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Sperm Quality, Kinematics, Membrane Integrity, and DNA Fragmentation of Frozen Sexed Semen in Holstein-Friesian Bulls

F. Safa[a], A. P. A. Yekti[a], P. Utami[a], H. A. Syah[a], N. Febrianto[a], A. Rachmawati[a], A. Yuswati[a], A. Amaliya[b], D. Sulistyowati[b], & T. Susilawati[a,*]
[a]Department of Animal Science, Universitas Brawijaya
Veteran Street, Universitas Brawijaya Malang 65145, East Java, Indonesia
[b]Singosari Artificial Insemination Center, Malang, Indonesia
*Corresponding author: tsusilawati@ub.ac.id
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ABSTRACT

Percoll density gradient centrifugation (PDGC) is a widely used method for sperm separation in Indonesia. This study evaluated sperm quality, membrane integrity, acrosome damage, and DNA fragmentation after PDGC-based sperm sexing and cryopreservation. This research was conducted as a laboratory experiment with six treatment groups: fresh semen (T1), post-sexing X-chromosome-bearing sperm (T2), post-sexing Y-chromosome-bearing sperm (T3), post-thawing unsexed semen (T4), post-thawing X-chromosome-bearing sperm (T5), and post-thawing Y-chromosome-bearing sperm (T6). The observed variables included individual motility (IM), viability, concentration, abnormality, total motile sperm (TMS), intact acrosome cap (IAC), membrane integrity (MI), DNA fragmentation, and sperm kinetic variables assessed using computer-assisted sperm analysis (CASA). The results showed that PDGC sexing significantly reduced IM, concentration, TMS, viability, MI, and IAC. Furthermore, the freezing of both unsexed and sexed semen further decreased IM, concentration, TMS, viability, MI, and IAC while increasing sperm abnormalities. DNA fragmentation increased post-sexing and freezing in unsexed semen, indicating potential DNA damage from these processes. Kinetic variable assessment using CASA showed that PDGC maintained sperm motility characteristics, whereas freezing led to a significant decline in sperm kinetics. In conclusion, PDGC is a viable method for sperm sexing in cattle, as it preserves sperm quality, membrane integrity, acrosome integrity, DNA integrity, and motility characteristics while being compatible with semen cryopreservation. These findings confirm that PDGC-processed semen is suitable for artificial insemination (AI).

Keywords: DNA fragmentation; intact acrosome cap; membrane integrity; percoll density gradient centrifugation

INTRODUCTION

Artificial insemination (AI) is a widely adopted biotechnology to improve genetic quality and expand livestock populations (Yekti *et al*., 2024). Artificial Insemination has advanced by incorporating sperm sexing technologies, which enable the separation of X- and Y-chromosome-bearing sperm (Uhm *et al*., 2023). Various methods have been developed for sperm sexing (Singh *et al*., 2019); however, in Indonesia, percoll density gradient centrifugation (PDGC) is one of the most commonly used sexing techniques due to its cost-effectiveness and proven efficacy in sperm separation (Resende *et al*., 2019). Susilawati (2014) reported that sperm sexing using PDGC with ten gradient layers and centrifugation speeds of 2250 and 1500 rpm resulted in 83% X-chromosome recovery. Yekti *et al*. (2023) further showed that post-thawing, PDGC-sexed sperm exhibited a motility rate of 45.42%, viability of 72.71%, abnormality rate of 6.7%, concentration of 28.27 million per straw, intact acrosome cap (IAC) of 74.70%, and chromatin damage of 7.7%. Despite these characteristics, the overall quality of sexed semen remained lower than that of unsexed semen. Reese *et al*. (2021) showed that AI using sexed semen resulted in a lower conception rate (43.9%) compared to unsexed sperm (56.1%). However, applying percoll gradient-selected frozen semen in dairy farms has increased the proportion of female offspring, reaching 81.25% (Yekti *et al*., 2023) and 71.4% (Promthep *et al*., 2016).

Semen quality is crucial for determining fertility rates (Khan *et al*., 2024). The use of low-quality sperm has been shown to result in reduced pregnancy rates, subsequently increasing the economic burden on farmers (Parisi *et al*., 2014). The sperm separation and cryopreservation processes have been observed to contribute to a decline in semen quality (Le *et al*., 2019; Susilawati, 2014). Cryopreservation causes ultrastructural injuries to sperm, oxidative and osmotic stress, reduced motility and viability, impaired

mitochondrial function and tail integrity, and increased DNA fragmentation (Khan *et al*., 2021).

Sperm quality assessment is based on multiple parameters, including individual motility, viability, abnormal morphology, concentration, and total motile sperm count. Evaluations of membrane integrity and IAC are conducted to enhance the overall assessment of semen quality. Several studies have confirmed a strong correlation between sperm quality parameters and fertility outcomes (Hallap *et al*., 2004; Gillan *et al*., 2008). The integrity of the sperm plasma membrane, acrosome, and DNA plays a vital role in determining sperm function, maintaining homeostasis, and ensuring the successful delivery of genetic material to the oocyte (Bollwein & Malama, 2023). Computer-assisted sperm analysis (CASA) has emerged as a reliable method for evaluating semen content due to advancements in reproductive biotechnology. Kathiravan *et al*. (2008) demonstrated that specific sperm motion characteristics analyzed using CASA, such as progressive motility and velocity parameters (e.g., path velocity and progressive velocity), can serve as predictive fertility indicators.

Sperm DNA fragmentation (SDF) is a critical factor influencing fertilization success and the development of healthy offspring (Agarwal *et al*., 2020). Le *et al*. (2019) reported that DNA damage in sperm could arise from various handling and processing treatments. Although sperm with fragmented DNA may still achieve fertilization, they are associated with an increased risk of pregnancy failure (Gosalvez *et al*., 2011). Moreover, Karoui *et al*. (2012) confirmed that DNA fragmentation significantly affects the outcomes of AI, with SDF levels between 7% and 10% in bulls indicating lower AI success rates. Despite extensive research on sperm DNA fragmentation, limited information is available regarding the effects of the PDGC method on bovine sperm DNA integrity (Demir *et al*., 2019). Furthermore, the extent to which cryopreservation influences DNA fragmentation remains unclear. Therefore, this study aimed to evaluate the differences in sperm quality, membrane integrity, intact acrosome cap, and DNA fragmentation following PDGC-based sperm sexing and freezing.

MATERIALS AND METHODS

This study was designed as a laboratory experiment utilizing a randomized block design. Semen samples were collected from five Holstein-Friesian bulls: Conan (5 years, 861 kg), Silver (2 years, 981 kg), GW Amish (2 years, 698 kg), Mate (2 years, 730 kg), and Master (2 years, 580 kg). All bulls were maintained at the Singosari Artificial Insemination Center (SAIC). Fresh semen samples were collected twice a week. Sperm were evaluated at six stages: fresh semen (T1), post-sexing X-chromosome-bearing sperm (T2), post-sexing Y-chromosome-bearing sperm (T3), post-thawing unsexed semen (T4), post-thawing X-chromosome-bearing sperm (T5), and post-thawing Y-chromosome-bearing sperm (T6).

Ethical Approval

This study received approval from the Health Research Ethics Committee at the Faculty of Medicine, Brawijaya University (ethical clearance number 48/EC/KEPK/12/2024).

Semen Processing

Semen in this study was processed following the protocol by SAIC (Prastiya *et al*., 2024). Semen was collected using an artificial vagina (AV; IMV Technologies, France). Only ejaculates containing more than 70% progressively motile sperm were selected for further processing. Sperm concentration was measured using a photometer (SDM 6, Minitube, Germany). The semen diluent consisted of Tris-aminomethane supplemented with 20% egg yolk. Both sexed and unsexed sperm were cooled to 5 °C for 22 hours before further processing. After cooling, sperm were packaged in 0.25 mL straws at 5 °C using a fully automated straw filling and sealing machine (MPP Quattro, Minitube, Germany). The straws were frozen in liquid nitrogen vapor at −140 °C for 12 minutes, then plunged into liquid nitrogen (−196 °C) and stored for at least 24 hours before analysis. Thawing was performed in a water bath (Memmert, Germany) at 37 °C for 30 seconds before evaluation.

Percoll Density Gradient Centrifugation

The sexing method applied in this study was PDGC based on the protocol developed by SAIC, a modified version of the sexing method described by Susilawati (2014). The sexing procedure was performed using a 10-gradient percoll medium (Sigma Aldrich, St. Louis, MO, USA) with concentrations ranging from 20% to 60%. A 0.5 mL portion of each gradient was arranged in test tubes from the highest to the lowest concentration (starting from the bottom), with a total gradient volume of 5 mL. A 2 mL semen sample was layered on top of the gradient and centrifuged (Hettich, Germany) at 291 × *g* for 7 minutes. Following centrifugation, 0.5 mL of the uppermost fraction (containing immotile sperm) was discarded. The remaining fractions were separated into 1.5 mL of the upper fraction (Y-chromosome-bearing sperm) and 1.5 mL of the lower fraction (X-chromosome-bearing sperm). Each fraction was mixed with 3 mL of Tris-aminomethane egg yolk diluent and then underwent a second centrifugation at 148.1 × *g* for 5 minutes. The supernatant was discarded, and the remaining sperm pellet was resuspended in Tris-aminomethane egg yolk diluent with the addition of glycerol. The sperm pellet was then collected for subsequent processing: cooling, filling, sealing, freezing, and thawing.

Variables

Individual motility. Individual motility (IM) was assessed using a modified method by De Jarnette *et al*. (2021). A 10 µL semen sample was placed on a

glass slide (One Lab, China) and covered with a glass coverslip (Herma, Germany). Furthermore, the sample was examined under a light microscope (Olympus CX-23, Japan) at 400× magnification across five fields of view. Motility was recorded as a percentage, rounded to the nearest 5%, and subsequently averaged.

Viability. Sperm viability was assessed according to Wysokińska *et al*. (2023) using eosin nigrosine staining. A 10 µL semen sample was mixed with eosin nigrosine solution (1:1) and smeared onto a glass slide. The slides were air-dried for 30 seconds before evaluation. Two hundred sperm were analyzed under a light microscope at 400× magnification. Sperm with pink-stained heads were classified as non-viable (dead), while those with unstained, colorless heads were classified as viable (live).

Abnormality. Sperm morphological abnormalities were evaluated using a modified method by Bernečić *et al*. (2021), employing the same smear slides prepared for the viability test. Abnormalities were categorized into head, acrosome, and mid-piece defects. Two hundred sperm were examined under a light microscope at 400× magnification to determine the percentage of abnormal sperm.

Concentration. The concentration of fresh semen and sexed sperm was determined using a photometer (SDM 6, Minitube, Germany). The concentration of sperm post-thawing was assessed following a modified method by Mahendra *et al*. (2016). A 10 µL sample of semen was diluted with 990 µL of physiological NaCl solution and homogenized in an Eppendorf tube (Onemed, Indonesia). From this suspension, 8 µL was loaded into a Neubauer counting chamber (Marienfield, Germany), which was covered with a glass coverslip. Sperm concentration was determined by counting the sperm in five large squares of the counting chamber: four in the corners and one in the center. Observations were performed in the upper (A) and lower (B) chambers. The sperm concentration was calculated using the following formula:

$$\text{Sperm concentration} = N \times FP \times 5 \times 10{,}000$$

Where N was the average number of sperm counted in chambers A and B, FP was dilution factor (1:100), 5 was the correction factor for counting five squares out of 25, and 10.000 was the correction factor for the chamber depth (0.0001 mL).

Membrane integrity. Membrane integrity (MI) was assessed using the method described by Baity *et al*. (2024). A 10 µL sperm sample was diluted with 100 µL of hypo-osmotic solution, prepared by dissolving 0.9 g of fructose (Merck, Israel) and 0.49 g of sodium citrate (Merck, Germany) in distilled water to a final volume of 100 mL. The suspension was then incubated in a water bath at 37 °C for 60 minutes. A drop of the semen suspension was placed on a glass slide, covered with a glass coverslip, and examined under a light microscope (Olympus BX-33, Japan) at 400× magnification. A total of 200 sperm were evaluated, with intact membranes identified by the presence of a swelling reaction in the circular tail end.

Intact acrosome cap. The assessment of the intact acrosome cap (IAC) was performed following a modified method by Tethool *et al*. (2022) with Giemsa stain (Merck, Germany). Sperm smears were air-dried and fixed in 5% formalin (Merck, Germany) at 37 °C for 5 minutes, then washed with distilled water and air-dried again. The smears were stained with a Giemsa solution prepared by mixing 3 mL of Giemsa stain, 2 mL of phosphate-buffered saline, and 35 mL of distilled water. This solution was incubated at 37 °C for 4 hours. After staining, the slides were washed with distilled water and air-dried. Two hundred sperm were evaluated under a light microscope at 400× magnification. A dark purplish-stained head identified sperm with intact acrosomes, while sperm with damaged acrosomes exhibited a light purple-stained head.

Sperm DNA fragmentation. Sperm DNA Fragmentation (SDF) analysis was conducted using the Halomax kit (Halotech HT-BT40, Spain), according to García-Macías *et al.* (2007). Sperm were diluted in phosphate-buffered saline (PBS) to achieve a final 15–20 million/mL concentration. The agarose solution was first placed in a water bath at 95–100 °C for 5 minutes, then transferred to a water bath at 37 °C for 5 minutes to equilibrate. A 25 µL aliquot of the sperm sample was pipetted (Socorex, Germany) into an empty Eppendorf tube and gently mixed with 50 µL of liquefied agarose, maintaining the tubes at 37 °C. A 2 µL droplet of the cell suspension was placed on a glass slide, covered with a glass coverslip, and incubated on a pre-cooled glass plate (2–8 °C) for 5 minutes. After incubation, the coverslip was carefully removed, and the sample was fully immersed in Lysis Solution, incubated for 5 minutes, and then thoroughly washed with distilled water for 5 minutes. The sample was dehydrated sequentially and flooded with 70% and 97% ethanol, followed by air drying. A 2 µL aliquot of the final mixture was placed on the slide and stained using the high-sensitivity fluorochrome FluoRed (Halotech HT-RFS100, Spain). Three hundred sperm were evaluated under fluorescence microscopy (Olympus BX-33, Japan) at 1000× magnification. Nucleoids with a large, spotty halo of chromatin dispersion characterized sperm with fragmented DNA. In contrast, sperm without DNA fragmentation exhibited nucleoids with a small, compact halo of chromatin dispersion.

Computer-assisted sperm analysis. Sperm motility and kinetic parameters were assessed using the method described by Utami *et al.* (2025) using the CASA IVOS system (CASA Hamilton Thorne IVOS II, France), operating at a frame rate of 60 Hz. The analysis settings were explicitly configured for bull semen (Bull IMV Set Up) following the manufacturer's guidelines. A 10 µL sperm sample was placed on a glass slide and loaded onto the IVOS II system. The 'jog in' and 'jog out'

buttons manually adjusted the field of view. The live configuration was set, followed by clicking 'accept' and selecting five fields of view. The auto-capture function was then activated for automated data collection. The CASA system measured the following parameters: motility, progressive motility (PM), distance average path (DAP), distance straight line (DSL), distance curved line (DCL), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), straightness (STR), linearity (LIN), beat cross frequency (BCF), and wobble (WOB).

Statistical Analysis

The DNA fragmentation test was conducted in triplicate, whereas all other variables were assessed in ten replicates. Results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using analysis of variance (ANOVA) to determine differences in semen quality parameters. The data were analyzed using R Studio (Version 4.3.3). When significant differences were detected, Duncan's multiple range test (DMRT) was applied for post-hoc analysis to identify specific differences between pairs of means.

RESULTS

Fresh Semen Quality

The fresh semen samples used in this study were of good quality (Table 1), with a progressive motility rate exceeding 70%, aligning with the standard criteria for semen suitability for further processing (Ax *et al*., 2000). The viability rate of 90.01 ± 2.91% was within the standard threshold of ≥80% (Garner & Hafez, 2000). The abnormality rate of 4.35 ± 0.7% was well below the acceptable limit of ≤20% (Ax *et al*., 2000), while the sperm concentration of 1,753 ± 208.1 × 10^6/mL was within the normal range (Garner & Hafez, 2000). Overall, the fresh semen samples in this study met optimal quality standards, ensuring that sperm could maintain viability and functionality during the separation process.

Table 1. Average quality of fresh semen Holstein-Friesian bull

Variables	Average ± SD
Macroscopic	
Volume (mL)	4.43 ± 1.80
Color	Milky white
pH	6.5 ± 0.16
Odor	Specific
Consistency	77.9 ± 3.6
Microscopic	
Individual motility (%)	75.58 ± 4.7
Concentration (10^6/mL)	1.753 ± 208.1
Viability (%)	90.01 ± 2.91
Abnormality (%)	4.35 ± 0.7

Sperm Quality

Table 2 shows that IM, concentration, TMS, and viability in fresh semen (T1) significantly decreased ($p<0.05$) post-sexing (X= T2, Y= T3) and freezing (T4). However, there was no significant difference between T2 and T3 ($p>0.05$). A further decline in IM, concentration, TMS, and viability was observed in sexed sperm post-thawing (X= T5, Y= T6). For IM and viability, no significant difference was noted between T5 and T6 ($p>0.05$). Meanwhile, for concentration and TMS, no significant difference was found among T4, T5, and T6 ($p>0.05$).

Sperm abnormalities in fresh semen (T1) did not increase significantly ($p>0.05$) post-sexing (X= T2, Y= T3). However, a significant increase ($p<0.05$) was observed post-thawing (T4). No significant difference was found between T2 and T3 ($p>0.05$). A further significant increase ($p<0.05$) in abnormalities was noted in sexed sperm post-thawing (X= T5, Y= T6). A significant difference ($p<0.05$) was identified between sexed sperm post-thawing, with T6 showing a higher abnormality rate than T5.

Table 3 shows that MI and IAC in fresh semen (T1) decreased significantly ($p<0.05$) post-sexing (X= T2, Y= T3) and freezing (T4). However, no significant difference was observed between T2 and T3 ($p>0.05$). A further decline in MI was noted in sexed sperm post-thawing (X= T5, Y= T6). Regarding MI, a significant difference was found between sexed sperm post-thawing, with T5 exhibiting higher MI than T6 ($p<0.05$). In contrast, for IAC, no significant difference was detected between T5 and T6 ($p>0.05$).

Sperm DNA fragmentation in fresh semen (T1) increased significantly ($p<0.05$) post-sexing (X= T2, Y= T3) and freezing (T4). However, no significant differences were observed among T2, T3, and T4 ($p>0.05$). Additionally, post-thawing sexed semen (T5 and T6) did not show any significant differences in SDF ($p>0.05$).

Table 2. Sperm qualities (mean ± SD) of Holstein-Friesian bull sperm post-sexing and post-thawing

Variables	Fresh semen	Post-sexing		Post-thawing		
	T1	T2	T3	T4	T5	T6
IM (%)	77.9 ± 3.6^a	65.0 ± 3.8^b	65.8 ± 1.9^b	51.9 ± 3.4^c	44.1 ± 2.4^d	45.0 ± 1.8^d
Concentration (10^6/mL)	1735.20 ± 208.1^a	805.90 ± 130.9^b	889.30 ± 244^b	122.13 ± 19.6^c	116.50 ± 20.7^c	115.88 ± 21.1^c
TMS (10^6/mL)	1362.86 ± 145.8^a	526.70 ± 107.5^b	586.40 ± 167,7^b	63.42 ± 11.2^c	51.38 ± 9.6^c	52.19 ± 9.6^c
Viability (%)	90.01 ± 2.9^a	83.00 ± 3.9^b	83.55 ± 3.3^b	63.51 ± 2.4^c	60.27 ± 2.2^d	58.88 ± 2.1^d
Abnormality (%)	4.35 ± 0.7^a	5.81 ± 0.9^{ab}	5.86 ± 1.4^{ab}	6.88 ± 2.0^{bc}	7.46 ± 1.7^{bc}	7.81 ± 1.5^c

Note: Means in the same row with different superscripts differ significantly ($p<0.05$). IM: Individual motility, TMS: Total motile sperm. T1: fresh semen, T2: post-sexing X-chromosome-bearing sperm, T3: post-sexing Y-chromosome-bearing sperm, T4: post-thawing unsexed semen, T5: post-thawing X-chromosome-bearing sperm, T6: post-thawing Y-chromosome-bearing sperm.

Table 3. Membrane integrity, intact acrosome cap, and sperm DNA fragmentation (mean ± SD) of Holstein-Friesian bull sperm after sexing and freezing

Variables (%)	Fresh semen	Post-sexing		Post-thawing		
	T1	T2	T3	T4	T5	T6
MI	88.39 ± 3.7^{a}	84.93 ± 2.8^{b}	84.08 ± 2.8^{b}	62.88 ± 2.33^{c}	60.25 ± 2.0^{cd}	59.62 ± 2.4^{d}
IAC	93.87 ± 1.3^{a}	89.19 ± 2.0^{b}	89.37 ± 2.1^{b}	79.80 ± 2.1^{c}	76.16 ± 2.4^{d}	76.69 ± 2.8^{d}
SDF	4.06 ± 0.37^{b}	5.53 ± 0.45^{a}	5.61 ± 0.43^{a}	6.25 ± 0.48^{a}	6.83 ± 0.70^{a}	6.66 ± 0.43^{a}

Note: Means in the same row with different superscripts differ significantly (p<0.05). MI: Membrane integrity, IAC: Intact acrosome cap, SDF: Sperm DNA fragmentation. T1: fresh semen, T2: post-sexing X-chromosome-bearing sperm, T3: post-sexing Y-chromosome-bearing sperm, T4: post-thawing unsexed semen, T5: post-thawing X-chromosome-bearing sperm, T6: post-thawing Y-chromosome-bearing sperm.

Table 4. Sperm motility character (mean ± SD) measured by computer-assisted sperm analysis of Holstein-Friesian sperm after sexing and freezing

Variables (%)	Fresh semen	Post-sexing		Post-thawing		
	T1	T2	T3	T4	T5	T6
PM	76.29 ± 4.5^{a}	77,03 ± 6.6^{a}	75.05 ± 7.2^{a}	53.17 ± 7.0^{b}	45.74 ± 9.2^{b}	47.72 ± 4.5^{b}
Motile	94.1 ± 3.0^{a}	84.43 ± 4.1^{b}	84.67 ± 8.0^{b}	76.45 ± 6.0^{c}	79.85 ± 4.6^{bc}	76.55 ± 6.2^{c}
DAP	43.29 ± 5.8^{b}	52.78 ± 5.4^{a}	52.33 ± 4.7^{a}	33.43 ± 2.7^{c}	35.33 ± 4.3^{bc}	34.40 ± 3.6^{bc}
DSL	36.09 ± 5.6^{b}	49.84 ± 5.3^{a}	49.14 ± 4.4^{a}	26.69 ± 2.0^{c}	25.72 ± 3.1^{c}	26.26 ± 3.2^{c}
DCL	66.85 ± 5.0^{b}	81.85 ± 9.6^{a}	79.68 ± 8.1^{a}	59.58 ± 9.2^{b}	66.09 ± 8.8^{b}	63.35 ± 8.3^{b}
VAP	128.84 ± 12.7^{a}	125.61 ± 11.7^{a}	128.71 ± 7.2^{a}	83.17 ± 9.5^{b}	88.98 ± 10.2^{b}	86.05 ± 10.6^{b}
VSL	117.16 ± 11.9^{a}	118.46 ± 11.2^{a}	120.65 ± 5.7^{a}	66.36 ± 4.8^{b}	65.07 ± 6.2^{b}	65.90 ± 8.1^{b}
VCL	200.07 ± 19.7^{a}	193.90 ± 22.3^{a}	196.50 ± 19.6^{a}	147.01 ± 28.2^{b}	164.08 ± 23.5^{b}	156.86 ± 23.6^{b}
STR	90.02 ± 2.5^{a}	94.15 ± 1.7^{a}	93.17 ± 2.0^{a}	80.73 ± 6.5^{b}	74.60 ± 5.2^{b}	78.06 ± 3.5^{b}
LIN	59.60 ± 5.9^{a}	63.61 ± 4.6^{a}	63.99 ± 4.1^{a}	48.37 ± 7.9^{b}	42.75 ± 5.3^{b}	45.39 ± 4.5^{b}
ALH	6.95 ± 1.2^{a}	5.95 ± 0.9^{a}	5.88 ± 0.9^{a}	10.82 ± 10.0^{a}	12.96 ± 11.1^{a}	8.70 ± 1.3^{a}
BCF	35.19 ± 3.3^{a}	40.37 ± 3.1^{a}	41.43 ± 3.7^{a}	23.18^{b}	20.01 ± 3.8^{b}	21.22 ± 2.6^{b}
WOB	65.54 ± 5.4^{a}	67.01 ± 4.0^{a}	67.93 ± 3.3^{a}	58.65 ± 5.1^{b}	56.16 ± 3.5^{b}	57.06 ± 3.5^{b}

Note: Means in the same row with different superscripts differ significantly (p<0.05). PM: Progressive motility, DAP: Distance average path, DSL: Distance straight line, DCL: Distance curved line, VAP: Average path velocity, VSL: Straight line velocity, VCL: Curvilinear velocity, STR: Straightness, LIN: Linearity, ALH: Lateral amplitude of head displacement, BCF: Beat cross frequency, WOB: Wobble. T1: fresh semen, T2: post-sexing X-chromosome-bearing sperm, T3: post-sexing Y-chromosome-bearing sperm, T4: post-thawing unsexed semen, T5: post-thawing X-chromosome-bearing sperm, T6: post-thawing Y-chromosome-bearing sperm.

Sperm Motility

Table 4 indicates that the sexing process (T2 and T3) significantly affected (p<0.05) motility reduction while enhancing distance parameters (DAP, DSL, and DCL). In contrast, the freezing process for both unsexed (T4) and sexed semen (T5 and T6) significantly decreased (p<0.05) most kinetic parameters, including progressive motility (PM), total motility, DAP, DSL, VAP, VSL, VCL, STR, LIN, BCF, and WOB. However, there was no significant difference in the amplitude of lateral head displacement (ALH) among all treatments (p>0.05).

DISCUSSION

The decrease in individual motility post-sexing can be attributed to the centrifugation process, which has been shown to cause physical damage and disrupt sperm metabolic activity (Ondho & Udrayana, 2018). The freezing process has been widely reported to decrease sperm motility further (Alm-Kristiansen, 2023). This reduction is primarily due to physical injury, cold stress, oxidative damage following cryopreservation, and decreased mitochondrial function and energy metabolism (Sun *et al*., 2020). Martin *et al*. (2004) also reported that cryopreservation induces sperm membrane dysfunction, preventing ATP regeneration and reducing energy availability and motility. Since only progressively motile sperm can fertilize an oocyte, immotile or abnormally moving sperm are unable to penetrate the cervical mucus barrier (Chakraborty & Saha, 2022).

A significant decline in sperm concentration and motility was observed following sperm sexing (Missio *et al*., 2018). This reduction occurs because X- and Y-chromosome-bearing sperm, initially present as a mixture, are separated into distinct fractions, effectively halving the sperm concentration. Percoll density gradient centrifugation sexing selectively removes non-viable and low-quality sperm, further reducing the overall sperm concentration (Keskin & Seda, 2017). Another contributing factor is the pipetting process, which may result in incomplete sperm retrieval from the upper and lower gradient layers, thereby decreasing the final sperm count.

Furthermore, a decrease in sperm concentration was observed post-cryopreservation. This reduction is attributed to the use of a semen diluent, which increases the total volume while helping to mitigate the detrimental effects of freezing and ensuring sperm survival during storage (Bustani *et al*., 2021). Despite these reductions, the sperm concentration achieved in this study met the minimum standard of 25 million sperm per straw, as specified by the Indonesian National Standard (2024).

The decrease in total motile sperm observed post-sexing and freezing closely correlates with the decrease in motility during these processes (Aldini *et al*., 2022). The reduction in total motile sperm in sexed semen primarily stems from decreased sperm motility, which arises from the centrifugation process. This decrease is also influenced by the decrease in sperm concentration that occurs during separation and freezing procedures. Given that total motile sperm is derived from the product of motility and concentration, a higher percentage of motile and concentrated sperm results in a higher TMS value (Yekti *et al*., 2023). In fresh semen, total motile sperm values are influenced by various factors, including season, ejaculation frequency, age, testicular size, and disease status (Ax *et al*., 2008). Despite these reductions, the total motile sperm obtained in this study met the minimum standard of 10 million sperm per straw, as established by the Indonesian National Standard (2024). Sperm viability is defined as the percentage of sperm capable of surviving during transit through the female reproductive tract and successfully fertilizing the oocyte (Rahman & Pang, 2020). Viable sperm are identified by a colorless (white) head, whereas non-viable sperm exhibit a pink-stained head (Figure 1). The decrease in sperm viability post-sexing is mainly attributed to the loss of seminal plasma, frictional forces generated during centrifugation, and increased free radical production (Rumende *et al*., 2013). Centrifugation creates frictional forces between sperm and the surrounding medium, which can compromise the structural integrity of the sperm plasma membrane (Mahfud *et al*., 2019).

The freezing process presents significant challenges to sperm, including oxidative injury, ice crystal formation, plasma membrane damage, DNA damage, cryoprotectant toxicity, and osmotic stress (Larasati *et al*., 2022). The sperm plasma membrane is particularly susceptible to damage, as external factors strongly influence its integrity. During cryopreservation, sperm experience extreme temperature fluctuations, which can lead to membrane damage (Prabowo *et al*., 2023). The freeze-thaw process induces temperature and osmotic stress, altering membrane organization, fluidity, permeability, and lipid composition (Le *et al*., 2009). Among the various forms of cryoinjury, plasma membrane damage is the most significant, leading to membrane swelling and loss (Khalil *et al*., 2018). Since sperm plasma membranes play a crucial role in maintaining motility, capacitation, and acrosome reaction or fusion, their structural integrity is essential for successful fertilization (Shan *et al*., 2021). Damage to the plasma membrane not only affects sperm function but also compromises fertilization potential, further emphasizing the detrimental impact of cryopreservation on sperm.

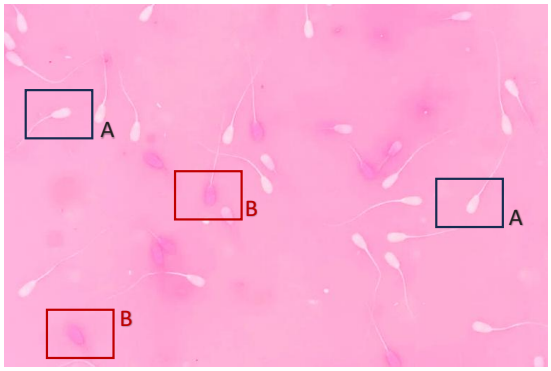

Figure 1. Viability of Holstein-Friesian sperm. Live sperm are marked with white head color (A), and dead sperm are marked with red head color (B). Image captured at 400× magnification.

The increase in sperm abnormalities post-sexing can be attributed to frictional forces generated during centrifugation, both between the sperm and the tube walls and among the sperm themselves. This mechanical stress can cause morphological damage, particularly at the junction between the head and tail (Susilawati *et al*., 2014). Furthermore, the increase in abnormalities post-thawing may be linked to oxygen exposure, which, while essential, can induce peroxidative damage when present in excess. Lipid peroxidation in sperm can lead to structural abnormalities, particularly in the midpiece or tail, thus increasing the rate of abnormalities following freeze-thawing (Solihati *et al*., 2024).

Multiple factors, such as reproductive organ health, age, management practices, and nutritional intake, affect the occurrence of abnormalities in fresh semen. In this study, the abnormality rates in all treatments adhered to the standards for AI. According to the Indonesian National Standard (2024), the maximum allowable sperm abnormality rate for AI is 20%. Morphologically abnormal sperm have a diminished ability to bind to the zona pellucida, likely due to defective membrane receptors involved in this binding process (Ducha *et al*., 2012).

Membrane integrity is a critical indicator of the sperm's ability to maintain its structural and functional stability, which is essential for successful fertilization (Ruiz-Díaz *et al*., 2020). The noted decrease in membrane integrity post-sexing can be attributed to mechanical stress, ice crystal formation, osmotic pressure fluctuations, and the potential toxicity of cryoprotectants, all contributing to plasma membrane damage (Küçük *et al*., 2020). Centrifugation-induced friction further intensifies oxidative stress, while extreme temperature variations during the freeze-thaw process heighten membrane vulnerability (Ugur, 2021). It has been extensively reported that Y-chromosome-bearing sperm display lower quality than X-chromosome-bearing sperm due to their smaller size and reduced biological resistance to environmental stress (Handarini *et al*., 2024). A reduction in membrane integrity adversely affects sperm viability and fertilization potential, ultimately affecting AI success rates.

The PDGC method, which separates sperm based on density, can reduce membrane integrity due to mechanical and osmotic stress (Afriani *et al*., 2022). The high centrifugal forces involved in this process increase the risk of plasma membrane damage, particularly in smaller and more fragile Y-chromosome-bearing sperm. Differences in osmotic pressure between the

percoll medium and sperm physiological conditions can lead to fluctuations in osmotic balance, compromising plasma membrane and acrosome integrity (Kumar *et al*., 2019). Mechanical stress from centrifugation has been shown to induce oxidative stress, contributing to lipid peroxidation and the deterioration of membrane lipid structures (Tiwari *et al*., 2021). Damage to the plasma membrane reduces the sperm's ability to survive in the female reproductive tract, thereby lowering conception rates (Peris-Frau *et al*., 2019).

A dark purplish-stained head characterizes sperm with intact acrosomes, while sperm with damaged acrosomes exhibit a light purple-stained head (Figure 2). The decrease in intact acrosome caps post-sexing may be linked to centrifugation-induced stress, which increases sperm susceptibility to acrosome damage. This process is triggered by the activation of amino acid oxidase, an enzyme recognized as a primary source of reactive oxygen species (ROS) production in sperm. Excessive ROS production can lead to premature capacitation (Arias, 2017) and oxidative stress, resulting in decreased motility, viability, and acrosome integrity due to a rapid reduction in intracellular ATP levels (Rawat & Sharma, 2020).

Percoll, which is used during sperm sexing, may attach to the sperm plasma membrane, releasing decapitating proteins and prematurely initiating the acrosome reaction (Chamberland *et al*., 2001; Oliveira *et al*., 2011). Furthermore, the decrease in intact acrosome cap post-thawing is primarily due to cold shock, which damages membrane integrity and acrosome organelles (Arvioges *et al*., 2021). Cold shock is a physiological stress response triggered by sudden temperature fluctuations during freezing and thawing (Lacalle *et al*., 2021). The biological mechanism of cold shock involves disruptions in membrane lipid fluidity, resulting in increased membrane permeability and the leakage of essential ions such as Ca^{2+} and K^{+} (Chen *et al*., 2024). These changes lead to damage in transmembrane proteins and phospholipids, resulting in plasma membrane instability and the loss of acrosome integrity, which is essential for fertilization (Bollwein & Malama, 2023).

The decrease in the intact acrosome cap directly affects the sperm's ability to fertilize oocytes (Neila-Montero *et al*., 2024). A damaged acrosome does not release the essential enzymes needed for the acrosome reaction, hindering zona pellucida penetration and reducing fertilization success (Nixon *et al*., 2021). Sperm DNA fragmentation is a crucial determinant of sperm fertility potential, affecting its ability to reach and fertilize the oocyte and to support early embryogenesis (Kumaresan *et al*., 2017). The assessment of sperm DNA fragmentation relies on the differential response of sperm chromatin to protein depletion. In sperm without significant DNA damage, the removal of nuclear proteins leads to intensely stained nucleoids surrounded by small, compact halos of DNA loops encasing a central chromatin core. In contrast, sperm with fragmented DNA display large, faintly stained halos, representing the diffusion of fragmented DNA strands from the residual chromatin core (Figure 3).

The increased sperm DNA fragmentation post-sexing may be associated with ROS production and mechanical DNA damage caused by centrifugation forces (Ali *et al*., 2022). Zini *et al*. (2000) reported that PDGC-based sperm separation does not selectively isolate sperm with intact DNA, suggesting that sexing does not improve DNA integrity. Gosálvez *et al*. (2011) found that sexed sperm had a higher sperm DNA fragmentation rate compared to unsexed frozen semen, indicating an increased susceptibility of sorted sperm to DNA fragmentation. However, these findings contradict previous studies that found no significant increase in sperm DNA fragmentation after sperm sexing using density gradient centrifugation (Malvezzi *et al*., 2014; Demir *et al*., 2019). This discrepancy may be due to methodological differences, sperm preparation protocols, or species-specific variations in sperm resilience to centrifugation and oxidative stress.

The increase in sperm DNA fragmentation post-thawing may be attributed to damage incurred during the freezing and thawing process, including thermal shock, intracellular and extracellular ice crystal formation, cellular dehydration, and osmotic shock. Rapid temperature fluctuations during freezing

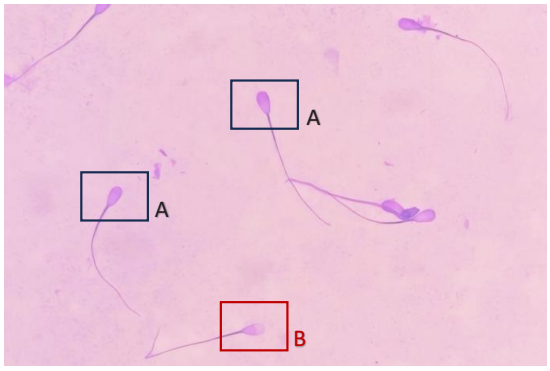

Figure 2. Holstein-Friesian sperm with intact acrosomes are characterized by a dark purplish-stained head (A), whereas sperm with damaged acrosomes exhibit a light purple-stained head (B). Image captured at 400× magnification.

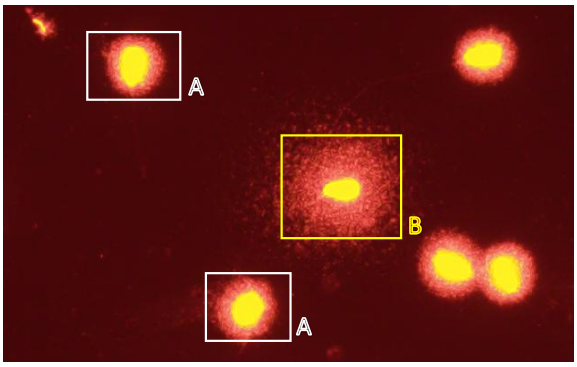

Figure 3. Sperm with unfragmented DNA is characterized by a small halo of chromatin dispersion (A), while sperm with fragmented DNA exhibits a large halo of chromatin dispersion (B). Image captured at 1000× magnification.

promote the formation of ice crystals that can rupture sperm plasma membranes and disrupt organelle function, thereby increasing DNA damage (Le *et al*., 2019). However, the impact of cryopreservation on increased sperm DNA fragmentation remains a subject of debate. Some studies have reported that freezing can increase DNA damage (El-Regalaty, 2017; Sangisapu & Sandeep, 2019; Lee *et al*., 2019; Ribas-Maynou *et al*., 2024), while contrasting findings from Martin *et al*. (2004) and Macente *et al*. (2019) concluded that freezing has no significant effect on sperm DNA fragmentation. Similarly, Brum *et al*. (2008) suggested that freezing does not directly induce sperm DNA fragmentation but instead influences cell apoptosis, which is often associated with DNA fragmentation.

Sperm DNA damage is commonly observed in semen post-freeze-thaw, a process known to increase ROS levels (Priyanto *et al*., 2018). However, Gürler *et al*. (2018) found that ROS does not directly compromise sperm DNA integrity. Instead, it is hydrogen peroxide (H_2O_2), a metabolic byproduct of sperm, that contributes to this damage. This indicates that oxidative stress leads to sperm DNA fragmentation through indirect mechanisms rather than ROS-induced DNA cleavage. Furthermore, sperm DNA fragmentation is affected by extrinsic factors such as oxidative stress and intrinsic factors related to sperm production and maturation. These intrinsic factors include defective spermatogenesis, impaired sperm transport within the male reproductive tract, abortive apoptosis, and defective chromatin remodeling (Baskaran *et al*., 2019). Furthermore, sperm resilience during freezing is also influenced by genetic factors, which may account for variations in cryotolerance among individual bulls (Morell *et al*., 2018).

Despite these potential risks, the sperm DNA fragmentation values observed in this study remained below the tolerance threshold for frozen bovine semen, as defined by Evenson (2016), which ranges from 10% to 20%. This indicates that the freezing and sexing protocols employed in this study effectively maintained sperm DNA integrity within acceptable limits for AI applications.

Low levels of DNA damage in sperm can be repaired by oocytes and embryos, enabling the birth of a healthy individual. However, high levels of DNA fragmentation may lead to early embryonic loss, abortion, or developmental disorders in successfully born offspring (Simoes *et al*., 2013). Additionally, sperm DNA fragmentation has been associated with implantation failure (Sedo, 2017). Mammalian cells possess complex mechanisms to detect DNA damage and activate appropriate responses to preserve genome integrity. DNA damage in sperm can be repaired during fertilization, particularly during pronuclei, DNA replication, and pronuclear fusion, which leads to the formation of a zygote (Jaroudi *et al*., 2007). However, DNA damage is the primary cause of cell death and injury, as cells are unable to avoid damage during replication, transcription, and translation (Wang *et al*., 2023).

Computer-assisted sperm analysis is a reliable and objective method that provides detailed information about sperm kinetic properties based on the individual motion characteristics of sperm (Gillan *et al*., 2008). The PDGC method effectively separates non-motile sperm, which accumulate in the upper layer and are discarded, thus preventing a decline in progressive motility after separation. In this study, the sperm kinetic parameters following separation using the PDGC method were well maintained. Similar findings were reported by Oliveira *et al*. (2011), who demonstrated that PDGC effectively selects motile sperm, allowing less active cells to remain in the upper fraction of the gradient.

Sperm with intact nuclear morphology exhibit higher density and settle in regions of greater density gradient. Moreover, motile sperm sediment faster than non-motile cells due to their alignment with centrifugal forces (Lee *et al*., 2009). The findings of this study are consistent with previous research, which has shown that percoll centrifugation effectively maintains sperm kinetic parameters (Utami *et al*., 2025) and is non-detrimental to gametes (Silva *et al*., 2019).

The kinetic parameters of sperm post-thawing suggest the occurrence of sperm hyperactivation, a phenomenon characterized by asymmetrical flagellar beating with a high beat amplitude (Sharif *et al*., 2022). Hyperactivation analysis using CASA requires kinetic measurements of sperm head movements, which are closely associated with characteristic changes in flagellar motion (Schmidt *et al*., 2004).

The observed low percentage of intact acrosome caps (Table 3) suggests that more sperm underwent capacitation, a prerequisite for fertilization. Hyperactivation and the acrosome reaction are integral parts of sperm capacitation, essential for successful fertilization (Stival *et al*., 2016). Moreover, Sharif *et al*. (2022) reported that one of the notable physiological changes sperm undergo during capacitation is the development of hyperactivated motility. While the precise triggers of hyperactivation remain incompletely understood, it is known to be induced by various extracellular factors (Schmidt *et al*., 2004). The decrease in velocity parameters (VAP, VSL, VCL) post-thawing is likely associated with mitochondrial damage, which occurs during cryopreservation and reduces flagellar activity (Utami *et al*., 2025). The kinetic parameters of sexed and frozen-thawed sperm indicate their potential ability to fertilize oocytes. Previous studies have shown that sperm capable of penetrating periovulatory cervical mucus in humans exhibit VAP values of ≥25.0 μm/s and ALH values of ≥4.5 μm (Akhtar *et al*., 2023). Sperm maintain vitality during cryopreservation due to the protective effects of egg yolk Tris-aminomethane diluent, which supports sperm viability and helps sustain sperm motility (Ratnawati *et al*., 2017).

Sperm sexing using PDGC resulted in a significant decrease in IM, concentration, total motile sperm, viability, membrane integrity, and intact acrosome cap. Similarly, freezing of both unsexed and sexed semen led to a further reduction in IM, concentration, total motile sperm, viability, membrane integrity, and intact acrosome cap, along with an increase in sperm abnormalities. Sperm DNA fragmentation increased after both sexing and freezing in unsexed semen,

suggesting that these processes contribute to sperm DNA damage. Assessment of kinetic parameters using CASA showed that sexing reduced motility while increasing distance parameters (DAP, DSL, and DCL). In addition, freezing unsexed and sexed spermatozoa decreased progressive motility, total motility, DAP, DSL, VAP, VSL, VCL, STR, LIN, BCF, and WOB. The PDGC method effectively removes non-motile spermatozoa but also induces membrane damage, primarily due to centrifugation-induced stress. Furthermore, the freezing process causes ultrastructural damage, which may impair the fertilization potential of spermatozoa. Despite these challenges, PDGC remains a viable method for sperm separation in cattle. It preserves sperm quality, membrane integrity, acrosome integrity, DNA integrity, and kinetic parameters while remaining compatible with semen cryopreservation.

CONCLUSION

Sperm sexing using PDGC resulted in a significant decrease in sperm quality, membrane integrity, intact acrosome hood, and DNA fragmentation. Furthermore, PDGC remains a viable method for sperm separation in cattle and is compatible with semen cryopreservation. This study confirms that PDGC-processed semen is suitable for artificial insemination.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest with stakeholders regarding the material presented in this manuscript.

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