyaz et al., 2018; Tudu, Sarkar, et al., 2023
Glutamine	Farshad and Hosseini, 2013; Singh et al., 2021
Taurine	Alomar, 2021; Kumar et al., 2022; Kumar, Khasatiya, et al., 2024
α-tocopherol	Motemani et al., 2017; Prastiya et al., 2021; Tudu, Mandal, et al., 2023
SOD	Forouzanfar et al., 2013; Das et al., 2021
Catalase	Das et al., 2021
Resveratrol	Silva et al., 2016; Lv et al., 2019; Falchi et al., 2020
Caffeine	Goswami et al., 2021; Bhut et al., 2024
L-carnitine	Heidari et al., 2022; Altyeb et al., 2022; Abd El-Hamid, 2024; Kamel et al., 2024
Fumaric acid	Saratsi et al., 2023
Ferulic acid	Zhang et al., 2023
Essential oils (Carvacrol and Thymol)	Kchikich et al., 2024

Table 3. Conception rates with frozen-thawed goat semen supplemented with different additives

Additives	Conception rate (%)	References
Ascorbic acid	42.85	Memon et al., 2012
Butylated hydroxytoluene (BHT)	35.71	Memon et al., 2012
Hypotaurine	33.33	Memon et al., 2012
Cysteine	33.33	Memon et al., 2012
Grape Seed Procyanidin Extract	72.5	Wen et al., 2019
Bull seminal plasma	88.24-94.73	Susilowati et al., 2020
Catalase	40	Ranjan et al., 2021
Platelet rich plasma	48-80	Salama et al., 2024
TEMPOL	61.4	Mara et al., 2007
Lycopene + Alpha Lipoic acid	52.5	Ren et al., 2018
Lycium barbarum and *Laminaria japonica*	54.88	Ren et al., 2019
Ferulic acid	80	Zhang et al., 2023
Ascorbic acid-2-glucoside (AA-2G), glutathione, melatonin, and vitamin C	65.78-74.15	Qin et al., 2018
Quercetin	80	Batool et al., 2024
Black *Cincau* extract	80	Wahjuningsih et al., 2021

membrane. Although it did not have a significant impact on the conception rates of ewes when using frozen-thawed semen. Gangwar, Ranjan, Kharche, et al. (2014), observed that the application of vitamin C at a concentration of 56.78 μM resulted in enhanced post-thaw motility and acrosomal integrity in cryopreserved goat spermatozoa. The utilisation of EDTA, a chelating agent for membranes at a concentration of 0.01%, was found to have advantageous effects in the process of cryopreservation of goat semen (Gangwar, Ranjan, Kumar, et al., 2014). Comprehensive compilation of various additives employed in the cryopreservation of goat semen has been tabulated (Table 2) along with conception rates (Table 3).

Freezing

The cryopreservation procedure has been shown to result in a reduction of over 50% in sperm viability. Throughout the process of cryopreservation, the spermatozoa experience various types of stresses, including biochemical, osmotic, thermal, and mechanical strains. These stresses become most evident during the stages of dilution, cooling, equilibration, freezing, and thawing. The diluted semen from male goats is subjected to a cooling process, where it is gradually brought down to a temperature range of 4-5°C, over a time frame of 1.5-4.0 hours. Following this, the semen is frozen using either pellets or straws as per earlier reports (Evans & Maxwell, 1987; Leboeuf et al., 2000). Studies have found a greater rate of sperm survival when semen was subjected to a cooling process at a temperature of 5°C within a time frame of 2.0-2.5 hours. A rapid cooling rate of less than 1.5 hours has been found to have a negative impact on the freezing capacity of goat semen (Purdy, 2006). Significant alterations were observed in goat spermatozoa when subjected to the cooling process within the range of 15°C to 5°C, with no discernible changes noted at temperatures below 0°C (Watson, 2000). The cryopreservation of sperm in straws is a more costly and labour-intensive method compared to the pellet freezing approach. However, it offers the advantage of allowing correct labelling of each sample for efficient inventory management. The process of freezing diluted and chilled semen samples involves loading them into straws with a volume of either 0.25 or 0.5 mL. These straws are then positioned on a rack approximately 3 to 4 cm above the liquid nitrogen (LN_2) for a duration of 7 to 8 minutes, utilising LN_2 vapour. This can be done either in a Styrofoam box or a programmable freezer. Subsequently, the straws are immersed in LN_2 for storage, following the procedure outlined by Evans and Maxwell in 1987. In pellet freezing protocol, which was developed by Pursel and Johnson (1976) for boar preservation of semen, aliquots of cooled semen sample ranging from 0.1 to 0.5 mL are frozen by dispensing them into indentations on a solid carbon dioxide block at a temperature of -79°C. The samples are frozen for a duration of 2-4 minutes, after which they are rapidly immersed in liquid nitrogen for long-term storage. In goats, this technique has been described by Chemineau et al. (1991), and Evans and Maxwell (1987). Sahni and Roy (1972) conducted groundbreaking research on the cryopreservation of goat semen using the original Cambridge method where Beltsville F5 (BF5) extender was used along with 1% glycerol, and samples were placed directly on a block of on dry ice (-79°C) and then plunged into liquid nitrogen (-196°C). Their findings showed that post-thaw motility of 30-40% was achieved when using citrate yolk and milk diluents containing 3-6% glycerol as a cryoprotectant. In a subsequent study, researchers observed a survival rate ranging from 35% to 50% in goat semen that had been frozen using the straw method at a temperature of -196°C (Maxwell et al., 1995). Nevertheless, Chemineau et al. (1991) observed that the freezing height above the liquid nitrogen should be determined by the size or diameter of the straw. Ritar et al. (1990), observed that the motility of sperm frozen in pellets after thawing was significantly higher (39%) compared to sperm frozen in straws. The observed variations in post-thaw motility, viability, and fertility could potentially be attributed to the distinct cooling rates generated by the pellet and straw techniques. Programmable freezers have emerged as a highly convenient solution for the freezing substantial quantities of semen straws. This is advantageous due to the ability to regulate the freezing rate and customise the freezing curve. For instance, the freezing curve can be tailored to follow a specific pattern, such as transitioning from 4 to -5°C at a rate of 4°C per minute, then from -5 to -110°C at a rate of 25°C per minute, and finally from -110 to -140°C at a rate of 35°C per minute. Subsequently, the semen straws can be immersed in liquid nitrogen. Esteso et al. (2018) conducted a study to examine the viability of cryopreserving goat semen using programmable freezers against vapour freezing. Researchers have found that using a programmed freezer produced sperm with increased percentage of motile sperm (38.7% vs 29.6%), progressive motility (6.7% vs 3.7%), and live sperm with intact acrosome (24% vs 17.9%) than using a vapour freezing approach. Nevertheless, it is crucial to consider various considerations when selecting a cryopreservation method, such as convenience of handling, manpower requirements, insemination process, inventory management, and the expense associated with acquiring a bio-freezer.

Thawing

The process of thawing semen samples depends on specific technique employed during the freezing of those samples. Evans and Maxwell (1987) recommended to thaw sperm pellets in a dry test tube at a temperature of 37°C. On the other hand, thawing straws can be done using different combinations of

Table 4. Conception rates in goats by different insemination protocols

Insemination protocol	Conception rate (%)	References
Vaginal insemination	58.3-85.5	Paulenz et al., 2005; Nordstoga et al., 2010
Intra-cervical insemination	38.7-78	Paulenz et al., 2005; Salvador et al., 2005 Leethongdee et al., 2013; Gangwar et al., 2023
Trans-cervical intra-uterine insemination	53.12-78.57	Sohnrey and Holtz, 2005; Gangwar et al., 2023
Laparoscopic intrauterine insemination	56.67-80	Anakkul et al., 2014; Gangwar et al., 2023

time and temperature. The process of thawing semen straws by immersing them in a water bath at a temperature of 37°C for a duration of 12-30 seconds demonstrated higher motility at 36.1% compared to a slower thawing approach (18.9% motility), which involved placing the straws in a water bath at 5°C for a period of 2 minutes (Deka & Rao, 1987). The thawing process of straws at a temperature of 70°C for a duration of only 7 seconds yielded notably higher levels of progressive motility (36.9%) and plasma membrane integrity (39.8%) in comparison to thawing straws at 37°C for 2 minutes (31.5% and 33.7% for progressive motility and plasma membrane integrity), respectively. Similarly, thawing straws at 40°C for 20 seconds resulted in 32.4% progressive motility and 33.5% plasma membrane integrity. In another study conducted on Savannah and Boer goats, Bezerra et al. (2012) observed that 0.5 mL straws thawed at 55°C for 7 s showed higher progressive motility (37.2%) compared to 0.25 mL straws thawed at 37°C for 1 min (32.2%).

Techniques of artificial insemination and site of semen deposition also impact the conception rate in goats. Generally, four AI techniques have been employed in goat breeding, viz. vaginal, intra-cervical, trans-cervical, and laparoscopic insemination. The variation in conception rates employing different AI techniques have been shown in Table 4.

Future scope

Future research endeavours may focus on the assessment of a straightforward, expeditious, and cost-effective approach for the processing and cryopreservation of goat semen. This investigation would involve determining the necessary temperature, optimizing the freezing process, and implementing measures to mitigate cryo-injury to the acrosome during the freezing-thawing procedure, all while ensuring satisfactory reproductive outcomes. It is imperative to conduct critical research in order to determine the minimum quantity of sperm required per inseminating dosage to achieve acceptable conception rates, often around 60%. Additionally, it is crucial to implement rigorous quality control measures for frozen semen throughout the various stages of manufacturing, processing, storage, and eventual utilization. Sex-sorted semen has demonstrated successful utilization in several species, such as cattle, horses, pigs, and sheep. Several genes and metabolomes have been identified in goats which are associated with freezability and regulation of several sperm parameters. Genes such as *DSCAML1* (Wang et al., 2020) is associated with sperm motility and spermatogenesis; *SMAD2* (Wijayanti et al., 2023) and *PPP3CA* (Bai et al., 2022) with litter size and semen quality, *SPAG11* (Harighi et al., 2019) with sperm motility. Several metabolomes have also been identified which are directly (L-glutamine, L-aspartate, and L-arginine) or indirectly (Benzoic acid, ketoisocaproic acid, and choline) regulating goat sperm freezability (Xu et al., 2023). In the future, it may be of significant value to conduct critical investigations on the utilization of these genes and/or metabolomes as fertility markers for selection of goats intended for semen cryopreservation.

Conclusion

The current status of goat-assisted reproductive technology demonstrates significant potential. The use of frozen-thawed goat sperm has the potential to be effectively employed in artificial insemination. However, the outcomes reported in scientific literature exhibit considerable variability. In order to address the variability, it is crucial for the scientific community to strive towards establishing a consensus on universally accepted techniques for freezing goat semen. This should include guidelines on the appropriate medium, cooling and freezing rates, sperm concentration, and other relevant factors. Furthermore, these practices should be applicable across different goat breeds. This study aims to enhance comprehension of goat sperm physiology and facilitate accurate research comparisons by ensuring consistent handling and freezing techniques for the goat sperm.

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