Review Article

Cryopreservation Techniques for Ram Sperm

Amit Saha, Mohammad Asaduzzaman, and Farida Yeasmin Bari

1Department of Surgery and Obstetrics, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh
2Department of Livestock Services, Farmgate, Dhaka 1215, Dhaka, Bangladesh

Correspondence should be addressed to Farida Yeasmin Bari; faridabari06@gmail.com

Received 15 March 2022; Accepted 18 April 2022; Published 30 April 2022

Academic Editor: Sumanta Nandi

Copyright © 2022 Amit Saha et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Germplasm storage and transportation in artificial insemination (AI) and other advanced technologies are facilitated by cryopreservation. In reproduction, the cryopreservation of sperm allows it to be transported across vast distances and used even after the sire’s death. However, the technique of cryopreservation might damage sperm and limit their activity. Several cryobiological investigations have reported that the integrity of the sperm membrane is frequently involved in the physical and biological elements that affect sperm survival at low temperatures during the cryopreservation process. However, successful cryopreservation of ram sperm is still a work in progress because a considerable percentage of sperm do not survive the freezing and thawing process. Sperms are destroyed during cryopreservation of semen due to varying concentrations of cryoprotective chemicals and if semen is not cooled at optimal cooling rates. Hence, it is crucial to know the optimum cooling rates with freezing and thawing protocols for maximum recovery of viable and functional sperm cells for a successful cryo-freezing of ram spermatozoa. Therefore, the current study compiled and compared the research on the impact of different cryopreservation procedures, cooling rates, equilibration time, and thawing protocols on post-thaw ram semen quality.

1. Introduction

Cryopreservation of sperm is one of the most important tools in animal research for improving reproductive technology [1]. Cryopreservation of mammalian sperm is a complicated technique that requires a proper balance of many factors to achieve optimal outcomes. The ram spermatozoa contain a low intramembrane cholesterol-to-phospholipid ratio compared to other species. Henceforth, cold-shock sensitivity in ram spermatozoa is higher than in other species [2]. Diluents, dilution-cooling-freezing, and thawing techniques all play a role in the success of ram semen cryopreservation [3, 4]. Moreover, the need to improve the reproductive efficacy of breeding with cryopreserved semen could involve using better freezing methods to improve post-thaw sperm quality. Sperm cells undergo biochemical and functional changes due to long-term spermatozoa storage, limiting their ability to fertilize [5]. The acrosome, nucleus, mitochondria, axoneme, and plasma membrane are also affected by rapid temperature changes, such as cold shock and the creation and dissolution of ice during the freezing-thawing process [6, 7]. To prevent intracellular crystallization, semen is routinely diluted with a cryoprotectant extender.

Egg yolk is employed in non-penetrating cryoprotectants that successfully protect sperm function (motility, viability, and acrosome integrity) after thawing, while glycerol is the most commonly used penetrating cryoprotectant [8–11]. In glycerol, the concentration that provides the best post-thaw survival rate is between 3% and 7%, while in egg yolk, it is between 5 and 20%. [12–14]. Sperm cryo survival is also influenced by freezing and thawing rates [15, 16]. To minimize cryoinjuries such as disruption of the sperm plasma membrane and DNA structure caused by considerable intracellular ice formation, as well as changes in intracellular pH and ionic composition, an adequate cooling rate must be identified [15, 17, 18].

The rapid cooling of sperm from 30 to 40 degrees Celsius causes “cold shock” damage [19]. Temperature variations during cooling produce stress on sperm membranes, causing phase shifts in lipids and a change in the functional condition of sperm membranes. Temperatures between 5° and
−15°C are known to cause a significant phase change [20], and this could be the perfect temperature range for temperature-dependent damage. The appropriate freeze rate to minimize intracellular ice crystal formation is slow enough to allow water to leave the cells yet fast enough to prevent severe cell dehydration and the solution effect [21]. For the first time, Polge [22] claimed that most spermatozoa destruction occurs in a crucial temperature zone between −15 and −30°C and that if cooling rates are not adequate, all cells may be killed by −80°C. The phase of supercooling (0°C to −5°C) and the development of ice crystals (−6°C to −15°C) are the two main temperature ranges where sperm are damaged during freezing [23]. Mazur [24] also believes that during freezing and thawing, damage to sperm membranes occurs in the temperature range of −15°C to −60°C, which is known as the crucial temperature range.

Standard cryopreservation techniques involve cooling spermatozoa contained in cryoprotective media in plastic straws in the vapor phase over liquid nitrogen. Different protocols have accurate data on freezing ram semen using programmable biofreezers, liquid nitrogen vapor, and dry ice. The techniques of collection of semen, category of semen extender, whether or not the seminal plasma should be separated before freezing, how lengthy the semen should be equilibrated before freezing (2 h or 4 h), the ultimate sperm concentration (ranging from $5 \times 10^6$/mL to $1000 \times 10^6$/mL), straw size (0.25 mL, 0.5 mL), and freezing methods (dry ice for pellets, vapor freezing or controlled freezing for the semen straws). All of these variables could impact the quality of sperm after they have been thawed. Because of these and other differences, this study aims to look at seminal plasma effects, semen cryopreservation media, cryoprotectants, semen dilution, cooling rate, and thawing rate, as well as the effect of the straw freezing method (vapor freezing, programmable freezing, or dry ice) on post-thaw ram semen quality.

2. Semen Collection

Semen collection aims to get the maximum sperm cells with the best quality possible from each ejaculate. Semen is collected mainly through artificial vaginal (AV) or electroejaculation (EE) in small ruminants. Semen collection by AV is the preferred method for sheep [25, 26]. The AV method of semen collection is more appropriate and simple to use and produces ejaculate with sperm quality comparable to natural ejaculate [27, 28]. Warm water in the AV provides thermal stimulation, while pressure in the AV stimulates the glans penis mechanically [27]. The main disadvantage of this procedure is that it necessitates a ram with good libido, the ability to mount females or treasure, and prior ejaculation training in AV [28]. As an alternative to AV, the EE method can be used [29]. An electrode probe (6–12V) is used to apply 3–5 bouts of short stimulation (3–8 sec) at 15–20 sec intervals to the nerve of the accessory glands of the reproductive organ in the EE method [30].

The EE method eliminates the need for ram training, boosts ejaculates quickly, and, most crucially, collects sperm from superior males who are unable to mount due to injury or old age [31]. The main disadvantage of this procedure is that it is stressful for the animal, which is a big problem in animal welfare [31, 32]. The qualities of sperm differ depending on the method of collecting; nevertheless, when AV is used, the results are more favorable [33]. The AV method of semen collection in rams yielded somewhat higher semen volume, sperm concentration, total sperm number, percentage of normal sperm, and wave motion than the EE method [32, 34]. The method of semen collection has an impact on the composition of seminal plasma and the volume and features of the sperm [32]. AV-collected spermatozoa in sheep were more resistant to cold shock than EE-collected spermatozoa [32]. Furthermore, it has been reported that seminal plasma proteins play an essential role in preventing cold shocks without harming the sperm cell membrane [35]. However, semen composition may change during the EE procedure, affecting sperm sample cryo resistance.

3. Sperm Dilution and Concentration

In order to achieve a high fertility rate with the fewest number of inseminations and sperm per insemination, a semen sample must be appropriately diluted to guarantee that sufficient amounts of sperm and diluent are present to accommodate the cells in an insemination straw. It was common practice to dilute farm animal sperm samples with precise amounts of diluents or to dilute them to a specified spermatozoa concentration. It has been effectively utilized dilution rates of $1:1$–$1:23$ (v/v; semen to diluent) [30, 36, 37]. For comparison, sperm concentration might be an ideal way to dilute semen. There have been instances of sperm being properly frozen and adequate fertility with samples ranging from 80 to $500 \times 10^6$ cells/ml [36–39].

4. Sperm Cryopreservation

Sperm cryopreservation is an optional approach for keeping spermatozoa from the best donor for unlimited periods, allowing for the preservation of genetic pools and the most efficient use of sperm dosages using AI. The principle of sperm cryopreservation is to halt the cellular metabolic rate of sperm cells frozen in LN2 (−196°C) and then thaw the sperm to restore their functional survival [40]. The entire procedure necessitates a high level of adaptation to several functions including dilution, incubation, cooling, freezing, and thawing [26, 41, 42]. The spermatozoa undergo varying degrees of ultrastructural, biochemical, and functional damage, resulting in decreased sperm motility, membrane integrity, and fertilizing ability [19, 43]. The production of extracellular and intracellular ice in the sperm cell is thought to cause these damages. The unfrozen solution induces efflux of intracellular water through ice crystallization, cell contraction, and possibly an influx of ions. The plasma, acrosome membranes, mitochondrial sheath, and axoneme of the sperm cell are more damaged in frozen-thawed semen [10, 19, 32].

The biochemical properties of sperm are also affected by the cryopreservation technique. There is a release of
glutamic-oxalacetic transaminase (GOT), inactivation of hyaluronidase, acrosin enzyme, and an increase in sodium. There are decreases in phosphatase activity, acrosomal proteolytic activity, cholesterol protein, potassium content, and adenosine triphosphate (ATP) and adenosine diphosphate (ADP) synthesis, as well as losses of amino acids and prostaglandins [2]. The total effect of cryopreservation on sperm cells is influenced by both internal and extrinsic variables. The inherent sperm cell properties, such as the cell geometry (diameter, volume, relation surface volume), sperm hydration condition, plasma membrane permeability to water and cryoprotectants, and spermatozoa age or maturation state, are all intrinsic variables [44, 45]. The cholesterol/phospholipids ratio, the number of lipids in the bilayer, the degree of hydrocarbon chain saturation, and the protein/phospholipid ratio are essential considerations [42]. In this context, sperm from bulls, rams, and boars has a greater unsaturated to saturated fatty acid ratio and is more susceptible.

In contrast, sperm from rabbits, dogs, and humans has a lower ratio and is more resistant. Cooling and freezing rates, cryoprotective agent type and concentration, extender composition, dilution rate, the temperature at which glycerol is added to the semen, equilibration duration, and thawing rate are all extrinsic factors [2, 45–47]. The osmotic tolerance limitations of ram sperm are similar to those of boar and bull sperm [48]. Ram sperm cells are more sensitive to extreme temperature changes during the freezing process and suffer more damage than bull sperm cells [10, 32, 49]. The cryopreservation of ram sperm is not a simple process. The motility of rams’ spermatozoa is better retained after slow and quick freezing than the morphological integrity of spermatozoa. The mitochondrial architecture is affected by freezing and thawing. However, the tail filament and fibrils do not display any changes [10].

5. Cryopreservation Diluents

Diluents are the media used to increase ejaculate volume and retain sperm fertility for the longest time possible, allowing multiple female animals to be inseminated over a long period [10, 50]. The semen diluent should be able to offer nutrients as an energy source, protect cells from temperature-related damage, provide a buffer to prevent damaging pH shifts [51], suppress bacterial development, and protect sperm cells during preservation [50, 52]. The diluent should have maximum solubility in water and minimal solubility in all other solvents, low salt effects, low buffer concentrations, low-temperature effects, well-behaved cation interactions, higher ionic strengths, and chemical stability [53]. The diluent composition is one of the essential elements impacting sperm quality and keeping sperm fertility for the longest time [54, 55]. Early diluents for ram sperm included citrates, egg yolk, and a monosaccharide such as glucose or fructose and milk [10, 55]. A variety of freezing diluents based on disaccharides (lactose, trehalose), trisaccharides (raffinose), complex polysaccharides (gum Arabic), or other complex compounds (polymethylpyrrolidone), as well as egg yolk and glycerol, were used to dilute and freeze the ram semen [10, 56]. Among the diluent combinations, tris-based or milk-based diluents are recommended for routine use in rams’ semen [8, 10, 11, 57]. To avoid the risk of microbiological contamination, many researchers use soybean lecithin instead of egg yolk in extenders [52, 58, 59]. According to Aboagla and Terada [60], the addition of trehalose or raffinose to the semen extenders plays an important role during the freezing stage of rams’ semen cryopreservation. The addition of trehalose or raffinose increases its cryoprotective activity. Bioxcell® (soybean lecithin-based) and AndroMed® were among the commercial diluents used to freeze the ram semen (Tris-based). These are Tris-based and contain phospholipids, citric acid, carbohydrates, antioxidants, and glycerol [13, 58]. However, various homemade diluents have been used to freeze ram sperm [8, 9, 11, 55, 56].

6. Cryoprotectant

The diluent used for cryopreservation should contain a cryoprotective preventing agent to protect the spermatozoa from cryogenic injuries during cooling and freezing [10]. Cryoprotectants protect frozen sperm cells by suppressing excessive salt concentrations, limiting cell shrinkage at a given temperature, minimizing intracellular ice formation, and reducing the fraction of the solution frozen at a given temperature [41, 61]. Penetrating (intracellular cryoprotectants) and non-penetrating (extracellular cryoprotectants) cryoprotective agents are the two types of cryoprotective agents. The distinction is due to their capacity to enter sperm cells [52, 62, 63]. Low molecular weight penetrating cryoprotectants (glycerol, dimethyl sulfoxide, ethylene glycol, and propylene glycol) induce membrane lipid and protein reorganization due to increased membrane fluidity, increased dehydration at lower temperatures, and lowered intracellular ice formation, leading to a higher sperm survival rate to cryopreservation [41]. Non-penetrating cryoprotectants (egg yolk, nonfat skimmed milk, trehalose, amino acids, dextrans, lactose, soybeans, and sucrose) get a high molecular weight, need not pass through the plasma membrane, and only function extracellularly; hence, they can affect cell dehydration. When ram sperm is frozen, glycerol is usually utilized as a cryoprotectant [8–11, 42]. It binds water and lowers the solution’s freezing point, reducing ice formation at any temperature [19]. Due to an osmotic stimulation and cell dehydration mechanism, glycerol has an extracellular effect, reducing the volume of intracellular water available for freezing and increasing the survival rate of cryopreserved cells [41]. Based on the concentration and temperature at which it is applied, glycerol is biologically harmful to spermatozoa and toxic to membrane integrity [59]. The toxicity of glycerol limits its use in diluents [19, 41, 52]. Glycerol in high concentrations is hazardous to sperm cells because it can cause osmotic damage. The concentration that provides the best post-thaw survival rate is between 4% and 7%. Because glycerol more easily enters the ram sperm, concentrations larger than 6% are harmful to sperm survival [10]. The amount of glycerol added to the diluents is determined by the extender composition, glycerol addition method, and cooling and freezing.
rate [2, 10, 41]. According to several studies, adding 3% to 7% glycerol to diluents comprising 5 to 20% egg yolk results in remarkable post-thaw motility restoration (44 to 85%) of ram sperm [12, 14, 64]. Soltanpour and Moghaddam [65] found that diluents having 7% glycerol and 20% egg yolk provided better sperm protection than extenders with 5% glycerol and 5% egg yolk. Higher egg yolk concentrations are used in the semen extender, which may lower glycerol levels. Nur et al. [7] examined the impact of glycerol (6%), propanediol (6%), sucrose (62.5 mM), and trehalose (62.5 mM) in a Tris-based extender with 20% egg yolk on post-thaw sperm quality in ram semen and noticed that all cryoprotectants had such a detrimental effect on sperm motility, morphology, and DNA integrity. Silva et al. [66] compared ram semen made by diluting in a Tris-egg yolk extender containing glycerol (5%), ethylene glycol (3%), or acetamide (3%) and did find that ethylene glycol, like glycerol, was capable of protecting progressive sperm motility, acrosome integrity, and oxidative stress during the freezing process. The usage of 5% glycerol, on the other hand, provided the best protection for plasma membrane integrity. Moustacas et al. [67] mentioned that the extender with 5% glycerol maintained plasma and acrosomal membrane integrity higher when assessing the efficiency of dimethylformamide alone or in combination with glycerol, a cryoprotectant for freezing ram semen. Moreover, when extenders including pure dimethylformamide or more than 2% in combination with glycerol were applied, sperm motilities were reduced to near zero. Egg yolk prevents sperm from cold shock, keeps them motile, prevents acrosomal enzyme loss, and keeps their mitochondrial membranes intact [10, 41]. Egg yolk is often used in freezing semen diluents because of its properties, such as phospholipid concentration, high molecular weight, and low-density lipoprotein fraction. During cryopreservation, the lipid component of egg yolk protects the sperm plasma membrane and acrosome from thermal damage [10, 42]. By forming a spermatozoa-lipoprotein complex, the egg yolk’s phospholipids and low-density lipoprotein (LDL) reduce sperm cold shock and hence the cooling injuries to the sperm [42]. When the egg yolk is utilized in conjunction with glycerol in the extender, the protection against cold shock may be increased [52]. Substantially higher egg yolk concentrations do not always imply improved sperm motility preservation. According to Gil et al. [13], a higher egg yolk concentration (over 5%) in a milk-based extender did not improve post-thaw motility. Although the impact depends on the extender composition, egg yolk concentrations ranging from 3–6% (as low as) to 15–20% (as high as) have been used to freeze ram semen [10, 60]. After AI, 15% egg yolk extended frozen-thawed semen increased fertility in sheep [55]. The use of egg yolk and skim milk and their performance after freezing and thawing have been shown to harm sperm quality. When compared to yolk citrates, it is believed that overall, milk used in ram semen preservation has a higher post-thaw survival rate. However, when the outcomes of several freezing procedures were compared, yolk citrates came out on top [68]. Alcay et al. [69] particularly compared lyophilized egg yolk to fresh egg yolk for the freezing of ram semen and reported that lyophilized egg yolk provided similar cryoprotective consequences as fresh egg yolk extender (see Table 1).

7. Semen Dilution and Cooling

During the freezing of semen, the selected semen samples are extended with the diluent and involve cooling gradually to 5°C. Dilution and cooling aim to prolong the lifespan and slow down the metabolic activity of the sperm. Semen dilution is done in specified ratios with the proper diluent to ensure that the volume of semen used for insemination has enough sperm per dosage to ensure high fertility without losing cells [119]. Due to an increase in the potassium content of the sperm cell, a too sperm concentrated sperm reduces sperm metabolic activity. When preparing semen for AI, the number of sperms per AI dose should be standardized and diluted using an extender. The cooling process should be done gradually and slowly, which is enough to save the sperm cells. Fast cooling between 30 and 0°C causes sperm damage, the so-called “cold shock” [19]. To prevent sperm cells from cold shock, the initial dilution of semen is done slowly down to 5°C over a few hours (1.5–2.0 h) by gradually adding the diluents, mixing them, and keeping the mixture close to body temperature [57, 120]. Before exposing cells to hypothermic conditions caused by dehydration, the cooling rate must be slow enough to allow sufficient cellular dehydration while still fast enough to freeze the remaining intracellular fluid [19, 42]. On the other hand, slow cooling allows water to escape the cells via osmosis, preventing the creation of fatal intracellular ice [121]. If cooled slower, ram sperms in the mid-piece and tail are most sensitive [79]. The optimal cooling rate (from body temperature to 5°C) was −10°C/h, and employing either egg yolk or milk as protective agents resulted in the least cold shock [42]. Semen extenders made with glycerol may be added initially or subsequently in a separate fraction during the dilution and cooling process. In the one-step method, the entire extender is added in the first condition after collecting semen. In the second case, a portion of the extender (without glycerol) is applied after semen collection, and the remainder (with glycerol) is added after cooling before semen freezing (two-step procedure) [30, 122]. Some authors obtained good results when the glycerol fraction was added at 4°C, but in other studies, better results were obtained after glycerol addition at 30–37°C [96, 123]. Although the difference is minor, adding glycerol at 5°C results in improved sperm survival than adding it at 30°C. The sperm stress is decreased to tolerable levels in the stepwise dilution procedure. The addition of cryoprotectants significantly enhances the fraction of surviving sperm compared to the single-step addition of cryoprotectants [19]. This could be owing to glycerol’s ability to permeate through the membrane at 30 degrees Celsius. Glycerol is less permeable to cell membranes at 4°C, making it less hazardous. Glycerol’s use is restricted due to its toxicity, as previously noted. Equilibration durations with glycerol should be balanced to take advantage of glycerol’s cryoprotective qualities while avoiding unnecessary sperm loss before cryopreservation.
| Breed                        | Collection method | Egg yolk (%) |
|------------------------------|-------------------|--------------|
| Merino ram                   | AV 6%             | 5%           |
| Bangladeshis rams            | AV 20%            | 7%           |
| Mehraban rams                | AV 7%             | 5%           |
| Zandi rams                   | AV 7%             | 5%           |
| Assaf rams                   | EE 10%            | 4%           |
| Zandi rams                   | EE 20%            | 8%           |
| Chal rams                    | AV 10%            | 5%           |
| Hems¸in rams                 | EE 15%            | 5%           |
| Dorper rams                  | AV 7%             | 3%           |
| Awassi rams                  | AV 6%             | 4%           |
| Indigenous Bangladeshi rams  | AV 10%            | 7%           |
| Leccese rams                 | AV 20%            | 5%           |
| Pampinta rams                | AV 10%            | 6%           |
| Bakhhtiar rams               | AV 20%            | 7%           |
| Santa Inês crossed rams      | EE 16%            | 5%           |
| Awassi rams                  | EE 20%            | 6%           |
| Santa Inês rams              | AV 20%            | 5%           |
| Sarda rams                   | AV 20%            | 7%           |
| Crossbred rams               | AV 20%            | 7%           |
| Pampinta rams                | AV 10%            | 3%           |
| Rahmani rams                 | AV 20%            | 6 or 3%      |
| Pampinta rams                | AV 10%            | 6%           |
| Merino rams                  | AV 15%            | 5%           |
| Bakhhtiar rams               | AV 20%            | 8%           |
| Suffolk rams                 | AV 15%            | 5%           |
| Merino rams                  | AV 15%            | 5%           |
| Akkaraman rams               | AV 10%            | 5%           |
| Akkaraman rams               | AV 10%            | 5%           |
| Churra rams                  | AV 20%            | 5%           |
| Santa Inês rams              | AV 10%            | 6%           |
| Île-de-France and lacaune rams | AV 20%        | 4%           |
| Leccese dairy breed of rams  | AV 20%            | 4%           |
| Crossbred rams               | AV 20%            | 2.5, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40% |
| Manchega ram                 | AV 10%            | 7%           |
| Bangladeshis ram             | AV 10%            | 7%           |
| Suffolk ram                  | AV 10%            | 7%           |
| Taleshi rams                 | AV 20%            | 7%           |
| Norwegian crossbred rams     | AV 5%             | 7%           |
| Dorper rams                  | AV 20%            | 5%           |
| Norwegian crossbred rams     | AV 5%             | 7%           |
| Merino rams                  | AV 10%            | 4%           |
| Dorset crossbred rams        | 20%               | 4%           |
| Santa Inês rams              | AV 20%            | 5%           |

| Thawing time and temperature | Post-thaw motility (%) | Reference |
|------------------------------|------------------------|-----------|
| 37°C for 30 s                | 44.5 ± 1.9             | [70]      |
| 37°C for 30 s                | 47.9 ± 2.0             | [70]      |
| 37°C for 20 s                | 41.7 ± 2.9             | [71]      |
| 37°C for 30 s                | 56.3 ± 2.0             | [72]      |
| 37°C for 30 s                | 46.2 ± 1.59            | [73]      |
| 37°C for 30 s                | 50.0 ± 1.27            | [69]      |
| 65°C for 6 s                 | 48.7 ± 20.9            | [74]      |
| 37°C for 30 s                | 48.43 ± 2.9            | [75]      |
| 37°C for 20 s                | 74.6 ± 7.8             | [76]      |
| 37°C for 30 s                | 29.8 ± 2.76            | [77]      |
| 37°C for 30 s                | 22.7 ± 4.5             | [78]      |
| 40°C for 30 s                | 33 : 3 ± 8 : 02        | [79]      |
| 37°C for 30 s                | 51.2 ± 1.9             | [7]       |
| 39°C for 14 s                | 62.0 ± 0.6             | [8]       |
| 37°C for 30 s                | 29.4 ± 2.9             | [81]      |
| 37°C for 10 s                | 29.2 ± 2.9             | [82]      |
| 37°C for 10 sec              | 51.8 ± 2.9             | [52]      |
| 37°C for 30 s                | 42.8 ± 8.8             | [84]      |
| 37°C for 30 s                | 48.0 ± 5.6             | [54]      |
| 37°C for 30 s                | 56.94 ± 0.79           | [87]      |
| 37°C for 10 s                | 39°C for 30 s          | 59.44 ± 0.79 | [87] |
| 37°C for 30 s                | 41 ± 2.9               | [89]      |
| 37°C for 10 s                | 41 ± 2.9               | [89]      |
| 37°C for 10 s                | 37°C for 10 s          | 58.3 ± 2.4 | [90] |
| 37°C for 10 s                | 37°C for 10 s          | 30.1 ± 2.1 | [92] |
| 37°C for 20–30 s             | 37°C for 30 s          | 50.9 ± 3.1 | [93] |
| 37°C for 30 s                | 37°C for 20 s          | 50.00 ± 1.58 | [94] |
| 37°C for 20–30 s             | 37°C for 20 s          | 39.5 ± 2.73 | [95] |
| 65°C for 6 s                 | 64.1 ± 10.8            | [12]      |
| 37°C for 30 s                | 38.1 ± 14.8            | [85]      |
| 37°C for 30 sec              | 38 ± 13.8              | [96]      |
| 37 to 38°C for 30 sec        | 38 ± 13.8              | [96]      |
| 39°C for 30 s                | 71 ± 13.8              | [97]      |
| 37°C for 20 s                | 55.06 ± 1.22           | [98]      |
| 38.5°C, 30 s                 | 44.2 ± 2.9             | [100]     |
| 37°C for 30 s                | 23.6 ± 0.6             | [101]     |
| 35°C for 15 s                | 64.4 ± 0.8             | [104]     |
| 35°C for 12 sec              | 65.0 ± 4.2             | [106]     |
| 37°C for 20 s                | 47.0 ± 1.2             | [3]       |
appropriate glycerol equilibration time has long been a point of contention among cryobiology professionals. Several studies have found that allowing sperm to equilibrate for several hours using glycerol increased post-thaw motility and fertility. Some writers, however, recommended a glycerol equilibration duration of 1.5–2.0 hours [57, 120].

8. Equilibration Time

The total time spermatozoa are in touch with glycerol before freezing is equilibration. However, the equilibration process is not limited to glycerol; it also applies to the other osmotically active extender ingredients. As a result, the equilibration approach can interact with the type of extender (buffer and cryoprotectant) utilized and other cryogenic processes [125, 126]. Different equilibration times ranging from 1 to 5 hours were employed in rams, with varying post-thaw seminal quality [9, 70, 71]. Sharma and Sood [127] and Ranjan et al. [128] found that a 4-hour equilibration period improved post-thaw semen quality. On the other hand, Baruah et al. [129] found no significant variations in sperm motility or acrosomal integrity in semen samples that had been equilibrated for 0.5, 1, or 1.5 hours.

9. Semen Freezing

The purpose of freezing semen is to gradually lower the temperature from 5 to −196°C to avoid injuring the sperm cells. When the temperature drops below 5°C and approaches −10°C, the intracellular water freezes, putting sperm cells at risk of forming ice crystals. Since the freezing rate regulates the extent and rate of cell dehydration, it should be as fast as possible. When sperm cells are rapidly cooled, water is not lost quickly enough to maintain balance, and cells that create intracellular ice during cryopreservation die [130, 131]. Suppose the cooling rate is very slow enough. In that case, the sperm cells will be exposed to high solute concentrations for an extended period, resulting in cell dehydration, volume contraction, and no intracellular freezing. All of these variables have an impact on sperm freezing success. The semen extender will determine the best cooling rate and the packaging utilized. Cells and their surrounding media stay unfrozen and superchilled when cooled to around −5°C. The exterior media freezes between −5 and −10°C, yet the cell contents remain unfrozen and supercooled. Because the supercooled water inside the cells has a more potent chemical potential than water in the partially frozen extracellular solution, water flows out of the cells osmotically, and it freezes outside [130]. A reasonable recommendation is to transfer the semen to liquid nitrogen (LN2) for storage at −15°C/min from +5°C to −100°C [132]. Sperm cells were frozen at a quick pace of 15–60°C/min and were found to have a reasonable survival rate. The effective cooling rate for ram sperm has been estimated to be around 20°C/min or more. Semen can be frozen faster or slower. Semen is cooled fast enough to avoid cooling damage yet slowly enough to allow for cell dryness without the production of intracellular ice. The cell dehydration associated with this slow freezing technique may help sperm cells survive, whereas rapid freezing rates are more likely to result in cellular death. A more stable thermodynamic equilibrium characterizes slow freezing. It employs low cryoprotectants, which are commonly linked to chemical toxicity and osmotic pressure [133]. On the other hand, slow freezing appears to be the most crucial aspect of the sheep preservation procedure [2, 133, 134]. Manual freezing or an automatically programmed biofreezer are both options for freezing semen. The semen straws are placed horizontally on a chilled rack and frozen for 8–10 minutes at 4–6 cm above the level of LN2 in the vapor phase (between −75°C and −125°C) in a manual freezer. After thawing, the initial freezing

| Breed | Collection method | Egg yolk (%) | Glycerol (%) | Thawing time and temperature | Post-thaw motility (%) | Reference |
|-------|-------------------|--------------|--------------|-----------------------------|------------------------|-----------|
| Indigenous rams | 20% | 5% | 37°C for 20 sec | 46.5 ± 5.3 | [107] |
| Portuguese Serra da Estrela and Saloa rams | AV | 15% | 5.3% | 37°C for 20 sec | 46.5 ± 5.3 | [55] |
| Suffolk rams | AV | 15% | 5% | 37°C for 20 sec | 46.5 ± 5.3 | [108] |
| Merino-Sakiz crossbreed rams | EE | 15% | 5% | 37°C for 20 sec | 46.5 ± 5.3 | [109] |
| Akkaraman rams | 10% | 5% | 37°C for 20 sec | 46.5 ± 5.3 | [110] |
| Merino of Palas rams | AV | 20% | 5% | 37°C for 20 sec | 46.5 ± 5.3 | [111] |
| Zandi rams | AV | 20% | 5% | 37°C for 20 sec | 46.5 ± 5.3 | [112] |
| Malpura ram | AV | 15% | 5% | 37°C for 20 sec | 46.5 ± 5.3 | [113] |
| Malpura and Bharat Merino | 50°C for 20 sec | 53.3 | 58.4 | [114] |
| Garole ram | 15% | 6% | 37°C for 20 sec | 46.5 ± 5.3 | [115] |
| Suffolk rams | 15% | 6% | 37°C for 20 sec | 46.5 ± 5.3 | [116] |
| Dorset rams | 15% | 5% | 37°C for 20 sec | 46.5 ± 5.3 | [117] |
| Moghan sheep | 5% | 5% | 37°C for 20 sec | 46.5 ± 5.3 | [118] |

EE = electroejaculation; AV = artificial vagina.

[124]. The appropriate glycerol equilibration time has long been a point of contention among cryobiology professionals. Several studies have found that allowing sperm to equilibrate for several hours using glycerol increased post-thaw motility and fertility. Some writers, however, recommended a glycerol equilibration duration of 1.5–2.0 hours [57, 120].
### Table 2: Comparison of various protocols for freezing of ram semen.

| Freezing step | Diluents | Equilibration time | Freezing rate | Concentration/dilution | Reference |
|---------------|----------|--------------------|---------------|------------------------|-----------|
| Three         | Tris/tes/glucose (TTG) solution | 5°C for 3 h | 40°C/min (+5°C to −35°C) | 100 × 10⁶ sperm/mL | [70] |
| Three         | Tris/tes/glucose (TTG) solution | 5°C for 3 h | 17°C/min (−35°C to −65°C) | 100 × 10⁶ sperm/mL | [70] |
| Two           | Tris/tes/glucose (TTG) solution | 5°C for 3 h | 3°C/min (−65°C to −85°C) and finally −196°C | 100 × 10⁶ sperm/mL | [70] |
| One           | Tris-citrate-fructose-egg yolk and Triladyl® | 5°C for 4 h | −5°C/min (+5°C to −8°C) | 400 × 10⁶ sperm/mL | [9] |
| Two           | Tris-citrate-fructose-egg yolk and Triladyl® | 5°C for 4 h | 5°C/min (+5°C to −8°C) | 100 × 10⁶ sperm/mL | [70] |
| Three         | Tris-citrate-fructose-egg yolk and Triladyl® | 5°C for 4 h | 5°C/min (+5°C to −10°C) | 100 × 10⁶ sperm/mL | [70] |
| One           | Tris-citrate-fructose media | 4°C for 2 h | Vapor freezing 5 cm above the LN2 for 12 min, then immersed directly into liquid nitrogen at −196°C | 4 × 10⁶ sperm/ml | [72] |
| Two           | Tris-citrate-fructose media | 4°C for 2 h | 15°C/min (−8°C to −120°C) & finally −196°C | 4 × 10⁶ sperm/ml | [73] |
| Two           | Tris-citrate-fructose-egg yolk | 5°C for 120 min | 3°C/min (+5 to −8°C) | 1:2 (semen/extender) | [69] |
| One           | Tris-citrate-fructose-egg yolk media | 5°C for 2 h | −20 °C/min (5°C to −100°C) & finally −196°C | 100 × 10⁶ sperm/ml | [74] |
| One           | Tris-citrate-fructose media | 4°C for 2 h | Vapor freezing 5 cm above the LN2 for 12 min, then immersed directly into liquid nitrogen at −196°C | 350 × 10⁶ sperm/ml | [75] |
| Two           | Tris-citrate-fructose-egg yolk | 5°C for 80 min | −25°C/min (−10°C to −150°C) & finally −196°C | 4 × 10⁶ sperm/ml | [76] |
| One           | Tris-citrate-fructose-egg yolk | 5°C for 3 h | Vapor freezing 4 cm above the LN2 for 15 min, then immersed directly into liquid nitrogen at −196°C | 4 × 10⁶ sperm/ml | [76] |
| One           | Tris-citrate-fructose-egg yolk | 5°C for 3 h | Vapor freezing 4 cm above the LN2 for 10 min, then immersed directly into liquid nitrogen at −196°C | 80 × 10⁶ sperm/ml | [77] |
| One           | Sterilyl® (Minitube, Germany) | 5°C for 2 h | Vapor freezing 7 cm above the LN2 for 15 min, then immersed directly into liquid nitrogen at −196°C | 100 × 10⁶ sperm/ml | [78] |
| Two           | Hepes–glucose buffer | 5°C for 4 h | −50°C/min (−5°C to −30°C) & finally −196°C | 20 × 10⁶ sperm/ml | [79] |
| Two           | TRIS-based extender | 5°C for 4 h | −5°C/min (+5°C to −8°C) & finally −196°C | 1:1 (semen/extender) | [7] |
| Two           | Skim milk and egg yolk | 5°C for 90 min | −5°C/min (−5°C to −25°C) & finally −196°C | 800 × 10⁶ sperm/ml | [80] |
| One           | Tris, fructose, egg yolk or Triladyl® | 4°C for 4 h | Vapor freezing 4 cm above the LN2 for 6 min, then immersed directly into liquid nitrogen at −196°C | 400 × 10⁶ sperm/mL | [9] |
| Freezing step | Diluents | Equilibration time | Freezing rate | Concentration/dilution | Reference |
|--------------|----------|--------------------|---------------|------------------------|-----------|
| One          | Tris or milk-based diluent | 5°C for 4 h | Vapor freezing at −75°C for 7 min, then immersed directly into liquid nitrogen at −196°C | 50, 100, 200, 400, 500, or 800 × 10⁶ sperm/mL | [97] |
| One          | TEST buffer | 5°C for 4 h | Vapor freezing 5–7 cm above the LN2 for 10 min, then immersed directly into liquid nitrogen at −196°C | 1:4 (semen/extender) | [82] |
| One          | Tris-citrate modified solution | 5°C for 2 h | Vapor freezing at −100°C, then immersed directly into liquid nitrogen at −196°C | 1 × 10⁸ sperm/mL | [83] |
| One          | Tris-citrate-fructose-egg yolk | 4°C for 2 to 3 h | Liquid nitrogen vapor for 12 min, then immersed directly into liquid nitrogen at −196°C | 1 × 10⁸ sperm/mL | [52] |
| One          | Tris-citrate-glucose-egg yolk-glycerol media | 5°C for 3 h | Vapor freezing 3 cm above the LN2 for 15 min, then immersed directly into liquid nitrogen at −196°C | 100 × 10⁶ sperm/mL | [67] |
| Two          | Bioxel | 5°C for 3 h | 5°C/min (5°C to −20°C) & finally −196°C | 1:5 (semen/extender) | [84] |
| One          | TRIS-egg yolk | 5°C for 4 h | Vapor freezing at −110°C for 10 min, then immersed directly into liquid nitrogen at −196°C | 1:1 (semen/extender) | [54] |
| One          | Tris-egg yolk-glycerol | 5°C for 3 h | −15°C/min (+5°C to −120°C) finally −196°C | 240 × 10⁶ sperm/mL | [140] |
| One          | Tris-citrate-fructose-egg yolk | 4°C for 140 min | Freezing on dry ice, then immersed directly into liquid nitrogen at −196°C | 400 × 10⁸ sperm/ml | [86] |
| Three        | Tris-citrate-fructose-egg yolk | 4–5°C for 4 h | −5°C/min (+4°C to −10°C) | 150 × 10⁶ per straw | [87] |
| One          | Tris-citrate-fructose-egg yolk-glycerol media | 5°C for 4 h | Vapor freezing at −100°C, then immersed directly into liquid nitrogen at −196°C | 1 × 10⁹ cells/ml | [88] |
| One          | Tris-citrate-fructose-egg yolk-glycerol media | 5°C for 4 h | Vapor freezing 5 cm above the LN2 for 10 min, then immersed directly into liquid nitrogen at −196°C | 200 × 10⁶ sperm/ml | [89] |
| One          | Tris-citrate-fructose-egg yolk | 5°C for 2 h | Vapor freezing, then immersed directly into liquid nitrogen at −196°C | 4 × 10⁸ sperm/ml | [90] |
| One          | Tris-citrate-glucose-egg yolk-glycerol media | 5°C for 2 h | Frozen in pellets on dry ice (−79°C), then immersed directly into liquid nitrogen at −196°C | 400 × 10⁶ sperm/ml | [91] |
| One          | Tris-citrate-fructose-egg yolk-glycerol media | 4°C for 2–3 h | Vapor freezing (−125°C to −130°C) for 3–4 min, then immersed directly into liquid nitrogen at −196°C | 100–200 × 10⁶ spz/ml | [92] |
| One          | Tris-citrate-fructose-egg yolk-glycerol media and AndroMed | 5°C for 3 h | Vapor freezing 4 cm above the LN2 for 15 min, then immersed directly into liquid nitrogen at −196°C | 250 × 10⁶ per ml | [58] |
| One          | Tris-citrate-fructose-egg yolk-glycerol media | 5°C for 2 h | Vapor freezing 4.5 cm above the LN2 for 15 min, then immersed directly into liquid nitrogen at −196°C | 4 × 10⁵ sperm/ml | [93] |
| One          | Tris-citrate-fructose-egg yolk-glycerol media | 5°C for 2 h | Vapor freezing (−100°C to −120°C), then immersed directly into liquid nitrogen at −196°C | 4 × 10⁵ sperm/ml | [94] |
| One          | Tris-citrate-fructose-egg yolk-media | 5°C for 2 h | Vapor freezing 5 cm above the LN2 for 10 min, then immersed directly into liquid nitrogen at −196°C | 100 × 10⁶ sperm/ml | [12] |
| One          | Tris-citrate extender | 5°C for 90 min | 12.5°C/min (5 to −120°C) | 100 × 10⁶ sperm/mL | [85] |
| Two          | Tris-egg yolk extender | 5°C for 4 h | 20°C/min to −100°C | 200 × 10⁶ sperm/ml | [99] |
| Freezing step | Diluents                                | Equilibration time | Freezing rate | Concentration/dilution | Reference |
|--------------|----------------------------------------|-------------------|---------------|------------------------|-----------|
| One          | Tris-fructose-citrate-egg yolk         | 4°C for 4 h       | 10°C/min (−100°C to −140°C) Vapor freezing 5–6 cm above the surface of the liquid nitrogen for 5–6 (temperature −80°C) minutes, then immersed directly into liquid nitrogen at −196°C | [100] |
| Four         | BullXcell® AndroMed®                   | 2 h at 4°C        | 9.5 cm, 5 min; 3rd phase, distance 5 cm, 6 min; 4th phase, distance 1.5 cm, 8 min | [101] |
| One          | Tris-citrate-glucose-egg yolk-glycerol media | 5°C for 2 h       | Vapor freezing 4.5 cm above the LN2 for 13 min, then immersed directly into liquid nitrogen at −196°C | 600 × 10⁶ sperm/ml | [102] |
| Two          | Milk-based extender                    | 60–90 min at 5°C  | 5°C/min (5°C to −10°C) | 400 × 10⁶ sperm/ml | [103] |
| One          | Tris-citrate-egg yolk-glycerol media   | 5°C for 120 min   | 20°C/min (5 to −120°C) | 100 × 10⁶ sperm/mL | [104] |
| Two          | Milk-based extender                    | 5°C for 90 to 120 minutes | 5°C/min (5°C to −10°C) | 1000 × 10⁶ sperm/mL | [105] |
| One          | Tris-citrate-glucose-egg yolk media    | 5°C for 90 min    | Vapor freezing −100°C for 10 min, then immersed directly into liquid nitrogen at −196°C | 1 × 10⁹ sperm/ml | [106] |
| One          | Tris-based extender                    | 20°C/min (5°C to −100°C) finally −196°C | 1:3 or 1:6 (semen/extender) | [3] |
| One          | Tris-based extender                    | 10°C/min (−80°C to −130°C) & finally −196°C | 1:3 or 1:6 (semen/extender) | [3] |
| One          | Tris-egg yolk-glycerol                 | 5°C for 3 h       | 15 min, then immersed directly into liquid nitrogen at −196°C | 400 × 10⁶ sperm/mL | [66] |
| Three        | Tris-citrate-fructose egg yolk media   | 5°C for 4 h       | 11.33°C/min (+5°C to −80°C) | 400 or 800 × 10⁶ sperm/mL | [107] |
| One          | Tris-citrate-fructose egg yolk media   | 4°C for 2-3 h     | 26.66°C/min (−80°C to −120°C) & finally −196°C | [108] |
| One          | Tris-based extender                    | 5°C for 2.5 h     | 13.33°C/min (−120°C to −140°C) & finally −196°C | [109] |
| One          | Tris-citrate—fructose-egg yolk-glycerol media | 4°C for 4-5 h     | Vapor freezing −125°C to −130°C for 3-4 min, then immersed directly into liquid nitrogen at −196°C | 800 × 10⁶ sperm/ml | [110] |
| One          | Tris-citrate—glucose-egg yolk-glycerol media | 5°C for 2.5 h     | 15 min, then immersed directly into liquid nitrogen at −196°C | [111] |
| One          | Soybean lecithin-based semen extender  | 4°C for 2 h       | Vapor freezing for 10 min, then | 400 × 10⁶ sperm/ml | [112] |
| One          | Soybean lecithin-based semen extender  | 4°C for 2 h       | Vapor freezing for 10 min, then | 400 × 10⁶ sperm/ml | [113] |
| One          | Soybean lecithin-based semen extender  | 4°C for 2 h       | 350 × 10⁶ sperm/ml | [75] |
| One          | Soybean lecithin-based semen extender  | 4°C for 2 h       | 350 × 10⁶ sperm/ml | [114] |
| One          | Soybean lecithin-based semen extender  | 4°C for 2 h       | 350 × 10⁶ sperm/ml | [115] |
| One          | Soybean lecithin-based semen extender  | 4°C for 2 h       | 350 × 10⁶ sperm/ml | [116] |
temperature has a considerable impact on spermatozoa motility and velocity; however, the best motility of spermatozoa may be attained at −125°C [135]. Nonetheless, freezing small-diameter straws caused a rapid drop in temperature, leading to intracellular water crystallization, which might induce fewer cell damage during the drop in temperature than delayed freezing, which produces severe dehydration [136]. The size of the straw should be used to determine the freezing level over the liquid nitrogen, according to Chemineau et al. [137]. 0.25 ml straws should be frozen 16 cm above liquid nitrogen for 2 minutes before being lowered to 4 cm for 3 minutes before being plunged into liquid nitrogen for storage, whereas 0.5 ml straws should be frozen 16 cm above liquid nitrogen for 2 minutes before being plunged into liquid nitrogen for storage. Alternative freeze positions and times have been mentioned, like 4–5 cm above liquid nitrogen for 4–5 min, with satisfactory results [26, 138]. Pontbriand et al. [3] found that temperature variations of 6 to 24°C per minute and 10 to 100°C per minute were bearable, implying that ram spermatozoa can endure a wide range of cooling rates. The temperature dropped at a controlled and programmed rate while utilizing an automatic freezing machine, from 4 to −5°C at 20°C/min, −5 to −110°C at 55°C/min, and −110 to −140°C at 35°C/min [80]. Sperm cells are often frozen at a high rate (15–60°C/min), resulting in the best post-thawing results [80]. Generally, sperm mixed in a freezing extender is slowly cooled at a rate of roughly 0.1°C/min from ambient temperature to 5°C and then frozen at a rate of 10–60°C/min-1 to temperatures as lower as −80°C before being stored in liquid nitrogen [139]. Before plunging into LN2, the ultimate temperature should be reduced to at least −130°C, regardless of whether the freezing is done slowly or quickly, to stop all metabolic processes, including thermally driven chemical changes [42] (see Table 2).

### 10. Semen Thawing

The process of thawing is the reversal of freezing, in which the solid phase transforms into a normal liquid phase [42, 141]. Cryopreservation methods and subsequent thawing have an impact on sperm cell survival. Sperm quality is harmed during freezing and thawing because sperm cells are exposed to two crucial temperature zones (−15 to −60°C): once during cooling to −196°C and again while thawing. In general, roughly 40–50 percent of the sperm population dies after freezing and thawing [19]. Survivability can be lowered from 85.6 to 34.3 percent in buck frozen-thawed sperm. While frozen-thawed ram semen may contain a large proportion of motile cells (40–60%), only around 20–30% of them are physiologically active [10, 19, 42]. The cells are pushed to retrace their route across the numerous environments encountered during thawing, and the influx of water that results can induce membrane breakage. The warming phase (thawing) is equally as critical to the spermatozoa’s survival as the initial cooling phase during freezing during the freeze-thawing of semen [10]. The combination of processing elements such as glycerol content, freezing rate, and packaging method determines the ideal thawing rate in most cases. It is worth noting if the cooling rate employed to freeze the sperm was high enough to induce intracellular freezing or low enough to cause cell dehydration [142, 143]. If the cooling rate was high, rapid thawing would be required to prevent any intracellular ice in the sperm cell from recrystallizing [136]. When sperms are thawed quickly, they are exposed to a concentrated solute and cryoprotectant for a short period of time, and the restoration of intracellular and extracellular equilibrium is faster than when they are thawed slowly. This topic was highlighted by Hammerstedt et al. [141], who noted that the rate of semen thawing must be adjusted for varied freezing rates in order to get the best sperm cell survival rate. Slow thawing (35°C for 12 sec) leads to 63% and 50% post-thawing sperm motility and membrane integrity, respectively [144]. Fast thawing (70°C for 5 sec) leads to higher post-thawing sperm motility and membrane integrity (67 and 50%, respectively).

Evans and Maxwell [30] discovered that thawing ram sperm at 38–42°C for 15–30 seconds results in sperm motility, acrosome integrity, and sperm cell fertility that are also similar. However, thawing at higher temperatures (60–75°C for 8 seconds) may result in equivalent sperm motility, acrosome integrity, and sperm cell fertility after thawing. Pontbriand et al. [3] showed no difference in spermatozoal motility, progressive status ratings, or acrosomal integrity when thawing straws in a water bath at a higher rate (60°C for 8 sec) versus a slower rate (37°C for 20 sec). When sperms were thawed at 50°C for 9 seconds instead of 70°C for 5 seconds, there was no difference in motility or membrane integrity, showing that thawing at 70°C for 5 seconds was superior to thawing at 37°C for 20 seconds. In farm conditions, thawing at a lower temperature may make it easier to use frozen-thawed ram semen. Several studies have

| Freezing step | Diluents | Equilibration time | Freezing rate | Concentration/dilution | Reference |
|--------------|----------|--------------------|---------------|------------------------|-----------|
| One          | TEST-yolk-glycerol extender | 5°C for 2 h | −25°C/min (5 to −125°C) finally −196°C | 1000 × 10⁶ sperm/ml | [114] |
| One          | Article | 5°C for 2 h | 25°C/min (5°C to −125°C) finally −196°C | 1 x 10⁹/ml sperms/ml | [115] |
| One          | Tris—citrate-fructose-egg yolk-media | 5°C for 2 h | 30°C/min (5°C to −150°C) finally −196°C | 400 × 10⁶ sperm/ml | [117] |
| One          | Tris–citrato-acid–fructose–yolk extender | 5°C for 3.5 h | Vapor freezing 4–5 cm above the LN2 for 10 min, then immersed directly into liquid nitrogen at −196°C | 1 × 10⁹ sperm/ml | [118] |

Table 2: Continued.
demonstrated that a longer thawing period is preferable to a shorter thawing period [145, 146]. A semen straw is usually thawed by immersing it in a 37°C water bath for 12–30 seconds [132]. At temperatures above 37°C, temperature and time become significantly more hazardous, as these high temperatures might result in massive sperm mortalities if thawing is done wrong [147]. When sperm cells are exposed to temperatures between −5 and 15 degrees Celsius, they are damaged [79]. Individual ejaculates of ram sperm are only suitable for preservation and insemination if the percentage of forward moving spermatozoa is greater than 40% after thawing and 30% after 5–6 hours of incubation [30].

11. Conclusions

During the cryo-freezing procedure, ram spermatozoa are more vulnerable to cold shock [2]. To achieve optimum sperm quality, various types of diluents, dilution methods, and cooling-freezing protocols have been endlessly tried by researchers worldwide. In this review, we summarized different cooling rates on ram semen cryopreservation. Personnel skills, quality chemical and cryo-protectants, and proper instrument function impede laboratory production of higher quality frozen ram sperm. The protocol that provides better advantages in ensuring less damage to sperms cells and better post-thaw quality should be adopted and optimized as a lab-specified protocol.

Conflicts of Interest

The manuscript is concerned with all authors and the authors declare no conflicts of interest.

Authors’ Contributions

A. Saha conceived the topic and reviewed it, while FY Bari supervised and read the paper. A. Saha tabulated all data, and FY Bari edited the final report. All authors read the manuscript.

Acknowledgments

While conducting the review study, the authors gratefully acknowledge the financial support provided by the Ministry of Science and Technology (MOST), People’s Republic of Bangladesh.

References

[1] J. D. Benson, E. J. Woods, E. M. Walters, and J. K. Critser, “The cryobiology of spermatozoa,” Theriogenology, vol. 78, no. 8, pp. 1682–1699, 2012.
[2] S. Salamon and W. M. C. Maxwell, “Frozen storage of ram semen I. processing, freezing, thawing and fertility after cervical insemination,” Animal Reproduction Science, vol. 37, no. 3-4, pp. 185–249, 1995.
[3] D. Pontbriand, J. G. Howard, M. C. Schieve, L. D. Stuart, and D. E. Wildt, “Effect of cryoprotective diluent and method of freeze-thawing on survival and acrosomal integrity of ram spermatozoa,” Cryobiology, vol. 26, no. 4, pp. 341–354, 1989.
[4] N. Tekin, O. Uysal, E. Akcay, and I. Yavas, “Effects of different taurine doses and freezing rate on freezing of ram semen,” Ankara Üniversitesi Veteriner Fakültesi Dergisi, vol. 53, 2006.
[5] S. Chełucci, V. Pasciu, S. Succi et al., “Soybean lecithin-based extender preserves spermatozoa membrane integrity and fertilizing potential during goat semen cryopreservation,” Theriogenology, vol. 83, no. 6, pp. 1064–1074, 2015.
[6] M. O’Connell, N. McClure, and S. E. M. Lewis, “The effects of cryopreservation on sperm morphology, motility and mitochondrial function,” Human Reproduction, vol. 17, no. 3, pp. 704–709, 2002.
[7] Z. Nur, B. Zik, B. Ustuner, H. Sagirkaya, and C. G. Ozguden, “Effects of different cryoprotective agents on ram sperm morphology and DNA integrity,” Theriogenology, vol. 73, no. 9, pp. 1267–1275, 2010.
[8] A. Rekha, B. F. Zohara, F. Y. Bari, and M. G. S. Alam, “Comparisons of commercial triladyl and locally manufactured extenders for the chilling of semen and their effects on pregnancy rates after transcervical AI in Bangladeshi Indigenous (Ovis aries) sheep,” Animal Reproduction, vol. 13, no. 4, pp. 735–742, 2016.
[9] A. Rekha, B. F. Zohara, F. Bari, and M. G. S. Alam, “Comparison of commercial triladyl extender with a tris-fructose-egg-yolk extender on the quality of frozen semen and pregnancy rate after transcervical AI in Bangladeshi Indigenous sheep (Ovis aries),” Small Ruminant Research, vol. 134, pp. 39–43, 2016.
[10] S. Salamon and W. M. C. Maxwell, “Storage of ram semen,” Animal Reproduction Science, vol. 62, no. 1–3, pp. 77–111, 2000.
[11] A. Azizunnessa, B. Fatema Zohara, F. Yeasmin Bari, and M. G. Shahi Alam, “Effects of proportion of egg yolk and preservation time on chilled semen from indigenous rams,” GSF Journal of Veterinary Science, vol. 1, no. 1, 2014.
[12] L. Anel, P. De Paz, M. Alvarez et al., “Field and in vitro assay of three methods for freezing ram semen,” Theriogenology, vol. 60, no. 7, pp. 1293–1308, 2003.
[13] J. Gil, N. Lundeheim, L. Söderquist, and H. Rodriguez-Martínez, “Influence of extender, temperature, and addition of glycerol on post-thaw sperm parameters in ram semen,” Theriogenology, vol. 59, no. 5-6, pp. 1241–1255, 2003.
[14] D. Moses, A. G. Martínez, G. Iorio et al., “A large-scale program in laparoscopic intrauterine insemination with frozen-thawed semen in Australian Merino sheep in Argentine Patagonia,” Theriogenology, vol. 48, no. 4, pp. 651–657, 1997.
[15] P. Mazur, S. P. Leibo, J. Farrant, E. H. Y. Chu, M. G. Hanna Jr., and L. H. Smith, G. E. W. Wolstenholme and M. O’Connor, Interactions of Cooling Rate, Warming Rate and Protective Additive on the Survival of Frozen Mammalian Cells, Churchill, London UK, 1970.
[16] A. Peña and C. Linde-Forsberg, “Effects of equox, one-or two-step dilution, and two freezing and thawing rates on post-thaw survival of dog spermatozoa,” Theriogenology, vol. 54, no. 6, pp. 859–875, 2000.
[17] M. E. Hammad, A. S. Askari, T. Georg, P. Rosenbaum, and W. Schmidt, “Effect of freeze-thawing procedure on chromatin stability, morphological alteration and membrane integrity of human spermatozoa in fertile and subfertile men,” International Journal of Andrology, vol. 22, no. 3, pp. 155–162, 1999.
N. Matthews, N. Bester, and L. M. J. Schwalbach, "A...

S. G. Giuliano, A. Director, M. Gambarotta, V. Trasorras,
and M. C. Wulster-Radcliffe and G. S. Lewis, "Development of a...

B. Leboeuf, B. Restall, and S. Salamon, "Production and...

Z. B. Azizunnesa, B. F. Zohara, F. Y. Bari, and M. G. S. Alam,
"Baseline study of reproductive performances of indigenous rams in Bangladesh," IOSR Journal of Agriculture and Veterinary Science, vol. 7, no. 6, pp. 83–89, 2014.

B. Leboeuf, B. Restall, and S. Salamon, "Production and storage of goat semen for artificial insemination," Animal Reproduction Science, vol. 62, no. 1–3, pp. 113–141, 2000.

A. Donovan, J. P. Hanrahan, T. Lally et al., Alp for sheep using frozen-thawed semen, Teagasc, Dublin, Ireland, 2001.

M. C. Wulster-Radcliffe and G. S. Lewis, "Development of a new transcervical artificial insemination method for sheep: effects of a new transcervical artificial insemination catheter and traversing the cervix on semen quality and fertility," Theriogenology, vol. 58, no. 7, pp. 1361–1371, 2002.

S. Giuliano, A. Director, M. Gambarotta, V. Trasorras, and M. Miragaya, "Collection method, season and individual variation on seminal characteristics in the llama (Lama glama)," Animal Reproduction Science, vol. 104, no. 2–4, pp. 359–369, 2008.

G. Evans and W. M. C. Maxwell, "Collection of semen: Handling and examination of semen; Dilution of semen; Frozen storage of semen; Insemination," in Salmon's Artificial Insemination of Sheep and Goats, pp. 85–166, Butterworths, Sydney, Australia, 1987.

M. Ortiz-de-Montellano, F. Galindo-Maldonado, E. O. Cavazos-Arizpe, A. M. Aguayo-Arceo, J. F. J. Torres-Acosta, and A. Orhuela, "Effect of electro-ejaculation on the serum cortisol response of Capriolo goats (Capra hircus)," Small Ruminant Research, vol. 69, no. 1–3, pp. 228–231, 2007.

F. Marco-Jiménez, S. Puchades, J. Gadea, J. S. Vicente, and P. M. Viudes-de-Castro, "Effect of semen collection method on pre-and post-thaw Guirra ram spermatozoa," Theriogenology, vol. 64, no. 8, pp. 1756–1765, 2005.

C. M. Malejane, J. P. C. Greyling, and M. B. Raito, "Seasonal variation in semen quality of Dorper rams using different collection techniques," South African Journal of Animal Science, vol. 44, no. 1, pp. 26–32, 2014.

N. Matthews, N. Bester, and L. M. J. Schwalbach, "A comparison of ram semen collected by artificial vagina and electro-ejaculation," South African Journal of Animal Science, vol. 4, pp. 28–30, 2003.

B. Barrios, M. Fernández-Juan, T. Muñoz-Blanco, and J. A. Cebrián-Pérez, "Immunocytochemical localization and biochemical characterization of two seminal plasma proteins that protect ram spermatozoa against cold shock," Journal of Andrology, vol. 26, no. 4, pp. 539–549, 2005.

A. J. Ritar, P. D. Ball, and P. J. O’May, "Artificial insemination of Cashmere goats: effects on fertility and fecundity of intravaginal treatment, method and time of insemination, semen freezing process, number of motile spermatozoa and age of females," Reproduction, Fertility and Development, vol. 2, no. 4, pp. 377–384, 1990.

A. J. Ritar, P. D. Ball, and P. J. O’May, "Examination of methods for the deep freezing of goat semen," Reproduction, Fertility and Development, vol. 2, no. 1, pp. 27–34, 1990.

J. M. Corteel and G. Baril, "Viabilité des spermatozoïdes de bouc conservés et congeles avec ou sans leur plasma seminal: effet du glucose," Annales de Biologie Animale, Biochimie, Biophysique, vol. 14, no. 4, pp. 741–745, 1974.

G. Karatzas, A. Karagiannidis, S. Varsakelis, and P. Brikas, "Fertility of fresh and frozen-thawed goat semen during the nonbreeding season," Theriogenology, vol. 48, no. 6, pp. 1049–1059, 1997.

J. Ruane, "A framework for prioritizing domestic animal breeds for conservation purposes at the national level: a Norwegian case study," Conservation Biology, vol. 14, no. 5, pp. 1385–1393, 2000.

W. V. Holt, "Basic aspects of frozen storage of semen," Animal Reproduction Science, vol. 62, no. 1–3, pp. 3–22, 2000.

C. M. O. Medeiros, F. Forell, A. T. D. Oliveira, and J. L. Rodrigues, "Current status of sperm cryopreservation: why isn’t it better?" Theriogenology, vol. 57, no. 1, pp. 327–344, 2002.

H. J. Bearden, Applied Animal Reproduction, Reston Publishing Company, Reston, VA, USA, 6th edition, 2004.

M. R. Curry, J. D. Millar, S. M. Tamuli, and P. F. Watson, "Surface area and volume measurements for ram and human spermatozoa," Biology of Reproduction, vol. 55, no. 6, pp. 1325–1332, 1996.

G. W. Salisbury, N. L. Vandemark, and J. R. Lodge, "Principles and techniques of freezing spermatozoa," in Physiology of Reproduction and Artificial Insemination of Cattle, W. H. Freeman, Ed., pp. 494–554, Freeman and Co, San Francisco, CA, USA, 1978.

L. Anel, M. Kaabi, B. Abroug et al., "Factors influencing the success of vaginal and laparoscopic artificial insemination in churra ewes: a field assay," Theriogenology, vol. 63, no. 4, pp. 1235–1247, 2005.

H. Paulenz, L. Söderquist, T. Ådne, A. Nordstoga, B. Gulbrandsen, and K. A. Berg, "Fertility results after different thawing procedures for ram semen frozen in mini-tubes and mini straws," Theriogenology, vol. 61, no. 9, pp. 1719–1727, 2004.

H. D. Guthrie, J. Liu, and J. K. Critser, "Osmotic tolerance limits and effects of cryoprotectants on motility of bovine spermatozoa," Biology of Reproduction, vol. 67, no. 6, pp. 1811–1816, 2002.

L. Gillan, W. M. C. Maxwell, and G. Evans, "Preservation and evaluation of semen for artificial insemination," Reproduction, Fertility and Development, vol. 16, no. 4, pp. 447–454, 2004.
Y. Fukui, H. Kohno, T. Togari, M. Hiwasa, and K. Okabe, C. F. B. Shipley, B. C. Buckrell, M. J. A. Mylne, J. Pollard, and H. Paulenz, T.˚Adnøy, O. H. Fossen, L. S¨oderquist, and S. S. Valente, R. M. Pereira, M. C. Baptista et al., “In vitro and K. J. Ellis and J. F. Morrison, “Buffers of constant ionic strength for studying pH-dependent processes,” Methods in Enzymology, vol. 87, pp. 405–426, 1982.

M. K. Soylu, Z. Nur, B. Ustuner et al., “Effects of various cryoprotective agents and extender osmolality on post-thawed ram semen,” Bulletin of the Veterinary Institute in Pulawy, vol. 51, pp. 241–246, 2007.

S. S. Valente, R. M. Pereira, M. C. Baptista et al., “In vitro and in vivo fertility of ram semen cryopreserved in different extenders,” Animal Reproduction Science, vol. 117, no. 1-2, pp. 74–77, 2010.

H. Paulenz, T. Ádnøy, O. H. Fossen, L. Söderquist, and K. Andersen Berg, “Effect of deposition site and sperm number on the fertility of sheep inseminated with liquid semen,” Veterinary Record, vol. 150, no. 10, pp. 299–302, 2002.

C. F. B. Shipley, B. C. Buckrell, M. J. A. Mylne, J. Pollard, and J. R. Hunton, “Artificial insemination and embryo transfer in sheep,” in Current Therapy in Large Animal Theriogenology, pp. 629–641, WB Saunders, Philadelphia, PA, USA, 2007.

Y. Fukui, H. Kohno, T. Togari, M. Hiwasa, and K. Okabe, “Fertility after artificial insemination using a soybean-based semen extender in sheep,” Journal of Reproduction and Development, vol. 54, no. 4, pp. 286–289, 2008.

J. Gil, M. Rodríguez-Irazoqui, N. Lundeheim, L. Söderquist, and H. Rodríguez-Martín, “Fertility of ram semen frozen in Biocecell® and used for cervical artificial insemination,” Theriogenology, vol. 59, no. 5-6, pp. 1157–1170, 2003.

E. M. E. Aboagla and T. Terada, “Effects of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa,” Theriogenology, vol. 62, no. 6, pp. 1160–1172, 2004.

K. Moore and A. Q. Bonilla, “Cryopreservation of mammalian embryos: the state of the art,” Annual Review of Biomedical Sciences, vol. 8, no. 0, pp. 19–32, 2007.

M. N. Bucak and N. Tekin, “Protective effect of taurine, glutathione and trehalose on the liquid storage of ram semen,” Small Ruminant Research, vol. 73, no. 1–3, pp. 103–108, 2007.

R. A. Tonieto, K. L. Goularte, G. D. A. Gastal, R. S. Schiavon, J. C. Deschamps, and T. Lucia Jr., “Cryoprotectant effect of trehalose and low-density lipoprotein in extenders for frozen ram semen,” Small Ruminant Research, vol. 93, no. 2-3, pp. 206–209, 2010.

J. Gil, L. Söderquist, and H. Rodríguez-Martín, “Influence of centrifugation and different extenders on post-thaw sperm quality of ram semen,” Theriogenology, vol. 54, no. 1, pp. 93–108, 2000.

F. Soltanpour and G. Moghaddam, “Effects of frozen diluents on storage of ram sperm,” International Journal of Advanced Biological and Biomedical Research, vol. 1, no. 12, pp. 1698–1704, 2013.

E. C. B. Silva, J. F. P. Cajuiero, S. V. Silva, P. C. Soares, and M. M. P. Guerra, “Effect of antioxidants resveratrol and quercetin on in vitro evaluation of frozen ram sperm,” Theriogenology, vol. 77, no. 8, pp. 1722–1726, 2012.

V. S. Moustacas, F. G. Zaffalon, M. A. Lagares et al., “Natural, but not lyophilized, low density lyoproteins were an acceptable alternative to egg yolk for cryopreservation of ram semen,” Theriogenology, vol. 75, no. 2, pp. 300–307, 2011.

B. H. Bean, B. W. Pickett, and R. C. Martig, “Influence of freezing methods, extenders, and storage temperatures on motility and pH of frozen bovine semen,” Journal of Dairy Science, vol. 46, no. 2, pp. 145–149, 1963.

S. Alcay, M. Berk Toker, E. Gokce et al., “Successful ram semen cryopreservation with lyophilized egg yolk-based extender,” Cryobiology, vol. 71, no. 2, pp. 329–333, 2015.

D. A. Galarza, A. López-Sebastián, H. Woelders, E. Blesbois, and J. Santiago-Moreno, “Two-step accelerating freezing protocol yields a better motility, membranes and DNA integrity of thawed ram sperm than three-steps freezing protocols,” Cryobiology, vol. 91, pp. 84–89, 2019.

P. K. Jha, M. G. Shahi Alam, A. A. Mansur et al., “Cryopreservation of Bangladeshi ram semen using different diluents and manual freezing techniques,” Cryobiology, vol. 89, pp. 35–41, 2019.

A. Najafi, M. H. Najafi, Z. Zanganeh, M. Shafai, F. Martinez-Pastor, and H. Adeldust, “Cryopreservation of ram semen in extenders containing soybean lecithin as cryoprotectant and hyaluronic acid as antioxidant,” Reproduction in Domestic Animals, vol. 49, no. 6, pp. 934–940, 2014.

A. Najafi, M. Zhandi, A. Towhidi et al., “Trehalose and glycerol have a dose-dependent synergistic effect on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender,” Cryobiology, vol. 66, no. 3, pp. 275–282, 2013.

M. Álvarez, J. Tamayo-Canal, C. Martínez-Rodríguez et al., “Specificity of the extender used for freezing ram sperm depends of the spermatozoa source (ejaculate, electroejaculate or epididymis),” Animal Reproduction Science, vol. 132, no. 3-4, pp. 145–154, 2012.

M. Emamverdi, M. Zhandi, A. Zare Shahneh, M. Shafai, and A. Akbari-Sharif, “Optimization of ram semen cryopreservation using a chemically defined soybean lecithin-based extender,” Reproduction in Domestic Animals, vol. 48, no. 6, pp. 899–904, 2013.

I. Ashrafi, H. Kohram, H. Naijian, M. Bahreini, and H. Mirzakhani, “Efect of controlled and uncontrolled cooling rate on motility parameters of cryopreserved ram spermatozoa,” BMC Research Notes, vol. 4, no. 1, pp. 547–556, 2011.

K. Demir, G. B. Öztürk, Ü. ÇIRIT, and H. H. Bozkurt, “Effects of cooling rate on membrane integrity and motility parameters of cryopreserved ram spermatozoa,” Kafkas Universitesi Veteriner Fakültesi Dergisi, vol. 21, no. 1, pp. 61–67, 2015.

M. C. Matias, A. R. R. Cézar, J. C. C. Marques, F. K. A. da Silva, V. N. F. G. Sandes, and D. Ribeiro, “Use of galactose to vitrify ram semen in straws,” Ciencia Animal Brasileira, vol. 22, 2021.

S. Kumar, J. D. Millar, and P. F. Watson, “The effect of cooling rate on the survival of cryopreserved bull, ram, and boar spermatozoa: a comparison of two controlled-rate cooling machines,” Cryobiology, vol. 46, no. 3, pp. 246–253, 2003.

G. P. Byrne, P. Lonergan, M. Wade et al., “Effect of freezing rate of ram spermatozoa on subsequent fertility in vivo and
in vitro," Animal Reproduction Science, vol. 62, no. 4, pp. 265–275, 2000.
[81] A. G. D’alessandro and G. Martemucci, “Evaluation of seasonal variations of semen freezability in Leccese ram,” Animal Reproduction Science, vol. 79, no. 1-2, pp. 93–102, 2003.
[82] A. A. Abdelhakeam, E. F. Graham, I. A. Vazquez, and K. M. Chaloner, “Studies on the absence of glycerol in unfrozen and frozen ram semen,” Cryobiology, vol. 28, no. 1, pp. 43–49, 1991.
[83] E. Aisen, M. Quintana, V. Medina, H. Morello, and A. Venturino, “Ultramicroscopic and biochemical changes in ram spermatozoa cryopreserved with trehalose-based hypertonic extenders,” Cryobiology, vol. 50, no. 3, pp. 239–249, 2005.
[84] N. U. R. Zekariya, Z. I. K. Berrin, B. Üstüner et al., “Effect of freezing rate on acrosome and chromatin integrity in ram semen,” Ankara Universitesi Veteriner Fakultesi Dergisi, vol. 58, no. 4, pp. 267–272, 2011.
[85] D. R. Câmara, S. V. Silva, F. C. Almeida, J. F. Nunes, and M. M. P. Guerra, “Effects of antioxidants and duration of pre-freezing equilibration on frozen-thawed ram semen,” Theriogenology, vol. 76, no. 2, pp. 342–350, 2011.
[86] S. Succu, F. Berlinguer, V. Pasciu, V. Satta, G. G. Leoni, and S. Naitana, “Melatonin protects ram spermatozoa from cryopreservation injuries in a dose-dependent manner,” Journal of Pineal Research, vol. 50, no. 3, pp. 310–318, 2011.
[87] M. N. Banday, F. A. Lone, F. Rasool, M. Rashid, and M. M. Awad, “Effect of sub-optimal glycerol concentration and cholesterol-loaded cyclodextrin in atrais-based diluent on cryopreserved ram spermatozoa,” Cryobiology, vol. 74, pp. 25–30, 2017.
[88] E. G. Aisen, V. H. Medina, and A. Venturino, “Cryopreservation and post-thaw fertility of ram semen frozen in different trehalose concentrations,” Theriogenology, vol. 57, no. 7, pp. 1801–1808, 2002.
[89] M. M. Awad, “Effects of sub-optimal glycerol concentration and cholesterol-loaded cyclodextrin in a tris-based diluent on cryopreserved ram sperm longevity and acrosomal integrity,” Small Ruminant Research, vol. 100, no. 2-3, pp. 164–168, 2011.
[90] E. G. Aisen, H. L. Alvarez, A. Venturino, and J. J. Garde, “Effect of trehalose and EDTA on cryoprotective action of ram semen diluents,” Theriogenology, vol. 53, no. 5, pp. 1053–1061, 2000.
[91] L. Anel, P. de Paz, M. Alvarez et al., “High pre-freezing dilution improves post-thaw function of ram spermatozoa,” Animal Reproduction Science, vol. 119, no. 1-2, pp. 137–146, 2010.
[92] M. Forouzanfar, M. Fazlitali, S. M. Hosseini et al., “Investigation of different glycerol and egg yolk concentration on freezing Bakhtiari ram semen.” Journal of Iranian Animal Anatomy Sciences, vol. 5, pp. 17–25, 2007.
[93] K. Cayan, N. Baspinar, M. N. Bucak, and P. P. Akalın, “Effects of cysteine and ergothioneine on post-thawed Merino ram sperm and biochemical parameters,” Cryobiology, vol. 63, no. 1, pp. 1–6, 2011.
[94] M. N. Bucak, A. Atescan, and A. Yuç, “Effect of antioxidants and oxidative stress parameters on ram semen after the freeze–thawing process.” Small Ruminant Research, vol. 75, no. 2-3, pp. 128–134, 2008.
[95] O. Uysal and M. N. Bucak, “Effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen,” Acta Veterinaria Brno, vol. 76, no. 3, pp. 383–390, 2007.
[96] G. Colas, “Effect of initial freezing temperature, addition of glycerol and dilution on the survival and fertilizing ability of deep-frozen ram semen,” Reproduction, vol. 42, no. 2, pp. 277–285, 1975.
[97] A. G. D’alessandro, G. Martemucci, M. A. Colonna, and A. Bellitti, “Post-thaw survival of ram spermatozoa and fertility after insemination as affected by pre-freezing sperm concentration and extender composition,” Theriogenology, vol. 55, no. 5, pp. 1159–1170, 2001.
[98] P. S. Fiser and R. W. Fairfull, “The effect of glycerol concentration and cooling velocity on cryosurvival of ram spermatozoa frozen in straws,” Cryobiology, vol. 21, no. 5, pp. 542–551, 1984.
[99] O. García-Álvarez, A. Maroto-Morales, M. Ramón et al., “Analysis of selected sperm by density gradient centrifugation might aid in the estimation of in vivo fertility of thawed ram spermatozoa,” Theriogenology, vol. 74, no. 6, pp. 979–988, 2010.
[100] B. B. A. Mahmuda, A. Nesa, B. F. Zohara, M. G. S. Alam, and F. Y. Bari, “Effect of preservation time on the quality of frozen semen in indigenous rams,” Bangladesh Journal of Animal Science, vol. 44, no. 1, pp. 10–15, 2015.
[101] M. Páček, M. Stádníková, F. Savvulidi, and L. Stádník, “Ram semen cryopreservation using egg yolk or egg yolk-free extenders: preliminary results,” Scientia Agriculturae Bohe- mica, vol. 50, no. 2, pp. 96–103, 2019.
[102] R. Motamedi-Mojdehi, M. Roostaei-Ali Mehr, and R. Rajabi-Toustani, “Effect of different levels of glycerol and cholesterol-loaded cyclodextrin on cryosurvival of ram spermatozoa,” Reproduction in Domestic Animals, vol. 49, no. 1, pp. 65–70, 2014.
[103] A. B. Nordstoga, L. Söderquist, T. Ådøy, and H. Paulenz, “Effect of different packages and freezing/thawing protocols on fertility of ram semen,” Reproduction in Domestic Animals, vol. 44, no. 3, pp. 527–531, 2009.
[104] R. T. Padilha, D. M. Magalhães-Padilha, M. M. Cavalcante et al., “Effect of insulin-like growth factor-I on some quality traits and fertility of cryopreserved ovine semen,” Theriogenology, vol. 78, no. 4, pp. 907–913, 2012.
[105] H. Paulenz, T. Ådøy, and L. Söderquist, “Comparison of fertility results after vaginal insemination using different thawing procedures and packages for frozen ram semen,” Acta Veterinaria Scandinavica, vol. 49, no. 1, pp. 26-27, 2007.
[106] V. Pelufo, M. López Armengol, V. Malcotti, A. Venturino, and E. G. Aisen, “Effects of glycerol and sugar mixing temperature on the morphologic and functional integrity of cryopreserved ram sperm,” Theriogenology, vol. 83, no. 1, pp. 144–151, 2015.
[107] P. K. Jha, M. G. S. Alam, M. A. Al Mansur et al., “Effects of number of frozen-thawed ram sperm and number of inseminations on fertility in synchronized ewes under field condition,” Journal of Animal Reproduction and Biotechnology, vol. 35, no. 2, pp. 190–197, 2020.
[108] T. Matsuoka, H. Imai, H. Kohno, and Y. Fukui, “Effects of bovine serum albumin and trehalose in semen diluents for improvement of frozen-thawed ram spermatozoa,” Journal of Reproduction and Development, vol. 52, no. 5, pp. 675–683, 2006.
[109] S. Gungor, A. Ata, and M. E. Inanc, “Effects of trehalose and catalase on the viability and kinetic parameters of cryopreserved ram sperm,” Acta Scientiae Veterinarieae, vol. 46, no. 1, p. 7, 2018.
[110] M. Moradi, H. Hajarian, H. Karamishabankareh, L. Soltani, and B. Soleymani, “Pre-treatment of ram semen extender
with magnetic nanoparticles on freeze-thawed spermatozoa," *Veterinary Medicine and Science*, vol. 8, no. 2, pp. 792–798, 2021.

[111] M. E. Inanc, O. Uysal, and A. T. A. Ayhan, "Cryopreservation and evaluation of Akkaraman ram semen with 7-dehydrocholesterol," *Ankara Universitesi Veteriner Fakultesi Dergisi*, vol. 65, no. 2, pp. 187–192, 2018.

[112] A. Anghel, S. Zamfirescu, D. Coprean, and E. Sogorescu, "The effects of cysteine, bovine serum albumin and vitamin e on the calititative parameters of frozen-thawed ram semen," *Annals of the Romanian Society for Cell Biology*, vol. 14, no. 2, 2009.

[113] R. K. Paul, K. Balaganur, D. Kumar, and R. Singh, "Pre-freezing equilibration for 22 h improves post-thaw sperm functions in cryopreserved ram semen by reducing cholesterol efflux," *Cryobiology*, vol. 96, pp. 76–84, 2020.

[114] S. Bag, A. Joshi, S. M. K. Naqvi, P. S. Rawat, and J. P. Mittal, "Effect of freezing temperature, at which straws were plunged into liquid nitrogen, on the post-thaw motility and acrosomal status of ram spermatozoa," *Animal Reproduction Science*, vol. 72, no. 3–4, pp. 175–183, 2002.

[115] A. Joshi, S. Bag, S. M. K. Naqvi, R. C. Sharma, P. S. Rawat, and J. P. Mittal, "Effect of short-term and long-term preservation on motion characteristics of Garole ram spermatozoa: a prolific microsheep breed of India," *Asian-Australasian Journal of Animal Sciences*, vol. 14, no. 11, pp. 1527–1533, 2001.

[116] M. K. Schmehl, S. P. Anderson, I. A. Vazquez, and A. F. Graham, "The effect of dialysis of extended ram semen prior to freezing on post-thaw survival and fertility," *Cryobiology*, vol. 23, no. 5, pp. 406–416, 1986.

[117] Ü. Cirit, H. Başış, K. Demir et al., "Comparison of cryoprotective effects of iodoxanil, trehalose and cysteamine on ram semen," *Animal Reproduction Science*, vol. 139, no. 1–4, pp. 38–44, 2013.

[118] M. Jafaroghli, B. Khalili, A. Farshad, and M. J. Zamiri, "The effect of supplementation of cryopreservation diluents with sugars on the post-thawing fertility of ram semen," *Small Ruminant Research*, vol. 96, no. 1, pp. 58–63, 2011.

[119] Z. Rasul, M. Anzar, S. Jalali, and N. Ahmad, "Effect of buffering systems on post-thaw motion characteristics, plasma membrane integrity, and acrosome morphology of bufallo spermatozoa," *Animal Reproduction Science*, vol. 59, no. 1–2, pp. 31–41, 2000.

[120] A. M. L. F. Chaveiro, L. Machado, A. Frijters, B. Engel, and H. Woelders, "Improvement of parameters of freezing medium and freezing protocol for bull sperm using two osmotic supports," *Theriogenology*, vol. 65, no. 9, pp. 1875–1890, 2006.

[121] P. Mazur, "Freezing of living cells: mechanisms and implications," *American Journal of Physiology-Cell Physiology*, vol. 247, no. 3, pp. C125–C142, 1984.

[122] L. J. Gil, "Evaluation of post-thaw sperm quality in the bull and ram with special emphasis on semen processing," Licenciate thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden, 1999.

[123] G. Colas, "Factors affecting the quality of ram semen," *Proceedings-Easter School in Agricultural Science*, University of Nottingham, Nottingham, England, 1983.

[124] T. G. Leite, V. R. do Vale Filho, R. P. de Arruda et al., "Effects of extender and equilibration time on post-thaw motility and membrane integrity of cryopreserved Gyr bull semen evaluated by CASA and flow cytometry," *Animal Reproduction Science*, vol. 120, no. 1–4, pp. 31–38, 2010.

[125] R. Muñoz, M. Fernandez, and A. I. Peña, "Post-thaw survival and longevity of bull spermatozoa frozen with an egg yolk-based or two egg yolk-free extenders after an equilibration period of 18 h," *Reproduction in Domestic Animals*, vol. 42, no. 3, pp. 305–311, 2007.

[126] R. Vishwanath and P. Shannon, "Storage of bovine semen in liquid and frozen state," *Animal Reproduction Science*, vol. 62, no. 1–3, pp. 23–53, 2000.

[127] A. Sharma and P. Sood, "Cryopreservation and the factors affecting it: an overview," *Veterinary Sciences: Research and Reviews*, vol. 6, no. 1, pp. 46–57, 2020.

[128] R. Ranjan, A. K. Goel, N. Ramachandran, S. D. Kharche, and S. K. Jindal, "Effect of egg yolk levels and equilibration periods on freezability of Jumapari buck semen," *Indian Journal of Small Ruminants (The)*, vol. 21, no. 1, pp. 32–36, 2015.

[129] C. K. Baruah, R. K. Biswas, B. C. Deka, and B. N. Borgohain, "Effect of glycerol equilibration periods on quality of frozen semen in Beetal x Assam local crossbred goats," *Indian Veterinary Journal (India)*, 2003.

[130] P. Mazur, "Equilibrium, quasi-equilibrium, and nonequilibrium freezing of mammalian embryos," *Cell Biophysics*, vol. 17, no. 1, pp. 53–92, 1990.

[131] K. Muldrew and L. E. McGann, "The osmotic rupture hypothesis of intracellular freezing injury," *Biophysical Journal*, vol. 66, no. 2, pp. 532–541, 1994.

[132] R. H. Foote, "Artificial insemination from the origins up to today," in *Proceedings of the International Symposium. From the First Artificial Insemination to the Modern Reproduction Biotechnologies: Traditional Ways and the New Frontiers of Animal Production*, pp. 23–68, Reggio Emilia, Italy, 1999.

[133] A. Arav, S. Yavin, Y. Zeron, D. Natan, I. Dekel, and H. Gacitua, "New trends in gametes' cryopreservation," *Molecular and Cellular Endocrinology*, vol. 187, no. 1–2, pp. 77–81, 2002.

[134] P. Thuwanut, K. Chatdarong, M. Techakumphu, and E. Axner, "The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of frozen-thawed epididymal cat spermatozoa," *Theriogenology*, vol. 70, no. 2, pp. 233–240, 2008.

[135] S. Bag, A. Joshi, P. S. Rawat, and J. P. Mittal, "Effect of initial freezing temperature on the semen characteristics of frozen-thawed ram spermatozoa in a semi-arid tropical environment," *Small Ruminant Research*, vol. 43, no. 1, pp. 23–29, 2002.

[136] A. K. Berg, "Artificial insemination in sheep in Norway," *Proceedings of Centre for Reproductive Biology (CRB): Special Symposium Aspects of Ovine Reproduction*, vol. 8, pp. 35–44, 1999.

[137] P. Chemineau, A. Daveu, F. Maurice, and J. A. Delgadillo, "Seasonality of estrus and ovulation is not modified by subjecting female Alpine goats to a tropical photoperiod," *Small Ruminant Research*, vol. 8, no. 4, pp. 299–312, 1992.

[138] C. G. Gravance, C. White, K. R. Robertson, Z. J. Champion, and P. J. Casey, "The effects of cryopreservation on the morphometric dimensions of caprine sperm heads," *Animal Reproduction Science*, vol. 49, no. 1, pp. 37–43, 1997.

[139] H. Sieme and H. Oldenhof, "Cryopreservation of domestic livestock semen," in *Cryopreservation and Freeze-Drying Protocols*, W. F. Wolkers and H. Oldenhof, Eds., vol. 1257pp. 277–287, Springer, New York, NY, USA, 3rd edition, 2015.

[140] S. V. Silva, A. T. Soares, A. M. Batista et al., "Vitamin E (trolox) addition to tris-egg yolk extender preserves ram
spermatozoon structure and kinematics after cryopreservation,” *Animal Reproduction Science*, vol. 137, no. 1-2, pp. 37–44, 2013.

[141] R. H. Hammerstedt, J. K. Graham, and J. P. Nolan, “Cryopreservation of mammalian sperm: what we ask them to survive,” *Journal of Andrology*, vol. 11, no. 1, pp. 73–88, 1990.

[142] J. M. DeJarnette, C. E. Marshall, R. W. Lenz, D. R. Monke, W. H. Ayars, and C. G. Sattler, “Sustaining the fertility of artificially inseminated dairy cattle: the role of the artificial insemination industry,” *Journal of Dairy Science*, vol. 87, pp. E93–E104, 2004.

[143] R. H. Foote, “Semen quality from the bull to the freezer: an assessment,” *Theriogenology*, vol. 3, no. 6, pp. 219–235, 1975.

[144] L. Söderquist, N. Madrid-Bury, and H. Rodriguez-Martinez, “Assessment of ram sperm membrane integrity following different thawing procedures,” *Theriogenology*, vol. 48, no. 7, pp. 1115–1125, 1997.

[145] J. O. Almquist, K. E. Grube, and J. L. Rosenberger, “Effect of thawing time on fertility of bovine spermatozoa in French straws,” *Journal of Dairy Science*, vol. 65, no. 5, pp. 824–827, 1982.

[146] C. Gaillard and H. Kupferschmied, “Thawing time and nonreturn rate of bovine semen frozen in fine French straws,” *Theriogenology*, vol. 18, no. 4, pp. 487–495, 1982.

[147] R. K. Tuli, R. Schmidt-Baulain, and W. Holtz, “Influence of thawing temperature on viability and release of glutamic oxaloacetic transaminase in frozen semen from Boer goats,” *Animal Reproduction Science*, vol. 25, no. 2, pp. 125–131, 1991.