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Impacts of Cryopreservation on Semen Quality and Sperm Protein Profiles of Pesisir Bulls

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ABSTRACT

This study aimed to evaluate the impacts of cryopreservation on semen quality and sperm protein profile in Pesisir bulls. Semen samples were collected from three bulls and analyzed in fresh and post-thaw conditions. The sperm motility and kinematic variables were assessed using Computer-Assisted Sperm Analysis (CASA), while sperm viability and plasma membrane integrity (PMI) were evaluated through eosin-nigrosin staining and the hypoosmotic swelling test (HOST), respectively. Subsequently, the total protein concentration (PC) and profile were examined using SDS-PAGE. The results showed that there was a significant decrease in semen quality after thawing, with sperm motility reducing from 81.10% to 70.22%, viability reducing fom 87.47% to 77.27%, and PMI reducing from 85.09% to 71.32% ($p<0.05$). Kinematic variables such as velocity, straightness, and beat cross frequency also decreased significantly. Protein analysis showed a reduction in total concentration from 1.78 mg/mL to 1.19 mg/mL and alterations in protein band distribution, with the loss of specific high- and low-molecular weight after freezing. These results suggested that cryopreservation negatively impacts semen quality and sperm protein integrity, potentially impairing fertility. Moreover, further studies were recommended to optimize cryopreservation protocols and mitigate adverse effects.

Keywords: cryopreservation; Pesisir bull; semen quality; sperm protein profile

INTRODUCTION

Pesisir bulls (*Bos indicus*) are one of Indonesia's indigenous cattle breeds, which has the smallest population. These bulls are primarily found in West Sumatra and are well adapted to the local environment, playing a crucial role in the rural economy (Sutarno & Setyawan, 2016). As a valuable genetic resource, maintaining the fertility and genetic diversity of Pesisir bulls is essential to enhance productivity and ensure sustainable livestock farming practices. One of the key methods in improving livestock genetic quality is assisted reproductive technology, particularly artificial insemination (AI). However, the success of AI depends significantly on the quality of the frozen semen used. Semen from Pesisir bulls is rarely used due to economic and production limitations. Farmers often favor crossbreeding Pesisir females with larger breeds, such as Ongole or Bali cattle, to produce offspring with higher market value. Consequently, data on the fertility performance of Pesisir bulls in AI programs remains limited.

Despite the smaller size, Pesisir bulls show several advantageous traits, including adaptability to tropical climates, resistance to low-quality feed, and stable genetic characteristics that support reproductive efficiency and resilience (Putra *et al*., 2016). Compared to larger breeds, such as Bali and Brahman cattle, Pesisir bulls produce lower carcass weight and grow at a slower rate, limiting their commercial appeal (Agung *et al*., 2018; Hartatik *et al*., 2019). Furthermore, the genetic diversity is more limited than Bali or Madura cattle, which can impact long-term adaptability in various farming systems.

To promote the conservation and use of Pesisir bulls in breeding programs, an effective semen cryopreservation method must be developed. Cryopreservation is a widely used method for the long-term storage of genetic material, which is essential for animal reproduction and conservation programs (Rana *et al*., 2020; Shanmugam & Mahapatra, 2019). Despite the numerous advantages, the freezing and thawing processes in cryopreservation often cause structural and functional damage to sperm, reducing motility, viability, and PMI. Additionally, cryopreservation affects kinematic parameters that describe sperm motility patterns, thereby influencing overall quality (Costa *et al*., 2019; Zhang, 2018). These adverse effects are primarily attributed to the formation of ice crystals, osmotic stress, and oxidative stress, which impair sperm function and reduce fertility (Kadirvel *et al*., 2012; Ryu *et al*., 2019).

In addition to causing physical damage, cryopreservation is believed to alter the molecular

composition of sperm, particularly protein expression. Proteomic studies have shown significant changes in the quantity and distribution of protein after freezing. These changes affect proteins that are crucial for energy metabolism, motility, and membrane stability (Fu *et al*., 2020; Ma *et al*., 2021). Key proteins, such as aquaporins and heat shock, are essential in maintaining sperm motility and resistance to cryopreservation-induced stress (Fujii *et al*., 2018; Prieto-Martínez *et al*., 2017). However, modifications or damage to protein during cryopreservation can reduce sperm quality in local breeds such as Pesisir bulls. This shows varying levels of cryotolerance, which require further investigation.

Although cryopreservation technology has advanced, comprehensive studies evaluating the impact on semen quality and sperm protein profiles in Pesisir bulls are limited. Therefore, this study aimed to assess the impact of cryopreservation on semen quality and sperm protein composition in Pesisir bulls. By integrating sperm kinematics analysis with protein profile mapping through electrophoresis, an analysis was conducted to elucidate the mechanisms underlying cryodamage. The results provided valuable contributions to the development of optimized cryopreservation protocols to enhance semen quality, improve reproductive efficiency, and support the sustainability of AI programs as well as the conservation of local cattle genetic resources in Indonesia.

MATERIALS AND METHODS

Ethical Clearance

All procedures in this study were approved by the Research Ethics Committee of the Faculty of Medicine, Universitas Andalas (Approval No: 422/UN.16.2/KEP-FK/2024). Ethical guidelines were followed for the collection and handling of semen samples from the Pesisir bulls.

Sample Collection and Evaluation

Semen samples were collected from three mature Pesisir bulls aged 3–4 years (Figure 1) obtained from Unit Pelaksana Teknis Daerah Balai Pengembangan Teknologi dan Sumber Daya (UPTD BPTSD) Tuah Sakato, Payakumbuh, West Sumatra, Indonesia. Each bull underwent 15 semen collections conducted twice a week, resulting in a total of 45 ejaculates. Immediately after collection, the semen was macroscopically evaluated for volume, pH, color, and consistency based on the procedure by Arifiantini (2012). Microscopic evaluation was also performed to assess sperm concentration, viabilities, abnormalities, PMI, and kinematics. Fresh semen was divided into two equal portions, one half was directly evaluated and processed for sperm protein analysis. Meanwhile, the other half was cryopreserved for further evaluation. Post-thaw semen was evaluated microscopically and for sperm kinematic parameters.

Sperm concentration was manually determined using a Neubauer hemocytometer before further assessments. Subsequently, sperm viability and abnormality were assessed using eosin-nigrosin staining. This was followed by evaluating PMI using the Hypoosmotic Swelling Test (HOST) (Arifiantini, 2012). Sperm kinematics were analyzed using a Computer-Assisted Sperm Analyzer (CASA: Sperm Vision™ Version 3.7.5 software (Minitube, Tiefenbach, Germany)). CASA was used to evaluate kinematic parameters, including sperm total motility (%), progressive motility (PM) (%), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), and amplitude of lateral head displacement (ALH). Initially, the CASA system was mounted on an Olympus BX 51 microscope (Olympus, Tokyo, Japan) and configured for bovine sperm analysis. The camera operated at 60 frames per second, with the microscope equipped with a 20× objective lens and a 10× eyepiece to ensure precise kinematic measurements (Hendri *et al*., 2024; Wahyudi *et al*., 2023).

Cryopreservation and Thawing Protocol

Fresh semen was diluted in a Tris-egg yolk-based extender containing 6% glycerol, achieving a final concentration of 100 million sperm/mL. The concentration was determined using a Neubauer hemocytometer prior to dilution, and diluted semen was equilibrated at 5 °C for 4 hours, as recommended by Masrizal *et al*. (2025). After equilibration, the samples were loaded into 0.25 mL straws and frozen in liquid nitrogen vapor (-120 °C) for 10 minutes before being stored in liquid nitrogen (-196 °C). For thawing, semen straws were immersed in a 37 °C water bath for 20 seconds, based on the results of Hendri *et al*. (2024), before evaluation.

Protein Profiling

Both fresh and frozen semen samples were centrifuged (3000 rpm, 45 mins, 4 °C). For fresh samples, centrifugation was performed to separate sperm cells

Figure 1. Pesisir bulls used in this study, with identification numbers: A. KJ, B. JLT, C. UPT.

from the seminal plasma, while frozen samples were used to remove the extender. Sperm pellets were washed three times with phosphate-buffered saline (PBS) by centrifugation at 3000 rpm for 15 minutes at 4 °C, following a modified protocol from Jaswandi *et al*. (2024), to effectively remove residual seminal plasma and extender components. Subsequently, sperm were lysed using PROP-PREP™ Protein Extraction Solution (iNtRON Biotechnology) and incubated at -20 °C for 15 mins. After incubation, the lysate was centrifuged at 13,000 rpm for 5 mins at 4 °C to remove cellular debris, ensuring a purified protein extract for further analysis.

Protein was quantified using the Bradford Protein Assay Kit (E-BC-K168-S, Elabscience®), and the optical density was measured at 595 nm using a spectrophotometer (UV-1800, Shimadzu). This was followed by separation on a 10% gel (Q-PAGE™ Precast Gels, SMOBIO®) and visualization with Fast Coomassie Blue Staining Solution (E-IR-R129, Elabscience®). Molecular weights were estimated using the ExcelBand™ 3-color Broad Range Protein Marker (PM2700, SMOBIO®). Furthermore, protein bands were analyzed using ImageJ software, and molecular weights were calculated based on a regression curve of migration distance, as described by Azizah *et al.* (2023), using the regression curve: $\log(y) = -10.1x^3 + 20.025x^2 - 14.31x + 5.195$, where y represented protein molecular weight (kDa) and x was the retention factor (Figure 2).

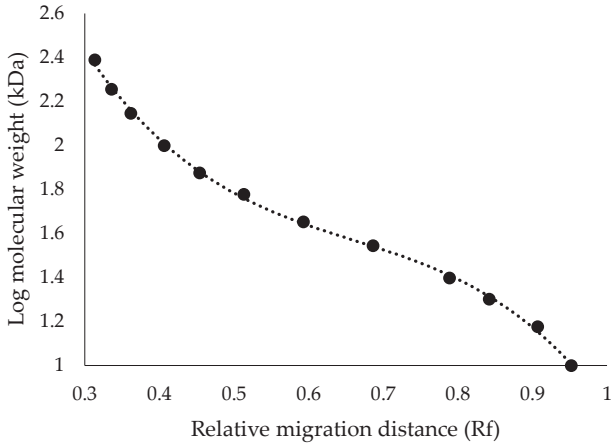

Figure 2. Regression curve for estimating the molecular weight (MW) of PM2700 protein marker bands

Data Analysis

Data were analyzed using SPSS version 25 (IBM Corp., Armonk, NY, USA). Paired t-tests were conducted to compare semen quality, sperm kinematic parameters, and total PC between fresh and post-thaw samples. Furthermore, protein band counts were analyzed descriptively. Pearson's correlation coefficients were calculated to assess the relationships between semen quality, sperm kinematics, and sperm PC. The results obtained were considered statistically significant at $p<0.05$.

RESULTS

Semen Quality of Pesisir Bulls

To assess the reproductive potential of Pesisir bulls, a comprehensive evaluation of fresh semen quality was conducted. The parameters observed were semen volume, pH, color, consistency, sperm mass movement, concentration, viability, abnormality, and PMI, with the results presented in Table 1.

The quality of fresh semen from the three Pesisir bulls (KJ, JLT, and UPT) showed consistent macroscopic and microscopic characteristics. The average semen volume was 3.94 ± 0.78 mL, with individual variations ranging from 3.84 ± 0.75 mL to 4.08 ± 0.77 mL, respectively. Meanwhile, the average semen pH was 6.59 ± 0.08, with a milky-white color and moderate consistency. The mass movement of sperm was graded as +2, showing good collective sperm motility. Microscopic analysis showed that fresh semen had a concentration of 1.86 ± 0.38 × 10^9 sperm/mL, with a viability rate of 87.47 ± 3.96% and an abnormal sperm count of 4.46 ± 0.40%. Additionally, PMI was at 85.09 ± 0.85%, showing that the sperm membrane remained in optimal condition.

Microscopic Quality, Sperm Kinematics, and Total PC in Fresh and Post-Thaw Pesisir Bull Semen

Microscopic quality and sperm kinematic parameters significantly decreased in Pesisir bull semen after cryopreservation (Table 2). Sperm viability, motility, and PMI decreased while abnormalities

Table 1. Fresh semen quality of Pesisir bulls

Variables	Bulls ID			Average ± SD
	KJ	JLT	UPT	
Semen volume (mL)	4.08±0.77	3.89±4.47	3.84±0.75	3.94±0.78
pH	6.8±0.08	6.59±0.08	6.59±0.09	6.59±0.08
Color	Milky-White	Milky-White	Milky-White	Milky-White
Consistency	Thin-Moderate	Thin-Moderate	Thin-Moderate	Thin-Moderate
Mass movement	++	++	++	++
Sperm concentration (10^9)	1.91±0.33	1.82±0.43	1.83±0.38	1.86±0.38
Sperm viability (%)	87.31±3.86	88.02±4.97	87.09±3.02	87.47±3.96
Sperm abnormality (%)	4.50±0.42	4.47±0.38	4.42±0.41	4.46±0.40
Sperm PMI (%)	85.17±0.88	85.06±0.85	85.02±0.89	85.09±0.85

Note: PMI=plasma membrane integrity.

Table 2. Comparison of microscopic quality and kinematics between fresh and frozen semen of Pesisir bulls

Variables	Semen	
	Fresh	Post-Thaw
Microscopics		
Sperm viability (%)	87.47±3,96[a]	77.27±6.99[b]
Sperm abnormality (%)	4.46±0.40[a]	5.09±0.85[b]
Sperm plasma membrane integrity (%)	85.09±0.85[a]	71.32±4.21[b]
Sperm kinematics		
Motility (%)	81.10±7.00[a]	70.22±9.30[b]
Progressive motility (%)	77.88±7.05[a]	57.39±7.63[b]
Distance average path (μm)	49.54±5.91[a]	28.19±10.78[b]
Distance curvilinear (μm)	77.87±7.97[a]	47.01±15.69[b]
Distance straight line (μm)	41.62±6.25[a]	22.35±11.43[b]
Velocity average path (μm/s)	115.93±13.63[a]	65.76±25.70[b]
Velocity curvilinear (μm/s)	181.87±18.40[a]	109.39±38.01[b]
Velocity straight line (μm/s)	97.83±14.4 [a]	52.33±27.24[b]
Straightness (%)	0.84±0.04[a]	0.77±0.07[b]
Linearity (%)	0.53±0.05[a]	0.46±0.07[b]
Wobble (%)	0.63±0.04[a]	0.59±0.04[b]
Amplitude of lateral head displacement (μm/s)	5.82±0.63[a]	4.87±0.80[b]
Beat cross frequency (hz)	35.36±3.70[a]	26.23±7.59[b]

Note: Different superscripts on the same row show differences in each treatment ($p<0.05$).

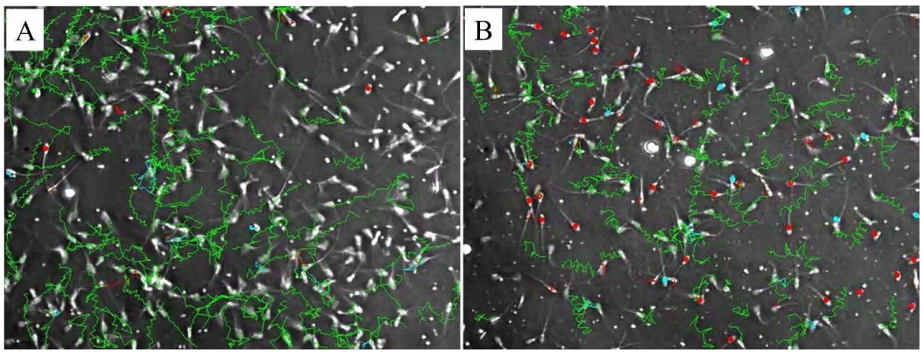

Figure 3. Motility of Pesisir bull sperm was analyzed using CASA. A: Fresh semen; B. Post-thaw semen.

increased slightly, showing a reduction in the overall quality. Furthermore, sperm viability declined from 87.47% to 77.27%, and motility dropped from 81.10% to 70.22% ($p<0.05$). PM and PMI also showed significant reductions. Kinematic analysis showed a significant decrease in VCL, VSL, and VAP post-thaw. Additionally, parameters such as STR, LIN, and BCF were found to be considerably reduced.

Motility analysis using CASA showed a significant reduction after cryopreservation. In fresh semen samples (Figure 3A), there was an indication of active and progressive movement, as demonstrated by dense and continuous green tracking lines. In comparison, post-thaw semen (Figure 3B) showed fewer and shorter motility tracks, reflecting a significant decrease in both motility and progressive movement. Furthermore, the study observed a significant decrease in the total PC in Pesisir bull sperm after cryopreservation. The average sperm total PC of fresh semen samples was recorded as 1.78 ± 0.39 mg/mL. Meanwhile, post-thaw samples showed a substantial decrease to 1.19 ± 0.18 mg/mL ($p<0.05$), as shown in Table 3.

Table 3. Sperm total protein concentration in fresh and post-thaw Pesisir bull semen

Semen	Sperm protein cocentration (mg/mL)	SD
Fresh	1.78	0.39
Post-Thaw	1.19	0.18

Correlation Between Microscopic Quality, Sperm Kinematic Parameters, and Total PC

Figure 4 presents heatmaps showing the correlations between microscopic quality parameters, kinematic variables, and total PC in fresh (left) and post-thaw (right) Pesisir bull sperm. The color gradient represents the strength and direction of correlations, where red shows positive correlations and blue indicates negative correlations, with intensity reflecting magnitude. In fresh semen, strong positive correlations were observed among several kinematic parameters, particularly between VAP, VCL, and VSL. This suggested that enhanced motility characteristics were interrelated. A similar trend was observed in post-thaw samples but with reduced correlation strength. This

showed that while motility-related parameters remain interlinked after freezing, cryopreservation negatively impacted overall movement. Total PC showed a moderate positive correlation with motility (Mot) and PM in both fresh and post-thaw semen, reinforcing the link between protein presence as well as quality.

In post-thaw semen, the correlations weakened, but the general patterns remained similar. Sperm viability (Via) and abnormalities (Abn) consistently correlated negatively. This showed lower viability was inherently associated with higher abnormalities, regardless of the cryopreservation process. However, post-thaw samples showed an increase in negative associations and a reduction in the correlation strength of motility parameters, implying a diminished functional capacity due to cryopreservation effects. Based on the results, the heatmaps underscore the subtle but significant influence of cryopreservation on sperm motility and protein dynamics in Pesisir bulls.

Protein Band Distribution Based on Molecular Weight in Fresh and Post-Thaw Pesisir Bull Sperm

SDS-PAGE analysis showed significant differences in sperm protein band distribution between fresh and post-thaw Pesisir bull semen. Fresh samples showed eight distinct protein bands, while post-thaw had only six bands (Table 4 and Figure 5). Protein with molecular weights of 60–75 kDa and >245 kDa was detected exclusively in fresh samples but absent in post-thaw semen (Table 5).

DISCUSSION

The semen quality of Pesisir bulls showed optimal physical and microscopic characteristics, with appropriate volume, pH, and consistency, serving as a suitable option for cryopreservation (Table 1). High sperm viability and low sperm abnormality percentages show strong reproductive potential influenced by age, physiology, and environment. Afriani *et al*. (2022) reported that bulls aged 36-48 months, weighing approximately 348 kg and with a scrotal circumference of 32 cm, showed superior semen quality. According to Isnaini *et al*. (2022), semen volume and sperm motility typically peak at approximately 7 years of age, followed by a gradual decrease. Comparisons between breeds also show that although fresh sperm motility tends to be stable, post-thaw samples vary significantly (Saranholi *et al*., 2021).

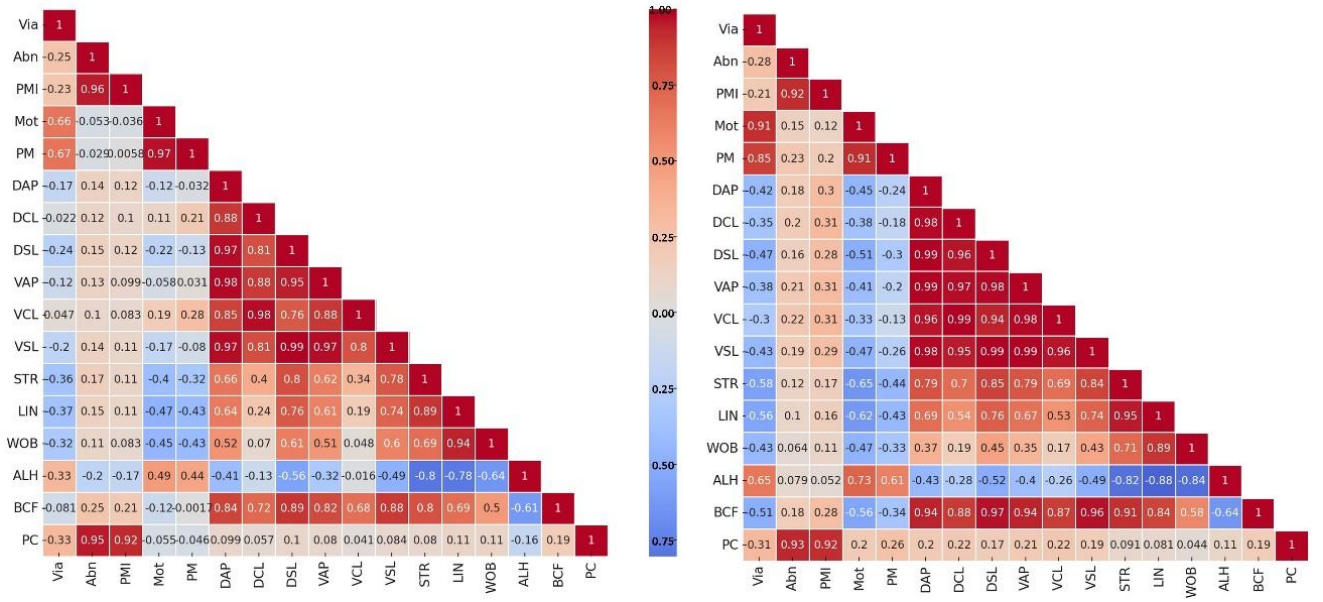

Figure 4. Heatmap of correlations between microscopic quality, sperm kinematic variables, and sperm total protein concentration in fresh (left) and post-thaw (right) Pesisir bull semen. The color scale shows the strength and direction of correlations, with red representing positive and blue representing negative correlations. Via=viability; Abn=abnormality; PMI=plasma membrane integrity; Mot=motility; PM=progressive motility; DAP=distance average path; DCL=distance curvilinear; DSL=distance straight line; VAP=velocity average path; VCL=velocity curvilinear; VSL=velocity straight line; STR=straightness; LIN=linearity; WOB=wobble; ALH=amplitude of lateral head displacement; BCF=beat cross frequency; PC=total protein concentration.

Table 4. Sperm protein band distribution based on molecular weight in fresh and post-thaw Pesisir bull semen

Semen	Molecular weight (kDa)												Number of sperm protein bands
	10-15	15-20	20-25	25-35	35-45	45-60	60-75	75-100	100-140	140-180	180-245	>245	
Fresh	-	-	+	-	+	++	+	+	+	-	-	+	8
Post-thaw	-	-	-	+	+	+	+	-	+	-	+	-	6

Note: ++=present (two bands); +=present (one band); -=absent.

In addition, several other factors contribute to semen quality, such as environmental conditions. Isnaini *et al*. (2022) found that climate change did not impact the semen quality of Madura bulls, one of Indonesia's local breeds. This suggested that Madura bulls had a higher degree of resilience to climate change than Pesisir bulls. Moreover, further studies were recommended to investigate the impact of climate, temperature, and other environmental conditions on the semen quality of Pesisir bulls. It is important to determine strategies that can be implemented to maximize reproductive potential. Semen quality is influenced by internal factors such as testosterone levels. Mahmoud *et al*. (2021) reported a robust correlation between testosterone levels and semen properties in buffaloes. Based on the analysis, it was observed that the combination of analytical tools, such as CASA, including treatments related to internal and external factors on the quality of Pesisir bull sperm, should be investigated in the future.

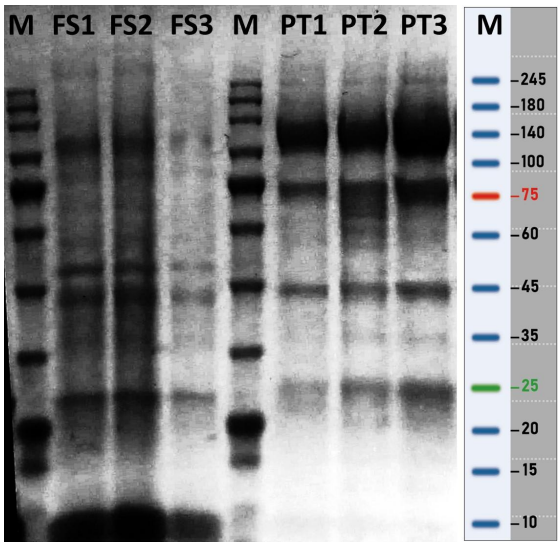

Figure 5. SDS-PAGE profile of sperm protein bands in fresh (FS1–FS3) and post-thaw (PT1–PT3) Pesisir bull semen. M=Marker.

Cryopreservation negatively affected the viability, motility, PMI, and protein profile of Pesisir bull semen (Table 2, Figure 3). The integrity of the sperm plasma membrane and PM experienced the same outcomes. Moreover, total PC decreased, showing that protein was degraded or lost due to cryodamage (Table 3). Cryopreservation disrupts the proteomic and kinematic properties of sperm. According to previous reports, freezing and thawing processes can change the expression of protein in energy metabolism and motility, which are critical for the functionality of post-thaw semen (Perez-Patiño *et al*., 2019; Wang *et al*., 2014). For instance, sperm resistance to freezing is related to the expression of heat shock protein (HSP), such as HSP90. This suggested that protein serves a protective function during cryopreservation (Wang *et al*., 2014).

Cryopreservation affects sperm protein profiles, morphology, and kinematic parameters such as VCL, VSL, and VAP, significantly decreasing post-thaw. This decrease is associated with reduced motility and fertility potential, limiting successful fertilization (Carreira *et al*., 2017; Fayyaz *et al*., 2022). Sperm with more abnormalities in the head, midpiece, and tail post-thaw can also fertilize less (Chaturvedi *et al*., 2022; Yoon *et al*., 2022). Therefore, an accurate assessment of sperm movement is carried out using CASA to measure motility parameters (Nagy *et al*., 2015;

Table 5. Identified sperm protein candidates, locations, and functions in fresh and post-thaw pesisir bull semen

Semen	MW (kDa)	Sperm protein candidate	Location	Function	References
Fresh	20-25	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Cytoplasm	Energy metabolism	Margaryan *et al*. (2015)
Post-Thaw	25-35	Annexin A5, Malate dehydrogenase 2 (MDH2)	Plasma membrane, Mitochondrial matrix	Membrane repair, motility	Ali *et al*. (2023); Byrne *et al*. (2012); Özbek *et al*. (2021)
Fresh and Post-Thaw	35-45	Tubulin, Clusterin (CLU)	Cytoskeleton, Cytoplasm	Maintains structural integrity, oxidative stress protection	Byrne *et al*. (2012); Cheema *et al*. (2016)
Fresh and Post-Thaw	45-60	Alpha-enolase (ENO1), Phosphoglycerate kinase 2 (PGK2)	Plasma membrane, Principal piece	Glycolytic enzyme for motility, ATP production	Díaz-Ramos *et al*. (2012); Huang *et al*. (2017); Özbek *et al*. (2021)
Fresh and Post-Thaw	60-75	Heat shock protein 70-2 (HSP70-2), T-complex protein 1 subunit 5 (CCT5)	Cytoplasm	Stress protection, spermatogenesis regulation	D'Amours *et al*. (2010); Rosyada *et al*. (2023)
Fresh	75-100	AKAP4, Aconitate 2 (ACO2)	Flagellum, Mitochondria	Regulates motility, tricarboxylic acid cycle	Luconi *et al*. (2004); Peris-Frau *et al*. (2020)
Fresh and Post-Thaw	100-140	T-complex protein 11 (TCP11), ATPase alpha 4 (ATP1A4)	Testis-specific, Plasma membrane	Regulates sperm morphology, motility, and capacitation	Liu *et al*. (2011); Rajamanickam *et al*. (2017); Castaneda *et al*. 2020)
Post-Thaw	180-245	Voltage-dependent anion channel (VDAC)	Mitochondria	Regulates mitochondrial function	Nibali *et al*. (2024)
Fresh	>245	Dynein heavy chain	Flagellum	Essential for flagellar motility	Byrne *et al*. (2012); Harima *et al*. (2025)

Note: MW= Molecular weight.

Oliveira *et al*., 2012). The results show that fresh and post-thaw samples have distinct expression patterns for protein included in motility and membrane integrity. For instance, proteins in the 35–50 kDa range related to enhanced motility in fresh sperm are significantly decreased (Baharun *et al*., 2024). The role of cryoprotectants and antioxidants in preserving sperm quality and minimizing cryodamage during freezing has been the subject of numerous studies (Alkhawagah *et al*., 2022; Fayyaz *et al*., 2022).

According to Hendri *et al*. (2024), thawing at 37 °C for 20 seconds produced optimal total and PM as well as superior sperm kinematics in comparison to 25 °C. Masrizal *et al*. (2025) reported that a 4-hour equilibration period before freezing provided an optimal balance between motility, viability, abnormality, and PMI, although longer equilibration could lead to increased abnormalities. These results emphasize the importance of optimizing thawing temperatures and equilibration periods to improve the quality of frozen Pesisir bulls' semen and the success of AI. The intricate interplay between microscopic quality, kinematic parameters, and total PC in sperm underscores the need for optimized cryopreservation strategies. Comprehending these relationships is essential for refining semen preservation methods and enhancing fertility outcomes in bovine reproduction.

The results of the correlation analysis showed strong positive relationships between viability, motility, and PMI with kinematic parameters (VCL, VSL, VAP) in fresh samples (Figure 4). Furthermore, total PC correlated positively with motility, viability, and membrane integrity. This showed the PC's role in energy metabolism and membrane stability, which is essential for sperm functionality. Based on the results, higher protein levels, viability, and intact membranes enhanced sperm motility and fertilization potential. However, these correlations showed a decrease post-thaw, as freezing caused a reduction of PC from 1.78 ± 0.39 mg/mL to 1.19 ± 0.18 mg/mL (Table 3), underscoring protein degradation and the corresponding effect on semen quality.

Previous studies have shown that HSPs and tubulin are vital for protecting sperm from oxidative stress and maintaining structural integrity during cryopreservation (Perez-Patiño *et al*., 2019; Wang *et al*., 2014). Post-thaw loss or downregulation of protein has also been shown to impair mitochondrial function and energy metabolism, thereby reducing motility and viability (Baharun *et al*., 2024; Carreira *et al*., 2017). Furthermore, protein expression is closely correlated with kinematic parameters, with high-fertility bulls expressing more motility- and metabolism-related protein, enhancing velocity and motility (Wang *et al*., 2014).

Marques *et al*. (2023) further observed that VCL and linearity (LIN) variations are key fertility indicators. Cryopreservation disrupts protein integrity, reducing the expression of fertility-related proteins essential for energy metabolism and motility. Morrell *et al*. (2018) and Somashekar *et al*. (2015) showed the need to assess post-thaw structural and protein integrity to improve reproductive outcomes. Significant correlations between microscopic quality, sperm kinematic parameters, and sperm total PC in fresh Pesisir bulls' semen underscore the role of protein integrity in maintaining functionality. Optimized preservation strategies, including tailored thawing protocols and cryoprotectants, are for quality preservation and enhancing fertility in Pesisir bulls.

The analysis of protein band distribution in fresh and post-thaw Pesisir bull sperm using SDS-PAGE showed significant differences in the number and intensity of protein bands. This showed that cryopreservation altered the protein profile of sperm cells. In this study, fresh semen showed more distinct protein bands than post-thaw samples, suggesting the loss or degradation of specific proteins during the freezing and thawing process (Figure 4). Protein bands with molecular weights of 60–75 kDa and >245 kDa were exclusively present in fresh samples but were absent in post-thaw semen. This loss showed that protein was highly susceptible to cryodamage and was critical in maintaining semen integrity and function.

Table 5 shows a comprehensive identification of protein candidates and their roles in sperm functionality. For instance, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (20–25 kDa), present exclusively in fresh samples, is for energy metabolism (Margaryan *et al*., 2015). Protein such as annexin A5 and malate dehydrogenase 2 (MDH2) (25–35 kDa), identified in post-thaw sperm, contributes to membrane repair and motility, showing adaptive responses to cryodamage (Ali *et al*., 2023; Byrne *et al*., 2012). Structural protein, including Tubulin and Clusterin (CLU) (35–45 kDa), is also essential for maintaining sperm structural integrity and providing protection against oxidative stress (Byrne *et al*., 2012; Cheema *et al*., 2016). Furthermore, enzymes included in energy production, such as Alpha-enolase (ENO1) and Phosphoglycerate kinase 2 (PGK2) (45–60 kDa), play crucial roles in ATP synthesis essential motility (Huang *et al*., 2017; Satrio *et al*., 2024). Stress-related protein, including Heat shock protein 70-2 (HSP70-2) and T-complex protein 1 subunit 5 (CCT5) (60–75 kDa), is essential for protecting against oxidative and thermal stress during cryopreservation (Rosyada *et al*., 2023).

Structural and motility-related proteins, including A-Kinase Anchoring Protein 4 (AKAP4) and Aconitate Hydratase 2 (ACO2) (75–100 kDa), have been identified as essential components for mitochondrial function and flagellar movement (Jumeau *et al*., 2018). Moreover, T-complex protein 11 (TCP11) and ATPase alpha 4 (ATP4A) (100–140 kDa) have been found to regulate sperm energy metabolism and capacitation (Castaneda *et al*., 2020; Liu *et al*., 2011). High molecular weight type, such as the Voltage-dependent anion channel (VDAC) (180–245 kDa), is included in mitochondrial bioenergetics (Hinsch *et al*., 2004; Shoshan-Barmatz *et al*., 2015). In line with previous reports, protein >245 kDa is essential for maintaining the structural framework of the flagellum (Shoshan-Barmatz *et al*., 2015).

Proteomic studies have shown that the cryopreservation process can lead to significant alterations in protein expression. This is particularly observed in proteins in critical processes such as

energy production, motility, and membrane stability (Ali *et al*., 2023; Mostek *et al*., 2018). The degradation or downregulation process has been shown to weaken sperm capacity to generate ATP and maintain membrane fluidity needed for motility and fertilization (Bogle *et al*., 2017; Harayama *et al*., 2010). Additionally, as analyzed using ImageJ, the reduction in band intensity in post-thaw samples underscores the extent of degradation. The partial loss of functional protein directly impacts sperm quality. Costa *et al*. (2023) emphasized that mitochondrial membrane integrity is crucial for ATP production, and its disruption leads to oxidative damage and reduced sperm function.

The results align with previous studies showing that cryopreservation has a detrimental effect on the proteome, causing the loss of essential protein required for fertilization. The implementation of strategies such as antioxidant supplementation and the optimization of cryoprotectant formulations has been shown to minimize protein degradation during freezing (Alkhawagah *et al*., 2022; Fayyaz *et al*., 2022). The significant disparities in sperm protein band distribution between fresh and post-thaw semen underscore the vulnerability of specific proteins to cryodamage. Therefore, protecting critical proteins implicated in energy metabolism and motility is essential to preserving semen quality. In this context, further studies on targeted cryoprotective strategies are essential to mitigate protein degradation and enhance the fertility outcomes of cryopreserved semen.

CONCLUSION

In conclusion, this study showed that cryopreservation significantly reduced semen quality and sperm protein integrity in Pesisir bulls, impairing motility, viability, and membrane stability. The results showed that the decrease in total PC and altered profiles suggested affected sperm function. Therefore, optimizing cryopreservation protocols, including improved thawing conditions and cryoprotectant use, was considered essential to preserve fertility and genetic resources.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest with any financial, personal, or other relationships regarding the material discussed in this study.

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