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Quality and Protein Profiles in Local Indonesian Ram Sperm Before and After Cryopreservation

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ABSTRACT

Sperm quality plays a crucial role in determining the success of fertilization. This study aimed to assess the quality of both fresh and frozen ram semen, as well as examine changes in semen protein content. Four local rams, aged 3-4 years, were selected for the study. Semen collection was conducted in the morning using an artificial vagina. The collected semen was divided into two parts: one for analyzing fresh semen and the other for freezing. The freezing process involved using a diluent consisting of Tris hydroxy amino methane, citric acid, egg yolk fructose, 20% egg yolk, and glycerol. Both fresh and frozen semen samples underwent analysis using the same method, including examination of concentration, motility, viability, and various kinematic motility parameters, such as VAP, VCL, VSL, LIN, and STR, with the assistance of CASA (AndroVision®). Following collection, both samples were centrifuged at 4500rpm for 10min, and the supernatant and precipitate were stored at -40°C until analysis. Protein concentration was determined using the Bradford kit, and semen protein profiles were evaluated through SDS-PAGE and Coomassie Blue staining. The study revealed a significant decrease in semen quality following cryopreservation ($P<0.05$). Protein analysis results indicated that fresh semen exhibited ten specific proteins, whereas frozen semen expressed six specific proteins. These findings suggested a correlation between protein content and sperm quality, with the cryopreservation process altering the composition of proteins in sperm.

Key words: Ram, Sperm, Fresh, Frozen, Protein

INTRODUCTION

Artificial insemination is a critical biotechnological tool for reproduction, enabling the management of infectious diseases, optimization of male genetic resources, and acceleration of genetic progress (Casali et al. 2017). Advancements in cryopreservation techniques have expanded the application of artificial insemination by enabling longer-term storage of spermatozoa (Kargar et al. 2017). However, despite these recent strides, the success rate of utilizing this technology with rams still requires improvement, as spermatozoa are particularly sensitive to freezing processes, particularly at temperatures below 4°C (Faigl et al. 2012).

Spermatozoa undergo various stresses during cryopreservation, including ice crystal formation, cold shock, chemical poisoning, oxidative stress, and osmotic pressure, all of which can lead to structural damage to the spermatozoa, including alterations to protein molecules. Research by Azizah et al. (2023) illustrates the close relationship between semen proteins and traits, with specific proteins associated with freezeability (Rego et al. 2016). Developing strategies to ensure high post-thaw sperm quality requires an understanding of how cryopreservation-induced stress impacts sperm biology (Ďuračka et al. 2023). While some research has focused on ram sperm proteins, including studies on Yunnan ram (Jia et al. 2022), there remains a lack of knowledge regarding tropical regions or local Indonesian ram. Therefore, this study aimed to investigate the effect of cryopreservation on the quality and protein status of ram semen, anticipating providing valuable insights into the application of artificial insemination and cryopreservation technology.

The identification of proteins in ram spermatozoa, both before and after cryopreservation, is essential because cryopreservation causes significant changes in the sperm

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proteome that affect post-translational modifications and abundance (Yoon et al. 2016; Bogle et al. 2017; Perez-Patiño et al. 2019). These modifications may affect the motility, capacitation, and functionality of sperm, all of which are essential for a successful fertilization process (Cormier and Bailey 2003; Kumaresan et al. 2012). Furthermore, cryopreservation may increase apoptotic markers, which indicate possible damage to sperm cells (Said et al. 2010). Understanding the molecular mechanisms behind these changes and developing strategies to mitigate cryoinjury require identifying the precise proteins impacted by cryopreservation. The sperm proteome dramatically changes when cryoprotectants are added during cryopreservation (Yoon et al. 2016). To minimize damage to sperm proteins, it is crucial to comprehend these changes to optimize cryopreservation protocols and choose the best cryoprotectants. Identifying the proteins linked to cryostress in spermatozoa is essential to protect sperm cells during cryopreservation (Yoon et al. 2016).

Furthermore, developing biomarkers that accurately predict sperm cryotolerance and post-thaw quality depends on identifying proteins before and after cryopreservation (Vieira et al. 2021). This has the potential to significantly improve reproductive outcomes in rams, in addition to facilitating the careful selection of quality sperm for use in assisted reproductive techniques. Finding the proteins that change during the cryopreservation process is crucial because it provides valuable information about how it affects sperm quality and fertility. This is important because it can enhance breeding efforts and genetic preservation programs in rams (Zhu et al. 2020). It is essential to meticulously identify the proteins in ram spermatozoa before and after cryopreservation in the Indonesian ram context, where unique obstacles and factors may apply. Acquiring this knowledge extends beyond academic pursuits; it forms the basis for developing focused strategies to reduce cryoinjury, improve cryopreservation procedures, and, most importantly, ensure the selection of superior sperm for assisted reproductive technologies.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Animal Ethics Committee of Universitas Andalas, West Sumatra, Indonesia.

Sample preparation

Four Garut rams, aged three to four, provided the fresh and frozen sperm utilized in this study. The animals were individually housed in separate cages, fed twice daily, and provided unrestricted access to water. Semen collection was performed twice a week in the morning using an artificial vagina. To compare the properties of frozen and fresh semen, the collected semen was divided into two portions. Frozen semen processing involved using Tris citrate egg yolk diluent (comprising Tris hydroxy amino methane, citric acid, fructose, egg yolk, and glycerol). The procedures adhered to the quality assurance standards of the institution, which the ISO 17025 national accreditation committee recognizes.

Sperm evaluation

The freshly obtained semen was macroscopically evaluated, considering volume, pH, color, and consistency, as well as microscopically assessed for mass movement, motility, concentration, viability and abnormality. Computer-assisted sperm analysis (CASA) was employed using AndroVision by Minitube. The motility parameters observed included curvilinear velocity (VCL; μm/sec), straight-line velocity (VSL; μm/sec), average path velocity (VAP; μm/sec), the amplitude of lateral head displacement (ALH; μm/sec), and straightness of path (STR; %), calculated as VSL/VAP × 100.

Subsequently, the semen underwent centrifugation at 4500 rpm for 10min at 4°C. The supernatant was separated, and the precipitate was stored at -40°C until used for quantitative and qualitative protein content analysis.

Sperm protein extraction

The ram semen obtained was placed into a 2mL microtube. Spermatozoa cells were separated from plasma by centrifugation at 3000rpm for 45min. Following the manufacturer's procedures, spermatozoan protein extraction was performed using PRO-PREP™ solution (Protein Extraction Solution, 17081 iNtRON Biotechnology, Korea). In summary, 500×10^6 spermatozoa cells from the sediment were combined with 1mL of PBS (Phosphate Buffer Saline) in a 1.5mL microtube and centrifuged at 13,000rpm for 15min. The supernatant was discarded, and the spermatozoa cells were mixed with 400μL of PRO-PREP™ solution and homogenized by vortexing. The solution mixture was then incubated at -20°C for 15min. After sonication for 20s three times, the diluent was centrifuged at 13,000rpm (4°C) for 5min. The supernatant was transferred into a 1.5mL microtube for measuring the protein content.

Spermatozoa protein concentration was assessed using the Bradford method (Bradford 1976). The Bradford analysis protocol involved using Bradford reagent (BR05, Eco Tech). The working solution was prepared by diluting reagent with aquabidest (1:4), and the standard solution was prepared by dissolving 0.563mg of Bovine Serum Albumin (BSA, Sigma) with PBS. Blank materials (3000μL working solution and 50μL PBS), standards (3000μL working solution and 50μL standard solution), and samples (3000μL working solution and 50μL sample) were each incubated at room temperature for 10min. The optical density (OD) was measured at a wavelength of 595 nm using a spectrophotometer (Shimadzu, UV 1800). Calculate the total concentration of dissolved protein using the formula:

$$Total\ Protein\ Concentration = \frac{\Delta A1}{\Delta A2} \times c \times f$$

ΔA1: OD sample – OD Blanko
ΔA2: OD standar – OD Blanko
c: Concentration standard (0.563mg/mL)
f: Sample dilution factor before testing

SDS-PAGE

Spermatozoa protein analysis was conducted using SDS-PAGE with a 20μg/mL sample. The sample amounts were determined with a Quibic fluorometer (Quibic, Invitrogen). The sample was placed into a microtube, added with 10μg of weight buffer, and homogenized by

vortexing for 1 minute. After being heated in a water bath for 10min at 70°C, the sample was centrifuged for 1 minute. The protein samples were run on a 10% polyacrylamide gel (Q-PAGE™ TGN Precast Gel, QP4210, SMOBIO® Technology, Inc. Taiwan) for 90min in Tris-glycine buffer solution (Tris-Glycin Running buffer, TGS 10 Eco Tech.), with voltage set at 110 volts and 110 mA.

Protein staining of samples on the gel was carried out using Coomassie Blue (Fast Coomassie Blue Staining Solution, E-IR-RI129) for 24 hours while shaking. The remaining stain was removed by soaking the gel in ddH2O in a closed container and washing it three times. Protein bands on the gel were observed with Gel Doc (iBright 1500, Invitrogen, Thermo Fisher Sci). The read sample protein bands were compared with the marker band (Excell band 3-color Broad Range Protein Marker 10-245kDa, SMOBIO® Technology, Inc. Taiwan) to determine the protein molecular weight.

SDS-PAGE photo data were employed to qualitatively determine the molecular weight of the sample (protein of frog skin secretion) by comparing the bands produced by the sample with those produced by the marker.

Data analysis

Data on spermatozoa characteristics are presented as averages and standard deviations. The kinematic data of fresh and frozen ram sperm were analyzed statistically using the T-test. The protein weight from the acrylamide gel was determined to obtain the molecular weight. Protein data were presented descriptively.

RESULTS AND DISCUSSION

Ram fresh sperm characteristics

Sperm characteristics play a pivotal role in male gamete fertility. Table 1 provides an overview of the fresh local ram semen characteristics, including volume, concentration, motility, viability, and abnormality.

Table 1: Characteristics of local ram semen

No	Characteristics	Mean	SE
1	Volume (mL)	1.50	0.55
2	Concentration (10^9)	1.97	0.3
3	Motility (%)	87.51	3.49
4	Viability (%)	90.35	3.57
5	Abnormality (%)	13.75	4.32

The seminal characteristics of local ram semen, including volume, concentration, motility, viability, and abnormality, serve as pivotal indicators determining semen quality. Notably, dietary components, particularly ingesting specific fatty acids such as omega-3, have been found to exert discernible influences on semen characteristics (Yuan et al. 2023). Furthermore, variations in semen quality have emerged among ram breeds, with disparities observed in sperm motility, pH, and concentration across different breeds (Al-Janabi et al. 2023). The complex interplay of age, body weight, and genetic factors contribute to discernible distinctions in testicular development and semen characteristics among rams (Elaref et al. 2021).

Fertility assessments highlighted seminal parameters' importance, including motility, viability, and concentration (Abdullah et al. 2022). Studies have examined the effects of seasonal variations, environmental factors, and supplement usage, with *Moringa oleifera* serving as an example, on semen quality (Fathy et al. 2022). Testicular size, testosterone levels, and semen characteristics in rams were influenced by the dynamic interaction of seasonal variations, social dominance, and breeding seasons (González-Maldonado et al. 2023). Substitute elements found in supplements and extenders, such as propolis ethanolic extract, have been investigated, revealing their potential influence on sperm properties (Abdelnour et al. 2023). Various factors, including age, breed, diet, environmental subtleties, and additional treatments, contribute to the complexity of ram semen quality. A comprehensive evaluation and improvement of ram reproductive performance necessitate a sophisticated understanding of these variables and their intricate effects on semen properties.

Kinematics of fresh and frozen ram sperm

Artificial insemination and breeding programs heavily rely on the quality of semen produced by rams. Studies have indicated notable variations in motility and other kinematic parameters between fresh and frozen ram semen (Table 2). Fresh semen generally exhibits higher progressive motility than frozen semen, encompassing progressive fast motility, progressive slow motility, and progressive circular motility. Furthermore, fresh semen demonstrates significantly higher levels of parameters compared to frozen semen, including VCL, VSL, VAP, DCL, DSL, DAP, ALH, BCF, HAC, WOB, LIN, and STR (Van de Hoek et al. 2022).

It has been discovered that cryopreserved ram sperm tends to have more heterogeneous and susceptible chromatin compared to fresh sperm, potentially leading to less effective fertilization (Ugur et al. 2019). Research has shown that ram semen, whether fresh or frozen, diluted with specific extenders, can yield satisfactory fertility outcomes (Bustani and Baiee 2021). Additionally, differences between fresh and frozen-thawed semen have been identified by comparing their motility, morphology, and velocity parameters using computer-assisted sperm analysis (Alm-Kristiansen 2023). According to Saha et al. (2022), cooled ram semen has been found to exhibit lower quality than fresh semen, impacting motility, morphology, survival ability, fertility, and embryonic loss. While fresh semen may not show variations in reproductive performance, frozen-thawed semen might, depending on the artificial insemination technique (Koch et al. 2022). Freezing-thawing processes in cattle and buffalo bull sperm have been demonstrated to have less impact on velocity and kinematic characteristics compared to absolute sperm motility (Pathak et al. 2019). Research has revealed differences in ram semen motility between frozen and fresh samples, with frozen semen demonstrating reduced motility rates (Gustina et al. 2023). Nevertheless, some studies have found no appreciable variations in motility between fresh and post-thaw semen samples from specific breeds (Baloch 2019). It has also been noted that fresh and frozen-thawed ram semen contain distinct subpopulations of sperm with differing swimming velocities and progressiveness (Peris-Frau et al. 2021), demonstrating how freezing - thawing

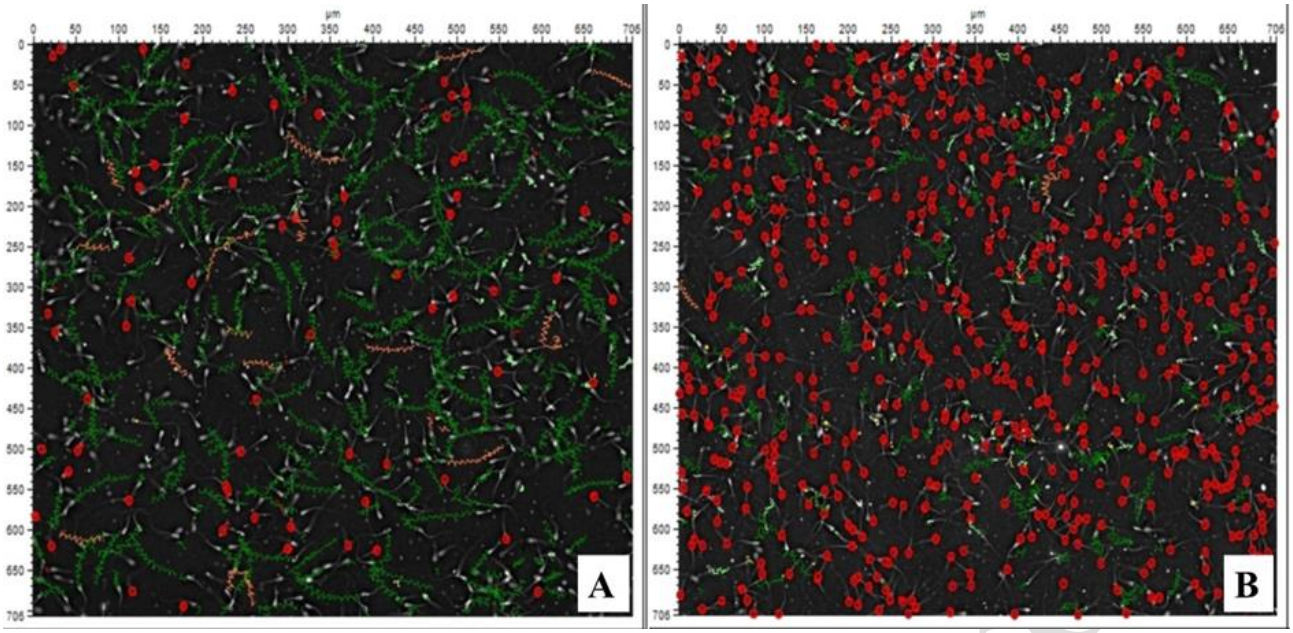

Fig. 1: Image obtained from computer-assisted sperm analysis (CASA): A. fresh sperm; B. frozen sperm.

processes can affect the kinematic parameters and motility of ram semen, potentially impacting fertility outcomes. Maintaining the reproductive potential of ram sperm and improving artificial insemination methods depend on understanding these distinctions.

Using computer-aided sperm analysis (CASA), several studies have examined the differences in ram spermatozoa motility between fresh and frozen semen. While CASA analysis may not be a direct predictor of ram field fertility, Pathak et al. (2019) concluded that the cryopreservation process significantly reduces the motility and kinematics of bovine spermatozoa (Pathak et al. 2019; Van de Hoek et al. 2022). However, Van de Hoek et al. (2022) highlighted that CASA analysis provides essential information for semen quality assessment. Furthermore, using CASA, Dessouki et al. (2022) examined buffalo semen and discovered variations in sperm motility and velocity parameters between fresh and frozen-thawed samples.

According to Alm-Kristiansen (2023), CASA makes it possible to evaluate different spermatozoa kinematic parameters, which helps to understand the efficiency of migration in the female genital tract. As shown in cattle and buffalo bull spermatozoa studies, the analysis entails assessing motility and velocity traits in fresh semen to predict post-thawed sperm motility and velocity (Pathak et al. 2019). Moreover, CASA's versatility has been demonstrated by its use in identifying distinct subpopulations of motile spermatozoa in donkey ejaculates (Catalán et al. 2020). According to Arzaluz et al. (2021), CASA systems evaluate sperm motility in the horizontal plane on the monitor, providing precise details on sperm motion characteristics.

The apparatus gathers subpopulations of motile sperm and assesses the quality of the sperm both before and after cryopreservation (Fig. 1). Additionally, the CASA settings for evaluating ram sperm have been standardized, ensuring the consistency and repeatability of this analytical method. When analyzing ram spermatozoa motility in fresh and frozen semen, CASA is a valuable tool that can assist in fertility assessments and provide insights into the sperm quality. The technology measures different aspects of motility, providing thorough knowledge of sperm behavior and supporting assisted breeding initiatives and reproductive research (Peris-Frau et al. 2021).

The velocity parameters of fresh ram semen are generally higher than those of frozen ram semen (Fig. 2). Research has indicated a noteworthy distinction in sperm concentration between fresh and frozen semen, with the former exhibiting a significantly greater spermatozoa concentration than the latter (Paventi et al. 2022). These variations have been clarified thanks to computer-assisted sperm analysis, or CASA. Rapid progressive motile sperm exhibit significant correlations with various velocity traits in both fresh and frozen-thawed semen, according to CASA, which also revealed significant relationships between the velocity traits of fresh and frozen-thawed semen (Pathak et al. 2019). Furthermore, spermatozoa's kinematic parameters have been measured using CASA. It has been demonstrated that freezing and thawing affect the structure of the sperm subpopulation in ejaculates in addition to impairing sperm motility (Alm-Kristiansen 2023).

According to Finelli et al. (2021), CASA systems provide an accurate and unbiased evaluation of semen parameters, making it possible to identify minute variations in sperm characteristics that might go undetected using traditional analysis techniques. The application of CASA has also been expanded to evaluate the effects of various cryoprotectants on the quality of post-thawed semen, showcasing the complementary actions of substances such as sucrose on the cryopreservation of chicken semen (Thananurak et al. 2019). When assessing the variations in velocity between fresh and frozen ram semen, CASA is an essential component. It offers insightful information about the kinematic characteristics of spermatozoa, emphasizing how cryopreservation affects the motility and subpopulation structure of sperm. Researchers can precisely evaluate semen quality, determine the best extenders for cryopreservation, and learn more about the variables affecting sperm velocity in ram semen using CASA.

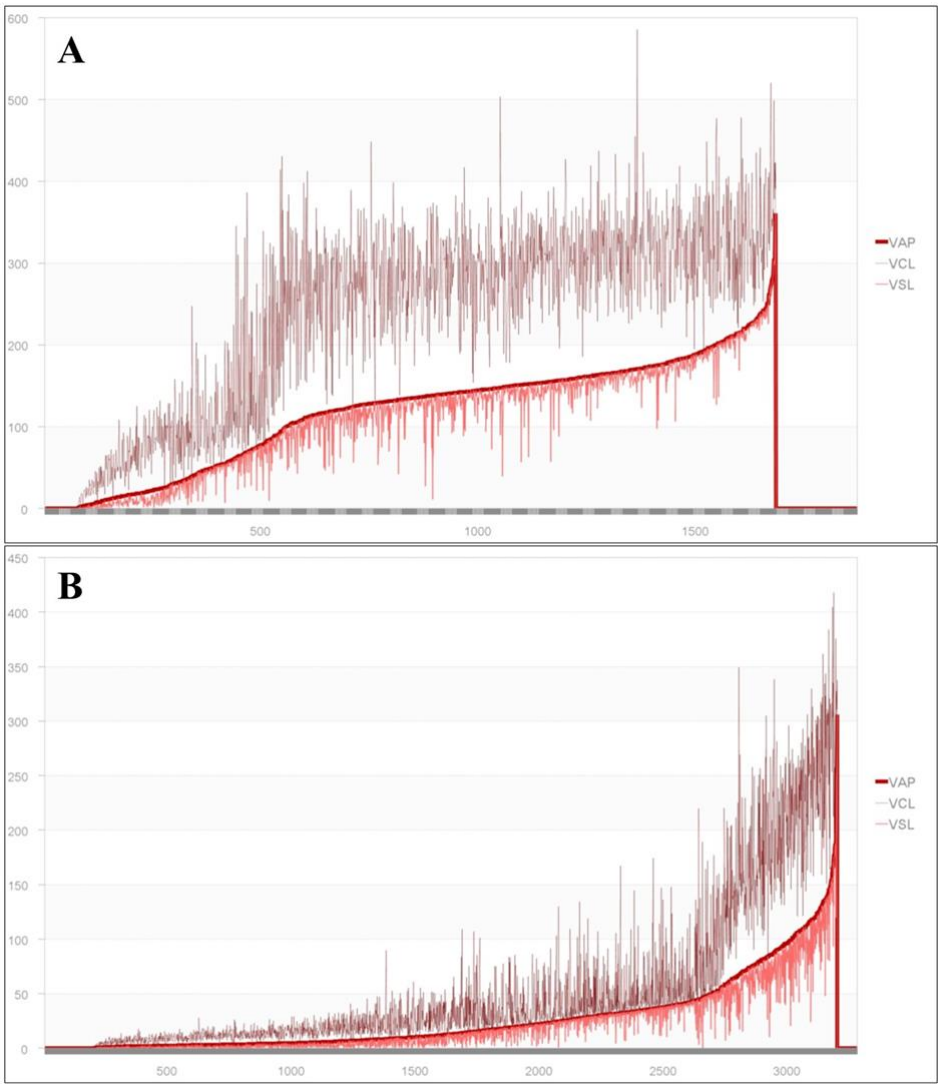

Fig. 2: Ram sperm velocity from computer-assisted sperm analysis (CASA): A. fresh sperm; B. frozen sperm.

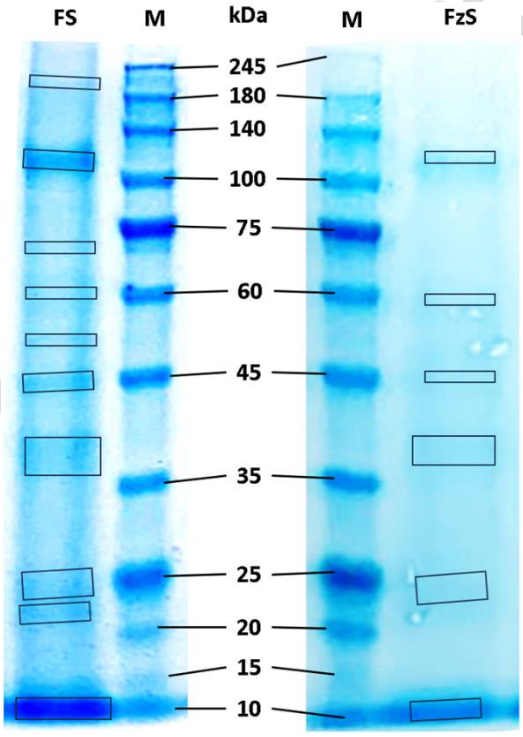

Fig. 3: 1D-SDS-Page fresh and frozen sheep sperm. FS: fresh sperm, M: marker, FzS: frozen sperm.

Ram sperm protein profile

Cryopreservation of semen is essential in animal reproductive research to preserve genetic material for future breeding. The protein content of semen is a crucial component that indicates the general well-being and efficiency of sperm cells.

The investigation unveiled a substantial contrast in the total protein content between fresh and frozen ram semen, with fresh semen exhibiting a concentration of 1.81±0.11mg/mL. In contrast, frozen semen displayed a concentration of 1.09±0.4mg/mL (Table 3). This decline in protein content within frozen semen suggests a profound impact of cryopreservation on the biochemical composition of seminal fluid. The freezing and thawing steps integral to the cryopreservation process may lead to protein denaturation or modification within the semen, potentially diminishing the levels of proteins essential for sperm viability and function. Ram sperm subjected to cryopreservation through freezing and thawing procedures undergo alterations in their biochemical composition, including denaturation and decreased concentration of crucial proteins, among other effects. These changes encompass shifts in the abundance levels of sperm proteins associated with membrane permeability, metabolism, flagella structure, motility, intracellular signaling, capacitation, apoptosis, and fertilization, all

Table 2: Kinematics of fresh and frozen ram sperm

Parameters	Semen	
	Fresh	Frozen
Progressive motility [%]	76.76±3.92[a]	41.31±10.47[b]
Progressive fast motility [%]	65.25±4.93[a]	15.53±8.85[b]
Progressive slow motility [%]	6.02±2.07[a]	15.45±4.89[b]
Progressive circular motility [%]	5.49±2.36[a]	0.33±0.44b[b]
VCL [µm/s]	274.75±63.77[a]	61.16±20.60[b]
VSL [µm/s]	146.10±47.74[a]	25.86±11.80[b]
VAP [µm/s]	156.24±44.27[a]	31.00±12.52[b]
DCL [µm]	53.07±36.51[a]	21.63±8.56[b]
DSL [µm]	26.68±17.52[a]	9.01±4.91[b]
DAP [µm]	29.40±19.62[a]	10.64±5.32[b]
ALH [µm]	1.93±0.59[a]	0.72±0.25[b]
BCF [Hz]	28.90±11.45[a]	10.42±3.20[b]
HAC [rad]	0.53±0.20[a]	0.13±0.04[b]
WOB (VAP/VCL)	0.57±0.11[a]	0.50±0.08[a]
LIN (VSL/VCL)	0.54±0.13[a]	0.41±0.09[a]
STR (VSL/VAP)	0.93±0.11[a]	0.83±0.18[a]

Different superscripts on the same row indicate differences in each treatment (P<0.05).

Table 3: Total protein of fresh and frozen ram sperm

Ram Sperm	Total Protein (mg/mL)	SD
Fresh	1.81	0.11
Frozen	1.09	0.4

Table 4: Distribution of protein bands of fresh and frozen sheep spermatozoa based on molecular weight

Ram Sperm	Molecule weight (kDa)										number of protein bands
	10-14	20-24	25	36-44	45	46-59	60	61-74	101-139	181-244	
Fresh	+	+	+	+	+	+	+	+	+	+	10
Frozen	+	-	+	+	+	-	+	-	+	-	6

ascribed to the freezing and thawing processes of cryopreservation (Arunkumar et al. 2022). Such modifications, indicative of biomedical and structural stress experienced by sperm, ultimately result in reduced post-thaw quality (Tamburrino et al. 2023).

Furthermore, cryopreservation significantly impacts the abundance of various proteins in frozen-thawed ram sperm, with some protein levels increasing while others decrease (Su et al. 2022). This process also alters the protein profile of ram sperm during in vitro capacitation, affecting sperm capacitation at the molecular level (Peris-Frau et al. 2019). Additionally, cryopreservation induces increased chromatin decondensation and reduced sperm viability, particularly evident in cryopreserved samples (Peris-Frau et al. 2020). Modifying proteins linked to ram sperm quality by cryopreservation suggests that the equilibration phase may serve as a crucial control point (Arunkumar et al. 2022). These findings collectively underscore the impact of freezing and thawing procedures on ram sperm protein composition, potentially reducing the concentration of vital proteins essential for sperm survival and function.

The protein gel electrophoresis analysis of ram sperm yielded an intriguing comparison between fresh and frozen conditions (Fig. 3). At the 10kDa molecular weight, protein bands were detected in fresh sperm and absent in frozen sperm. At 14kDa, both conditions exhibited protein bands. At molecular weights 24, 25, 36, 44, 45, 46, 59, 60, 61, 74, 101-139, and 181-244kDa, fresh sperm displayed protein bands, while frozen sperm showed no bands at specific molecular weights (Table 4). These results suggest that sperm freezing may influence the protein pattern, with certain bands remaining undetected under freezing conditions. This finding holds crucial implications in sperm conservation and the reproductive success of rams utilizing semen freezing techniques.

Based on the literature review, several candidate proteins in fresh and post-thawing sperm from the ram were identified (Table 5). Proteins expressed across all age groups serve fundamental functions in sperm, including metabolism (glycolysis and the tricarboxylic acid cycle) utilized for motility, hyperactivation, capacitation, acrosome reaction, and fertilization.

The expression of the proteome, as depicted in Table 5, was revealed to exert a crucial role in governing various facets of sperm function, encompassing motility, metabolism, capacitation, and fertilization. Glycolysis and the oxidative phosphorylation (OXPHOS) pathways emerged as pivotal players in the intricate regulation of sperm metabolism, as evidenced by studies (Magdanz et al. 2019). Significant protein candidates linked with sperm metabolism included AK1 (21–24kDa), associated with the tricarboxylic acid cycle, and GPI (61–74kDa) and PGAM2 (25–34kDa), implicated in glycolysis (Peris-Frau et al. 2020). Specifically, GPI (61-74kDa) played a crucial role in the second phase of glycolysis, converting glucose-6-phosphate to fructose-6-phosphate, and collaborated with proteins such as ATP synthase and GAPDHS to support motility functions (Gomes et al. 2020). Glucose-6-phosphate isomerase (GPI), a key enzyme in glycolysis, proved to be a reliable predictor of human sperm freezability (Guo et al. 2019). The lower abundance of GPI in fresh ram semen compared to frozen semen suggests a potential correlation between GPI levels and ram sperm quality. GPI overexpression may be a protective mechanism against oxidative stress in semen, indicating its role in safeguarding sperm from oxidative damage (Kurkowska et al. 2020).

Furthermore, the association between GPI levels and sperm-washing methods was evidenced by GPI leakage during the procedures. However, its presence in sperm cytoplasmic droplets suggested a functional role in sperm function (Henning et al. 2021). Research exploring how compounds like palmitoleic acid impact sperm quality by enhancing ram spermatozoa's kinematics and antioxidative parameters has contributed to a deeper understanding of GPI's complex function (Eslami et al. 2017). The dynamic presence of GPI in frozen semen, its reduction in fresh ram semen, and its role in shielding sperm from oxidative stress collectively point to a multifaceted interaction between ram sperm quality and GPI levels. Beyond its potential as a marker for sperm health in ram semen, further investigation into the precise mechanisms underlying GPI's influence on sperm quality holds promise for enhancing ram fertility outcomes and semen preservation techniques.

Additionally, through glycolysis, the pivotal protein Phosphoglycerate Mutase 2 (PGAM2) was expressed in the energy metabolism pathway (Guo et al. 2019). According to Peña et al. (2022), PGAM2 catalyzes the glycolysis pathway's transition from 3-phosphoglycerate to 2-phosphoglycerate. In the cytosol of sperm, a process known as glycolysis converts sugar into the final products lactate and pyruvate (Magdanz et al. 2019). Following their diffusion into the mitochondria, these substrates served as

Table 5: Candidate sperm proteins based on molecular weight (Mol. Wt)

Sperm	Mol. Wt	Protein candidate	Location	Functions	References
Fresh and Frozen	10-14	Cytochrome C (CYT C)	Post acrosome and mid piece of the sperm	Capacitation and acrosome reaction	Rahman et al. (2017)
Fresh	20-24	Adenylate kinase isoenzyme 1 (AK1)	Mitochondria	tricarboxylic acid cycle - Motility	Luconi et al. (2004)
Fresh and Frozen	25	Phosphoglycerate mutase 2 (PGAM2)	Cytoplasm	Glycolysis pathway	Peris-Frau et al. (2020)
Fresh and Frozen	36-44	Clusterin (CLU)	Cytoplasm, mitochondria secreted	Protection against oxidative stress	Cheema et al. (2016)
Fresh and Frozen	45	Phosphoglycerate kinase 2 (PGK2)	Principal piece	Motility	Huang et al. (2017)
Fresh	46-59	Alpha-enolase (ENO 1)	Cytoplasm, Plasma membrane	Motility	Díaz-Ramos et al. (2012); Aslam et al. (2018)
Fresh and Frozen	60	T-complex protein 1 subunit 5 (CCT5)	Cytoplasm	Indicators of impaired spermatogenesis processes	D'Amours et al. (2010); Özbek et al. (2021)
Fresh	61-74	Glucose-6-phosphate Isomerase (GPI)	Cytoplasm	Glycolysis, Motility	Peris-Frau et al. (2020)
Fresh and Frozen	101-139	ATPase Na+/K+ transporting subunit alpha 4 (ATP1A4)	Plasma membrane	Motility, Capacitation	Rajamanickam et al. (2017)
Fresh	181-244	Sperm-associated antigen 17 (SPAG17)	microtubules	sperm flagellar	Kazarian et al. (2018)

the primary source of material for the tricarboxylic acid cycle (Costa et al. 2023). PGAM2 was found in both fresh and frozen ram sperm cells, and it was an essential enzyme for glycolysis. Converting glucose into pyruvate, which produces ATP and NADH and is necessary for sperm motility and successful fertilization, requires PGAM2. Using mass spectrometry, a proteomic analysis verified PGAM2's existence in ram sperm cells, highlighting the protein's significance in sperm cryopreservation. PGAM2 is essential for preserving sperm motility and viability after thawing, as well as for successfully storing, optimizing cryopreservation procedures, and enabling the efficient use of ram sperm for reproduction. Moreover, PGAM2 participated in the glycolytic pathway by interconverting 3-phosphoglycerate and 2-phosphoglycerate. It also produced reducing power, coordinated the synthesis of amino acids and nucleotide precursors, and produced energy. According to studies, PGAM2 controlled glycolysis and biosynthesis, which aided in the regulation of metabolism and the growth of tumors (Li et al. 2021).

The OXPHOS pathway's electron transport chain converts adenine into ATP with the help of the tricarboxylic acid cycle, which produces adenine, then moved to microtubules, enabling various sperm activities (Magdanz et al. 2019). AK1 (21–24kDa) plays a crucial role among the protein candidates connected to the tricarboxylic acid cycle pathway. AK1 is prominently present in the midpiece (proximal tail region) of sperm, visible in the outer dense fiber-outer microtubular doublet of the tail and mitochondria (Özbek et al. 2021). Responsible for maintaining equilibrium in the energy system, AK1 facilitates the transformation of two ADP molecules into a single molecule each of ATP and AMP (2ADP↔ATP+AMP) via a reversible transphosphorylation process (Fujisawa 2023). Sperm then utilize these adenine nucleotide molecules to adjust their motility (Freitas et al. 2016). Highly fertile cattle exhibit high levels of AK1 protein, active in sperm with high motility (Harayama et al. 2017; Kasimanickam et al. 2019). Fresh ram sperm cells express the protein Adenylate Kinase Isoenzyme 1 (AK1), as Özbek et al. (2021) reported. This ubiquitous enzyme, crucial for maintaining cellular energy homeostasis, in the flagella of bovine and murine sperm implies its role in sperm motility.

Abundant AK1 was found in spermatozoa from bulls with higher artificial insemination (AI) and fertility rates (Harayama et al. 2017). Furthermore, D'Amours et al. (2010) discovered that AK1 was more expressed in the high-fertility group than in the low-fertility group in a proteomic comparison of sperm proteins extracted using a detergent. Xie et al. (2020) also demonstrated AK1's importance for myocardial energetic homeostasis, neuronal maturation and regeneration, and cellular phosphotransfer networks. To understand how cryopreservation affects the presence of this protein, more research is necessary to comprehend the specific lack of AK1 in frozen ram sperm cells.

ATP1A4 (101-140kDa) and CYT C (10-14kDa) are pivotal protein candidates for various sperm functions, including motility, hyperactivity, capacitation, and the acrosome reaction. ATP1A4, also known as NKAα4, is abundantly produced by testis germinal cells and is prominently present in the sperm midpiece (Syeda et al. 2020). Its role in motility extends to controlling intracellular pH and calcium levels through secondary transport mechanisms (NCX and NHE), which is crucial for planning sperm migration (Rajamanickam et al. 2017; Syeda et al. 2020). Moreover, ATP1A4 actively participates in sperm capacitation through signal kinase activity mechanisms, underscoring its multifaceted involvement in essential sperm functions (Rajamanickam et al. 2017). The prevalence of ATPase Na+/K+ transporting subunit alpha 4 (ATP1A4) in spermatozoa underscores its critical regulatory role in ram sperm function and fertility. Elevated ATP1A4 expression correlates with improved sperm motility, which is essential for successful fertilization (Syeda et al. 2020).

Furthermore, CYT C is upregulated during the acrosome reaction and capacitation, facilitated by tyrosine phosphorylation pathways. Found in the apical region, post-acrosome, and midpiece of sperm, CYT C's presence suggests its involvement in critical sperm functions. Essential for cellular respiration, CYT C transfers electrons from the cytochrome bc1 complex to cytochrome c oxidase in fresh and frozen sperm cells. Additionally, CYT C's role in

triggering the intrinsic cell death pathway, apoptosis, highlights its critical involvement in cellular processes (Abramczyk et al. 2022). Further research is needed to elucidate CYT C's precise role in sperm function and fertility.

Sperm motility was significantly enhanced by the proteins PGK2 and ENO1, which generate ATP through glycolysis. PGK2, predominantly expressed in the testes, particularly in post-meiotic germ cells and sperm, converts 1,3-biphosphoglycerate and ADP into 3-phosphoglycerate and ATP. Its reduced expression in older human sperm and low expression in immature and asthenozoospermia sperm underscore its critical role in sperm motility, directly correlating with male fertility (Huang et al. 2017; Liu et al. 2019). Phosphoglycerate kinase 2 (PGK2) was detected in fresh and frozen ram sperm cells, consistent with its role in sperm metabolism and energy production. As a crucial component of the glycolytic pathway, PGK2 is indispensable for ATP production, the primary energy source for sperm motility. This study emphasizes the importance of glycolytic enzymes specific to sperm, like PGK2, in metabolism, protein phosphorylation, and sperm function (Huang et al. 2017). Understanding PGK2's activity and expression in the context of cryopreservation and assisted reproductive technologies is essential, as its presence in ram sperm cells supports sperm motility and viability through energy production and metabolism.

According to Díaz-Ramos et al. (2012) and Jiang et al. (2015), ENO1, abundant in the mature sperm tail, is indispensable for sperm function as it catalyzes the dehydration of 2-phosphoglycerate into phosphoenolpyruvate in the final stage of glycolysis, a critical step in ATP production. Its significance in successful reproduction is underscored by its notable expression in highly fertile cattle sperm and its identification as a fertility biomarker in cattle (Harayama et al. 2017; Aslam et al. 2018). Furthermore, it was discovered that melatonin supplementation in ram semen freezing extenders protected against cryopreservation injuries in a dose-dependent manner. This finding implies that cryopreservation may impact factors such as ENO1, necessitating further protective measures to maintain sperm function (Alevra et al. 2022). Given its critical role in cellular processes essential for sperm function, the absence of ENO1 in frozen ram semen raises concerns about sperm quality and fertility. Refining cryopreservation methods and protecting ram semen fertility require a thorough understanding of factors such as ENO1.

The investigation discovered more proteins linked to defense against oxidative stress-induced apoptosis, such as Clusterin (CLU / 36–44kDa) and CCT5 (60kDa), associated with abnormalities in the spermatogenesis developmental process. These results were consistent with earlier studies showing that male aging increased reactive oxygen species (ROS) production, leading to oxidative stress events in sperm (Aitken 2020). Cheema et al. (2016) found a correlation between the expression of the CLU protein and its ability to protect sperm from oxidative stress-induced apoptosis through the caspase cascade mechanism. According to research by Kant et al. (2019), there was a significant amount of CLU expression in the seminal plasma of elderly male humans compared to adults. Kasimanickam et al. (2019) found a similar amount of CLU expression in the sperm and seminal plasma of cattle with low fertility, implying that the sheep semen in the study may have already aged. Fresh and frozen ram sperm cells have been found to contain CLU, consistent with its known presence in seminal plasma and its function in various biological processes, such as sperm maturation, motility, and fertilization. Although Cheema et al. (2016) did not specifically address CLU in ram sperm cells in their study, which focused on the purification and characterization of heparin-binding proteins from the seminal plasma of crossbred cattle bulls, CLU could be identified in ram sperm due to general knowledge of its presence in seminal plasma. Furthermore, considering the similarities in reproductive physiology among mammals, the discovery of CLU in the seminal plasma of cattle suggested its potential presence in ram semen. The correlation between CLU and sperm motility and capacitation highlighted the various functions of CLU in sperm function. CLU's role in maintaining sperm quality during cryopreservation was demonstrated by its ability to protect against apoptosis, especially in oxidative stress (Cheema et al. 2016). Finding CLU in fresh and frozen ram sperm cells suggested that it might mitigate the adverse effects of cryopreservation on sperm viability and functionality, potentially improving fertility results.

A molecular chaperone called CCT5 plays a crucial role in protein folding, particularly during spermatogenesis and sperm function. Studies have consistently shown a strong correlation between CCT5 expression levels and male infertility, with lower levels of CCT5 associated with abnormal sperm morphology and decreased sperm motility. Furthermore, the presence of CCT5 in fresh and frozen ram sperm cells suggests its potential involvement in preserving sperm structure and function during cryopreservation, thereby contributing to maintaining the quality of post-thaw sperm (Ďuračka et al. 2023). Research has underscored the significance of TCP1 subunit 5 (CCT5), containing chaperonins, in various cellular processes. For instance, CCT5 plays a role in ciliogenesis by regulating vesicle transport to cilia through BBSome assembly (Wu et al. 2020). Additionally, studies have highlighted CCT5 and AK1 as predictors of a significant portion of the variation in male fertility, emphasizing their critical roles in successful reproduction (Kasimanickam et al. 2012). Furthermore, aberrations in protein folding and turnover can accumulate defective proteins in sperm, potentially compromising sperm function (Samanta et al. 2019). A comparison between fresh and cryopreserved bovine spermatozoa revealed the importance of CCT5 in correctly folding cytoskeletal proteins to preserve sperm structure and function (Ďuračka et al. 2023).

Proteomic analysis of frozen and fresh ram sperm cells has unveiled crucial insights into the intricate network of proteins governing various aspects of sperm function. GPI, PGAM2, AK1, ATP1A4, CYT C, PGK2, ENO1, CLU, and CCT5 are identified proteins essential for sperm motility, capacitation, and defense against oxidative stress. Their differential expression in frozen and fresh samples illustrates how cryopreservation impacts the sperm proteome, underscoring the need for further investigation to enhance ram fertility outcomes and optimize cryopreservation techniques. The study's findings enrich our understanding of sperm biology and offer new avenues for advancing assisted reproductive technologies in sheep breeding programs.

Conclusion

This study provided comprehensive information on the kinematics, candidate sperm proteins, protein composition, and characteristics of ram sperm. Properties of fresh ram semen, including volume, concentration, motility, viability, and abnormality, served as crucial markers of material quality, essential for fertility evaluations. Moreover, notable variations in motility and velocity parameters were observed in the kinematics of fresh and frozen ram sperm, suggesting that cryopreservation affects sperm quality. The analysis results indicated a significant decrease in protein concentration in frozen semen, suggesting biochemical changes due to cryopreservation. Additionally, protein gel electrophoresis revealed variations in protein patterns between fresh and frozen conditions, with implications for sperm conservation and reproductive success. Furthermore, identifying potential sperm proteins sheds light on the molecular processes governing sperm fertility and function. Overall, these findings advanced our understanding of ram sperm biology and provided valuable insights for enhancing artificial insemination methods and maintaining sperm quality.

Conflict of interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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Author's contribution

Jaswandi and Ananda analyzed the data and wrote the manuscript. Jaswandi, Sigit Prastowo, and Rini Widyastuti designed the concept, searched for funding, and compiled and reviewed the paper. Ananda oversaw field and laboratory work, conducted field and laboratory work, and performed data tabulation.

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