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Diluent C and Liquid Nitrogen Fumigation Optimize Cryopreservation of Hu Ram Semen for Farm-Level AI

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ABSTRACT

This research aimed to compare the impact of various semen extenders and freezing techniques on the post-thaw quality of sperm following cryopreservation, with the goal of identifying an affordable and effective approach for preserving Hu ram semen suitable for use on farms. Semen was collected from five Hu rams. In *Experiment I*, samples were diluted using eight distinct extenders (formulas A–H). After dilution and gradual cooling, 0.25 mL straws were filled and frozen by exposure to liquid nitrogen vapors. In *Experiment II*, diluent C was selected as the base formula, and semen was cryopreserved through liquid nitrogen fumigation and two different program-controlled freezing systems. The frozen semen was analyzed for motility indicators (total motility (TM), progressive motility (PM)), sperm kinetics (straight-line velocity (VSL), average path velocity (VAP), curvilinear velocity (VCL), amplitude of lateral head movement (ALH), wobble coefficient (WOB), and mean angular displacement (MAD)), levels of reactive oxygen species (ROS), and membrane and acrosome integrity.

In *Experiment I*, diluent C demonstrated superior TM, PM, and structural integrity of both membrane and acrosome and lower ROS levels ($p < 0.05$) compared with the other extenders except A. It also exhibited greater ($p < 0.05$) values for VCL, VAP, ALH, WOB, and MAD compared with B, D, E, and F. In *Experiment II*, there were no significant differences ($p > 0.05$) among the three freezing systems for TM and the biokinetic indicators. Nevertheless, liquid nitrogen fumigation achieved higher ($p < 0.05$) PM, membrane and acrosome, integrity, and reduced ROS levels compared with the programmed systems. Overall, semen diluted with formula C produced higher-quality sperm after thawing. The liquid nitrogen vapor method yielded better cryopreservation outcomes than the program-controlled approaches when diluent C was applied.

Keywords: Extender, Freezing process, Sperm motility, Functional sperm integrity, ROS

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Introduction

In China, the improvement of breeding stock quality has become a cornerstone of modern intensive livestock farming. Among domestic breeds, the Hu sheep is classified as a national first-level protected animal and is one of the world's few white lambskin breeds [1]. It is highly prized by farmers for its prolific reproductive performance, continuous breeding cycles, and strong resistance to heat, humidity, and environmental variation [2]. Hu sheep are also recognized for high carcass yield and excellent, protein-rich meat quality [3], providing promising prospects for meat and lamb product industries [4]. Consequently, the scale of Hu sheep production continues to grow rapidly. Artificial insemination (AI) and semen freezing technology have become essential in

achieving large-scale breeding efficiency. Semen cryopreservation is a fundamental component of reproductive biotechnology and an integral aspect of AI [5].

The cryopreservation process involves collecting semen, diluting, cooling, equilibrating, and storing it in liquid nitrogen to suppress metabolic activity and ensure long-term viability [6]. Once thawed, sperm regain partial motility and maintain fertilization potential [7]. This technology eliminates spatial and temporal barriers, facilitates the use of elite males, minimizes breeding costs, and enhances reproductive outcomes [8]. Additionally, it helps prevent disease transmission and contributes to the conservation of rare or endangered species [9].

The success of cryopreservation is heavily influenced by the composition of the diluent and the freezing procedure [10, 11]. During freezing and thawing, the extender protects sperm by providing energy, minimizing cryo-damage, and stabilizing the cell membrane [12, 13]. Two major cooling methods are commonly utilized: liquid nitrogen fumigation and controlled-rate freezing [14]. The fumigation technique requires minimal apparatus, making it cost-efficient and convenient, particularly when processing small sample volumes [15]. Controlled-rate cooling, on the other hand, regulates nitrogen vapor injection to achieve steady temperature reduction, resulting in consistent sperm quality [16]. Despite these advantages, its practical application remains limited because of its complexity, high cost, and dependence on specialized temperature control devices. Therefore, the fumigation method continues to be favored for its simplicity and lower operational expense.

An appropriate combination of diluent composition and freezing procedure is crucial for optimal sperm preservation. However, few comparative studies have explored these factors, and existing results show variable post-thaw quality. For example, Falchi [17] used a Tris–citric acid–glucose extender and liquid nitrogen fumigation for buck semen, obtaining 61% total motility. Igbokwe [18] reported 36% motility for goat semen using a Tris-based formula and fumigation. İnanç [19] achieved 28% TM in Sonmez ram semen using a Tris–citric–fructose base, whereas Pradiee [20] observed 15% TM for mouflon rams with a Tris–citric–glucose mixture. In contrast, Vozaf [21] used a commercial product (Triladyl®) combined with controlled-rate cooling for Wallachian ram semen, yielding 62% TM.

Hence, this study aimed to determine the influence of different diluents and freezing techniques on post-thaw sperm characteristics in Hu rams.

Materials and Methods

Experimental design

Experiment I was conducted to evaluate the effects of eight base extenders (A–H) on sperm motion (TM, PM), kinetic behavior, ROS levels, and structural integrity of the membrane and acrosome after thawing. *Experiment II* investigated the influence of three freezing techniques—two controlled-rate programs and one liquid nitrogen fumigation—using diluent C as the standard extender. The same post-thaw parameters were assessed to determine the optimal freezing procedure for Hu ram semen.

Animals and semen collection

The experiment used healthy Hu rams housed at the Yangzhou University sheep unit, all kept under identical feeding and management routines. Each 2–3-year-old animal received a daily ration containing measured amounts of concentrate and alfalfa hay, with unrestricted access to water. Semen was obtained three times weekly by means of an artificial vagina. Between June and August, a total of 80 ejaculates were gathered from five males previously confirmed to be fertile. Each sample was inspected for semen characteristics, and only those meeting the following standards were selected: ejaculate volume within 0.5–1.5 mL, sperm concentration above 2.5×10^9 sperm/mL, and total motility exceeding 80%. Qualifying ejaculates were then mixed in equal proportions to produce pooled samples for later analyses.

Preparation of semen diluents

Six fundamental extenders (A–F) were prepared in the laboratory following the compositions summarized in **Table 1**. Among these, diluent E corresponded to phosphate-buffered saline (PBS), whereas diluent F was simple isotonic saline (NS). Two commercial media—G (Shengyuan, Zhengzhou, China) and H (Beiteshuang, Beijing, China)—were also used; both lacked cryoprotective additives.

Freezing extender I was obtained by mixing 20% (v/v) egg yolk with 80% of the chosen basal extender, while freezing extender II contained 6% glycerol blended with 94% of extender I. The basic solutions were refrigerated at 4 °C until use; egg yolk and glycerol were incorporated immediately prior to semen dilution.

Table 1. Composition of the six basal extenders used for semen dilution.

Constituent	A	B	C	D	E (PBS)	F (NS)
Tris (Sangon Biotech, Shanghai, China)	1.82 g	-	1.82 g	0.28 g	-	-
Glucose (Sangon Biotech, Shanghai, China)	0.25 g	-	-	0.25 g	-	-
Fructose (Sangon Biotech, Shanghai, China)	-	0.25 g	0.25 g	-	-	-
Citric acid (Sangon Biotech, Shanghai, China)	0.91 g	-	0.91 g	-	-	-
Sodium citrate (Sangon Biotech, Shanghai, China)	-	1.20 g	-	0.35 g	-	-
Sodium bicarbonate (Sangon Biotech, Shanghai, China)	-	-	-	0.05 g	-	-
Sodium chloride (Sangon Biotech, Shanghai, China)	-	-	-	-	400.33 mg	0.45 g
Potassium chloride (Sangon Biotech, Shanghai, China)	-	-	-	-	10.06 mg	-
Dibasic Sodium Phosphate (Sangon Biotech, Shanghai, China)	-	-	-	-	56.78 mg	-
Potassium dihydrogen phosphate (Sangon Biotech, Shanghai, China)	-	-	-	-	12.25 mg	-
Pen Strep (Thermo, Waltham, MA, USA)	10,000 IU	10,000 IU	10,000 IU	10,000 IU	10,000 IU	10,000 IU
Total volume	50 mL	50 mL	50 mL	50 mL	50 mL	50 mL

Cryopreservation and thawing procedures

Fresh semen was diluted with extender I in a 1:3 proportion and cooled gradually at 4 °C for 2.5 hours while wrapped in a towel to avoid thermal shock. Extender II was then added in a 1:2 ratio, followed by another 2.5-hour equilibration at the same temperature. The equilibrated mixture was filled into 0.25 mL plastic straws, which were heat-sealed with sealing powder.

For vapor-phase freezing, the straws were positioned roughly 2 cm above the surface of liquid nitrogen for 20 minutes and then plunged directly into liquid nitrogen for storage.

For controlled-rate cooling, a programmable freezing unit (TF-PA-II, Tianfeng Industrial, Shanghai, China) was used to regulate the descent in three steps:

- Program 1: 4 °C → −10 °C at 5 °C/min; −10 °C → −100 °C at 40 °C/min; −100 °C → −140 °C at 20 °C/min.
- Program 2: 4 °C → −10 °C at 5 °C/min; −10 °C → −100 °C at 20 °C/min; −100 °C → −140 °C at 20 °C/min.

Once the cycles were completed, all straws were directly submerged in liquid nitrogen for long-term preservation. After seven days, selected samples were thawed at 55 °C in a water bath for 8 seconds and used immediately for analysis.

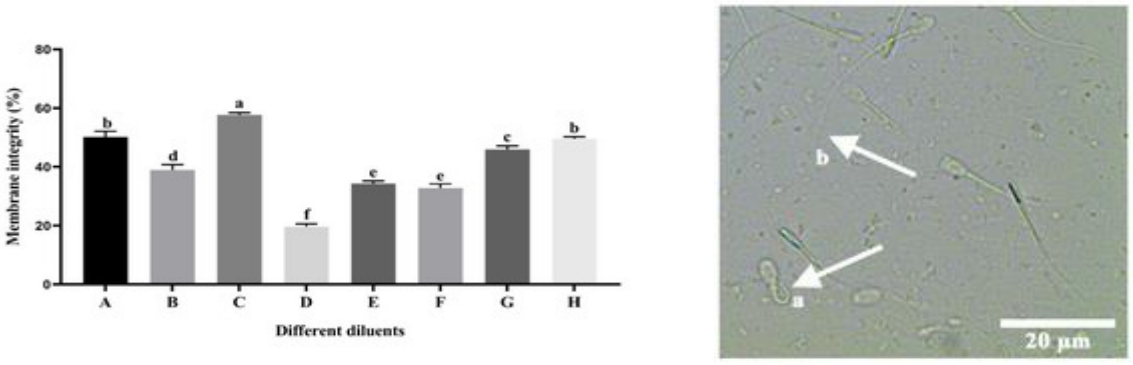
Assessment of sperm motility and kinematic traits

The motion characteristics of thawed sperm were analyzed using a computer-assisted sperm analysis system (CASA; ML-608JZ II, Mailang, Nanning, China). Thawed semen was diluted 1:4.5 with the basic extender, and 1.4 µL was loaded into a counting chamber (YA-1, Yucheng, Nanjing, China). At least 500 spermatozoa from several microscopic fields were evaluated for total motility (TM), progressive motility (PM %), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), wobble coefficient (WOB %), and mean angular displacement (MAD, °/s).

Determination of membrane integrity

Integrity of the sperm plasma membrane was analyzed through the hypo-osmotic swelling test (HOST) following the method described by [22]. A 20 µL portion of thawed semen was combined with 200 µL of hypo-osmotic

medium containing 9 mg/mL fructose and 4.9 mg/mL sodium citrate. The mixture was incubated at 37 °C for 30 minutes. Evaluation was performed under a phase-contrast microscope (CX31, Olympus, Tokyo, Japan) at 400× magnification, counting 200 sperm cells. Sperm with coiled tails were recorded as having intact membranes, whereas straight-tailed sperm indicated damaged membranes **(Figure 1b)**.



a) b)

Figure 1. Assessment of membrane integrity.
(a) Comparison of thawed sperm membrane integrity among the various extenders. Different letters represent significant differences ($p < 0.05$), while identical letters indicate no significant variation ($p > 0.05$).
(b) Microscopic images used for membrane integrity evaluation: curled sperm (a, arrow) exhibit intact membranes, whereas straight sperm (b, arrow) represent compromised membranes.

Assessment of acrosomal integrity in thawed sperm

The post-thaw acrosome condition of sperm cells was determined through dual fluorescent staining using propidium iodide (PI; Solarbio, Beijing, China) and fluorescein isothiocyanate–peanut agglutinin (FITC–PNA; Sigma, St. Louis, MO, USA) [23]. In this procedure, semen samples were first diluted in the basic extender at a ratio of 1:6. A 100 µL portion of the diluted sample was then mixed with FITC–PNA at a concentration of 200 µg/mL and supplemented with 2 µL of PI (0.5 mg/mL). The mixture was maintained in darkness at 37 °C for 10 min. After incubation, 700 µL of phosphate-buffered saline (PBS) was added, and the samples were immediately analyzed using a FACSCalibur flow cytometer (Beckman Coulter, Shanghai, China). Sperm cells exhibiting $FITC^{-}/PI^{-}$ and $FITC^{-}/PI^{+}$ fluorescence patterns were classified as having intact acrosomes. For each test, 10,000 sperm cells were recorded by flow cytometry.

Determination of intracellular ROS levels

Reactive oxygen species (ROS) generation in thawed spermatozoa was quantified using the fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime, Shanghai, China) [24]. The semen was diluted in the basal medium (1:6), and a 50 µL aliquot of this mixture was incubated with DCFH-DA (10 mM). The reaction was carried out at 37 °C for 30 min under light-protected conditions. At the end of incubation, the samples were washed once with PBS and resuspended in 400 µL of the same buffer. Fluorescence was then measured with a multi-mode microplate reader (PerkinElmer, Waltham, MA, USA) at excitation and emission wavelengths of 488 nm and 525 nm, respectively. The relative fluorescence intensity was used as an indicator of ROS content.

Statistical evaluation

All collected data were analyzed using IBM SPSS Statistics (version 25.0, Armonk, NY, USA; 2017). One-way analysis of variance (ANOVA) was applied, followed by Duncan's multiple range test for comparison of means. A significance level of $p < 0.05$ was used. Results are expressed as Mean ± SEM, and all experiments were repeated four times per treatment group.

Results and Discussion

Impact of different diluents on motility and movement dynamics of thawed sperm

Table 2 shows that total motility (TM) and mean angular displacement (MAD) values were significantly higher ($p < 0.05$) in sperm frozen with diluents A and C than in all other groups. Progressive motility (PM) was also

markedly greater in samples preserved with diluent C ($p < 0.05$). Sperm stored in diluents A and H displayed higher PM ($p < 0.05$) than those treated with diluents B, D, E, F, and G. The post-thaw curvilinear velocity (VCL), average path velocity (VAP), and amplitude of lateral head displacement (ALH) were significantly enhanced ($p < 0.05$) in samples from diluents A, C, G, and H compared to the rest. The wobble coefficient (WOB) also exhibited a significant rise ($p < 0.05$) in diluents A and C relative to B, D, E, F, and G.

Table 2. Effects of different extenders on motility and kinematic characteristics of thawed sperm.

Diluent	TM (%)	PM (%)	VSL (µm/s)	VCL (µm/s)	VAP (µm/s)	ALH (µm)	WOB (%)	MAD (°/s)
A	74.82 ± 1.22 a	60.32 ± 1.02 b	40.98 ± 0.93 b	60.33 ± 1.73 a	42.66 ± 1.22 a	17.67 ± 0.51 a	0.53 ± 0.02 a	39.10 ± 2.60 a
B	54.09 ± 0.44 d	39.56 ± 1.01 d	41.12 ± 1.24 b	53.77 ± 0.62 b	38.02 ± 0.44 b	15.75 ± 0.18 b	0.39 ± 0.04 bc	20.75 ± 0.82 c
C	76.81 ± 1.04 a	64.42 ± 0.84 a	41.18 ± 1.41 b	62.05 ± 0.52 a	43.88 ± 0.37 a	18.18 ± 0.15 a	0.58 ± 0.06 a	43.54 ± 4.79 a
D	3.81 ± 0.14 g	2.57 ± 0.21 g	29.14 ± 2.46 c	43.71 ± 1.14 c	30.90 ± 0.81 c	12.80 ± 0.34 c	0.36 ± 0.02 c	2.78 ± 0.27 e
E	39.12 ± 0.49 e	24.98 ± 0.92 e	40.54 ± 1.90 b	53.20 ± 0.99 b	37.62 ± 0.70 b	15.58 ± 0.29 b	0.36 ± 0.01 c	11.44 ± 0.27 d
F	31.47 ± 1.79 f	19.38 ± 0.90 f	40.88 ± 1.00 b	53.44 ± 0.95 b	37.79 ± 0.68 b	15.66 ± 0.28 b	0.40 ± 0.04 bc	9.60 ± 0.14 de
G	66.37 ± 2.78 c	51.57 ± 0.88 c	47.65 ± 0.30 a	60.43 ± 0.51 a	42.73 ± 0.36 a	17.70 ± 0.15 a	0.36 ± 0.02 c	28.21 ± 2.44 b
H	70.68 ± 0.82 b	57.92 ± 1.37 b	43.88 ± 0.19 b	60.39 ± 0.75 a	42.70 ± 0.53 a	17.69 ± 0.22 a	0.48 ± 0.03 ab	29.61 ± 2.21 b

Note: Distinct superscript letters indicate significant differences ($p < 0.05$); identical letters show no statistical difference ($p > 0.05$). TM = total motility; PM = progressive motility; VSL = straight-line velocity; VCL = curvilinear velocity; VAP = average path velocity; ALH = lateral head amplitude; WOB = wobble coefficient; MAD = mean angular displacement.

Influence of diluents on sperm plasma membrane integrity

Figure 1 illustrates that sperm maintained in diluent C exhibited the highest post-thaw membrane integrity ($p < 0.05$) among all treatments. Samples in diluents A and H also showed greater integrity ($p < 0.05$) than those in B, D, E, F, and G, though no significant difference was detected between A and H ($p > 0.05$). Moreover, sperm frozen in diluent B had higher ($p < 0.05$) membrane integrity than those in D, E, and F. In contrast, diluent D produced the lowest ($p < 0.05$) membrane preservation among all formulations.

Effect of diluents on sperm acrosomal integrity during freezing

According to **Figure 2**, the post-thaw acrosome integrity was greatest in spermatozoa frozen with diluent C ($p < 0.05$), with comparable results in diluent A ($p > 0.05$). Diluents A and C outperformed B, D, E, and F ($p < 0.05$). In addition, sperm preserved with commercial diluents G and H showed higher integrity ($p < 0.05$) than those frozen with D and F. The lowest acrosomal integrity was found in samples from diluent D ($p < 0.05$).

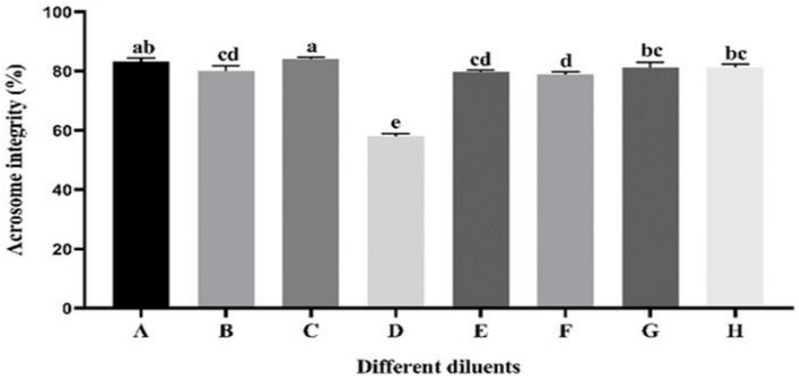


Figure 2. Comparison of post-thaw acrosomal integrity in sperm stored with various diluents.
Note: Different letters indicate significant ($p < 0.05$) and identical letters non-significant ($p > 0.05$)

differences.

Effect of diluents on intracellular ROS generation

As shown in **Figure 3**, ROS concentrations in sperm preserved with diluents A and C were markedly lower ($p < 0.05$) than in other treatments, though no significant variation occurred between these two groups ($p > 0.05$). Sperm stored in diluents B, E, and F exhibited significantly less ROS ($p < 0.05$) than those preserved with D, G, and H. No statistical differences were detected among B, E, and F ($p > 0.05$).

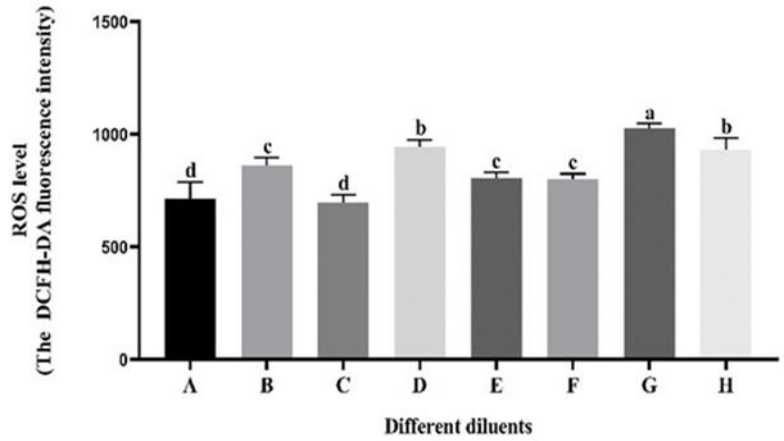


Figure 3. Influence of various extenders on ROS concentration in thawed sperm.

Note: Groups marked with different letters differ significantly ($p < 0.05$), while identical letters show no significant difference ($p > 0.05$).

ROS: reactive oxygen species; DCFH-DA: 2,7-dichlorodihydrofluorescein diacetate.

Comparison of freezing procedures on sperm motility and movement dynamics after cryostorage

Data in **Table 3** indicate that sperm from the *liquid nitrogen vapor exposure* treatment exhibited the greatest post-thaw total motility (TM), though the difference was not statistically significant ($p > 0.05$) compared with *programs 1* and *2*. Post-thaw progressive motility (PM) was notably higher ($p < 0.05$) in *program 1* and *liquid nitrogen vapor* groups than in *program 2*. Among all treatments, no meaningful variation ($p > 0.05$) appeared in sperm kinetic properties.

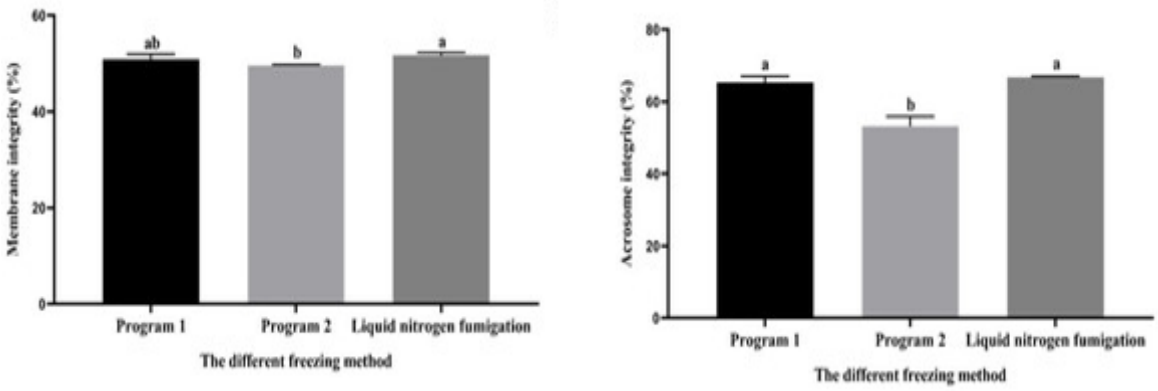
Table 3. Influence of freezing strategy on sperm motility and kinetic parameters.

Freezing Method	TM (%)	PM (%)	VSL (μm/s)	VCL (μm/s)	VAP (μm/s)	ALH (μm)	WOB (%)	MAD (°/s)
Program 1	66.72 ± 1.36	54.62 ± 0.98 a	40.00 ± 0.46	60.10 ± 0.81	42.50 ± 0.57	17.60 ± 0.24	0.53 ± 0.01	32.51 ± 3.57
Program 2	64.09 ± 2.42	50.58 ± 0.89 b	39.60 ± 0.51	58.81 ± 0.64	41.58 ± 0.45	17.23 ± 0.19	0.52 ± 0.03	36.89 ± 5.85
Liquid nitrogen fumigation	69.24 ± 0.58	55.52 ± 0.55 a	40.27 ± 0.76	60.56 ± 1.24	42.82 ± 0.87	17.74 ± 0.36	0.56 ± 0.01	36.77 ± 1.99

Note: Columns with differing letters are significantly distinct ($p < 0.05$); identical letters indicate no significant difference ($p > 0.05$).

Influence of freezing techniques on sperm membrane and acrosome stability after thawing

As shown in **Figure 4a**, sperm preserved through *liquid nitrogen vapor exposure* had markedly greater membrane integrity ($p < 0.05$) compared with *program 2*, but no significant difference ($p > 0.05$) was noted relative to *program 1*. In **Figure 4b**, sperm acrosome integrity in *program 1* and *liquid nitrogen vapor* groups was significantly lower ($p < 0.05$) than that of *program 2*, yet the difference between those two groups themselves was non-significant ($p > 0.05$).



a) b)

Figure 4. Variation in thawed sperm membrane and acrosome stability under different freezing approaches. (a) Membrane integrity. (b) Acrosome integrity.

Note: Distinct letters imply significant differences ($p < 0.05$); identical letters denote non-significance ($p > 0.05$).

Changes in sperm ROS concentration under various freezing methods

As illustrated in **Figure 5**, sperm thawed from the *liquid nitrogen vapor* treatment demonstrated a substantially reduced ROS level ($p < 0.05$) in comparison with other techniques. Likewise, sperm from *program 1* exhibited lower ROS ($p < 0.05$) than *program 2*.

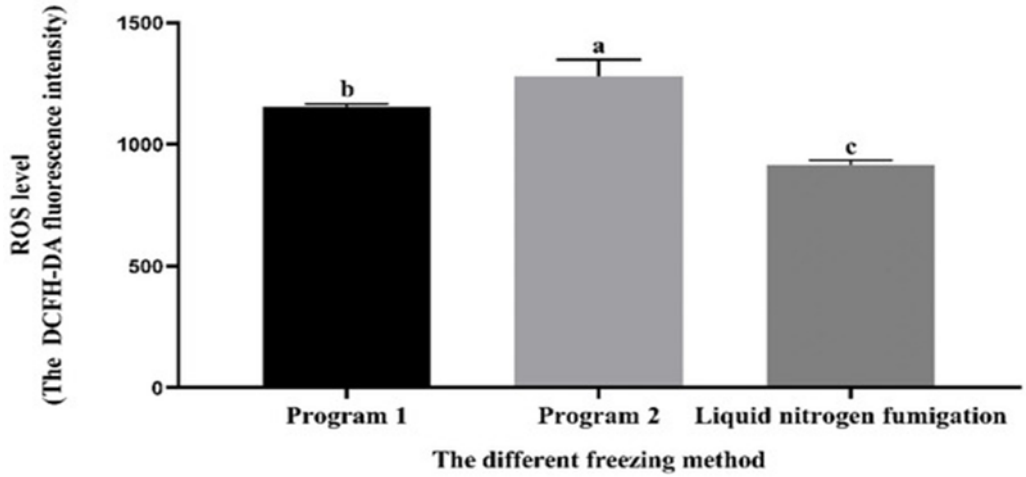


Figure 5. Influence of freezing method on post-thaw sperm ROS concentration.

Note: Groups with different letters differ significantly ($p < 0.05$); identical ones show no significant variation ($p > 0.05$).

Establishing a straightforward and efficient extender and cryostorage method is essential for advancing artificial insemination (AI), protecting genetic diversity, and minimizing inbreeding. Factors such as diluent makeup, dilution rate, equilibration time, freezing process, and thawing conditions all affect the outcome of semen preservation [25]. This experiment analyzed eight base diluents (A–H) and three freezing systems for their impact on thawed *Hu ram* sperm quality. Results clearly indicated that *liquid nitrogen vapor exposure* (2 cm above liquid nitrogen for 20 min) in combination with *diluent C* (Tris–citric acid–fructose formulation containing 20% egg yolk and 6% glycerol) represents a simple, inexpensive, and highly effective cryopreservation protocol, superior to other diluents or programmed cooling.

During freezing, an appropriate extender composition provides metabolic support, maintains a balanced environment, and limits cold-induced cellular damage [26]. Typically, extenders include buffering agents (Tris, citric acid, sodium citrate), cryoprotectants (egg yolk, glycerol), and energy-providing sugars (fructose, glucose) [27]. In this study, sperm stored in *diluents E* and *F* showed significant declines ($p < 0.05$) in TM, PM, kinetic

motion, membrane, and acrosome integrity compared with *A* and *C*, indicating that egg yolk and glycerol alone are insufficient. The results confirm that buffering systems such as Tris and citric acid, combined with carbohydrates, are key to maintaining sperm quality during freezing.

According to Bravo [28], semen preserved in Tris-based diluents yielded better outcomes than phosphate-buffered saline (PBS). Tris serves as a reliable buffering agent, a conclusion supported by Graham's [29] findings in bovine sperm, where Tris was deemed the most effective buffer for semen preservation. Sperm metabolism can alter environmental pH and suppress certain enzymatic reactions [30]; therefore, compounds like citric acid and sodium citrate stabilize pH conditions. Carbohydrates act not only as an energy reserve but also as protectants, maintaining both sperm structure and ultrastructure during cryostorage [31, 32].

Compared with diluent C, diluent B performed better under low-temperature storage [33]. Nonetheless, semen thawed from diluent B displayed lower ($p < 0.05$) TM, PM, motility kinetics, and membrane and acrosome integrity, while exhibiting a higher ($p < 0.05$) ROS level than that treated with diluent C. These discrepancies might result from stronger cryogenic stress and the superior buffering effect of Tris and citric acid in diluent C, whereas diluent B contained only sodium citrate as a single buffer [34].

Among all extenders, diluent D showed the weakest cryoprotective efficiency, likely due to insufficient buffer content to maintain solution stability. Similarly, Lv's study [35] confirmed that adding citric acid to the diluent achieved better cryoprotection than using sodium citrate alone. Diluent C also produced higher ($p < 0.05$) PM and membrane integrity values than diluent A, while other traits were not significantly different ($p > 0.05$). The likely cause was that Hu sheep utilize fructose more efficiently than glucose. Prior research has likewise demonstrated that fructose outperforms glucose in semen preservation for both bucks [36] and wolves [37].

Furthermore, diluent C yielded higher ($p < 0.05$) TM, PM, MAD, membrane, and acrosome integrity, along with reduced ($p < 0.05$) ROS values compared with two commercial extenders. The compositions of these commercial formulations remain unspecified, possibly because they were designed for general use in both sheep and goats, rather than tailored for a specific breed, thus reducing cryopreservation efficiency. Consequently, the chemical composition of the freezing diluent plays a decisive role in maintaining sperm motility during the freezing–thawing process.

Avdatek [38] and Bucak [39] used the same basic extender as the one tested in this study for crossbred sheep and Merino rams, but the freezing technique differed, resulting in thawed PM values of only 13% and 10%, respectively. These outcomes highlight that only an appropriate diluent combined with an optimized freezing method can achieve effective cryopreservation.

During dilution and freezing, sperm cells undergo temperature-related stress, leading to various degrees of injury. The range between 0 and −60 °C—often termed the *critical temperature zone*—is particularly harmful [40]. In this region, the cooling rate can cause ice crystal formation, leading to irreversible cell damage [41]. In the present study, Program 2 yielded significantly lower ($p < 0.05$) PM compared to Program 1 and the liquid nitrogen vapor approach. This reduction likely stems from the slower cooling rate of Program 2, which promotes the creation of damaging ice crystals, impairing sperm function and reducing motility.

Sperm membrane damage at any stage of cryopreservation compromises fertilization potential [42]. The acrosome houses hydrolytic enzymes necessary for penetrating the cumulus cells and zona pellucida during fertilization [43]. Ice crystal formation can also denature lipoproteins, disrupting membrane architecture and leading to cytoplasmic loss [44]. The liquid nitrogen fumigation technique produced higher ($p < 0.05$) membrane and acrosome integrity compared with Program 2, with no significant difference ($p > 0.05$) from Program 1.

Under normal physiological conditions, sperm maintain a balance of ROS levels; moderate ROS concentrations are necessary for capacitation and acrosomal reactions, but excessive levels are detrimental [45]. In this experiment, Program 2 displayed higher ($p < 0.05$) ROS values compared with Program 1 and liquid nitrogen fumigation, likely due to the slower cooling rate, increased crystal formation, impaired motility, and ROS leakage from damaged sperm [46].

Research by Stuart [47] demonstrated that rapid freezing yields higher semen quality than slow freezing, consistent with the current results. Similarly, Galarza [48] observed a 61% TM in Merino ram semen using program-controlled cooling. In contrast, Esteso [49] found that in Iberian ibex, the program-controlled method produced higher semen quality than liquid nitrogen fumigation. Collectively, these findings confirm that semen quality is highly dependent on species, extender composition, and cooling procedure.

Nonetheless, liquid nitrogen fumigation remains more cost-effective and simpler to implement than program-controlled systems, making it more accessible globally. Hence, combining diluent C with liquid nitrogen

fumigation offers a practical, low-cost, and efficient cryopreservation protocol, eliminating the need for sophisticated equipment or complicated formulations. This approach could significantly enhance the utilization of elite breeding males, accelerate genetic improvement, and boost reproductive efficiency.

Conclusion

Based on assessments of CASA, membrane, and acrosome integrity, as well as ROS levels, the thawed semen from Hu rams treated with diluent C exhibited superior quality relative to other extenders. Furthermore, when paired with liquid nitrogen fumigation, diluent C achieved better cryopreservation outcomes than the program-controlled cooling approach. In addition to being technically simpler, the liquid nitrogen fumigation method avoids the need for costly laboratory setups while maintaining excellent post-thaw results. However, further validation through in vitro fertilization or artificial insemination studies is recommended to confirm its effectiveness under practical conditions.

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Conflict of Interest: None

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Ethics Statement: None

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