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X/Y-Bearing Spermatozoa Enrichment and Semen Quality Following Egg White Albumin Sedimentation Using Different Gradients and Incubation Durations in Holstein-Friesian Bulls

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ABSTRACT

Sperm sexing is a reproductive technology for controlling offspring sex ratios by separating X- and Y-bearing spermatozoa. This study evaluated the effectiveness of egg white albumin sedimentation using different gradients and incubation durations in maintaining semen quality and enriching X- and Y-bearing spermatozoa from Holstein–Friesian (HF) bull semen. A 2 × 2 factorial design was applied, comprising four treatments: G2I20 (two gradients, 20 min), G3I20 (three gradients, 20 min), G2I40 (two gradients, 40 min), and G3I40 (three gradients, 40 min), each with 10 replicates. Variables included semen quality such as motility, viability, abnormality, sperm concentration, plasma membrane integrity (PMI), total motile spermatozoa (TMS), and sperm kinematics, which were assessed by computer-assisted sperm analysis (CASA), and the proportions of X- and Y-bearing spermatozoa. Most semen quality variables were not significantly affected by the treatments ($p>0.05$). However, gradient number significantly affected TMS and sperm concentration in the lower fraction ($p<0.05$). CASA analysis showed significant effects on straight line velocity (VSL), curvilinear velocity (VCL), and beat cross frequency (BCF) in the upper fraction, but not in the lower fraction ($p>0.05$). The proportion of X-bearing spermatozoa in the upper fraction ranged from 70.70% to 74.00%, while Y-bearing spermatozoa in the lower fraction ranged from 74.20% to 80.10%. Chi-square analysis indicated significant deviations from the expected ratios only in G2I40 in the upper fraction and in all treatments in the lower fraction. G2I20 exhibited numerically higher sperm concentration, PMI, and TMS. In conclusion, egg white albumin sedimentation maintained comparable semen quality across treatments, while G2I20 provided a shorter processing duration with favorable numerical outcomes for semen quality.

Keywords: albumin; egg white sedimentation; Holstein-Friesian; semen quality; sexing sperm

INTRODUCTION

Reproductive technologies, particularly artificial insemination (AI), play an important role in improving the productive efficiency and genetic dissemination of dairy cattle populations. In Holstein-Friesian (HF) dairy farms, the preferential production of female calves is particularly valuable in dairy herds because they serve as replacement heifers and support herd expansion and future milk production (Shabirah *et al*., 2025). Spermatozoa bearing the X chromosome produce female offspring, whereas those bearing the Y chromosome produce male offspring (Bai *et al*., 2025). Sex determination relates to the presence of the Y chromosome, which carries the sex-determining region Y gene responsible for initiating testis development and the male sexual differentiation pathway (Houzelstein *et al*., 2024; She & Yang, 2017). Based on these biological differences, several technologies for spermatozoa separation have been developed to increase the proportion of offspring of the desired sex. Flow cytometry is the most established commercial approach and separates X- and Y-bearing spermatozoa based primarily on differences in DNA content and fluorescence intensity. However, its application requires advanced equipment, specialized expertise, and relatively high operational costs (Yodrug *et al*., 2025). Although Percoll density gradient centrifugation (PDGC) can effectively separate spermatozoa in HF cattle, this method may reduce sperm quality, including membrane integrity and kinematic characteristics, particularly after sex sorting and cryopreservation (Safa *et al*., 2025; Yekti *et al*., 2023). More recently, Syah *et al.* (2026) showed that PDGC processing was associated with reduced progressive motility and altered sperm kinematic parameters after cryopreservation, with the magnitude of these effects varying among bulls and sperm fractions. Safa *et al.* (2025) also reported

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significant decreases in sperm quality from fresh semen to post-cryopreservation, with reductions of up to 39.67% in motility, 2.36% in viability, and 93.19% in sperm concentration, along with an approximately 69.73% increase in sperm abnormality following separation and freezing. These limitations emphasize the need for a simpler, more accessible sex-selection approach that preserves spermatozoa function.

Albumin sedimentation has been investigated as an alternative approach for separating X- and Y-bearing spermatozoa based on differences in motility, density, size, and mass, which are related to variations in DNA content and other physical characteristics (Rahmat *et al*., 2024). In addition to the separation medium, the extender used during semen processing can influence spermatozoa preservation. Conventional extenders containing Tris, egg yolk, or skim milk provide buffering, energy, and membrane protection during cooling and freezing (Schoelerman *et al*., 2026; Ugur *et al*., 2019). Pramudhita *et al.* (2026) reported that Trico egg-yolk extender was able to maintain several conventional post-thaw sperm quality parameters at levels comparable to those obtained with conventional Tris egg-yolk extender, while also showing potential to reduce bacterial contamination, particularly *Staphylococcus* spp. These findings indicate the potential of Trico extender as an alternative extender for bovine semen preservation and provide a basis for its application in subsequent semen processing and reproductive biotechnology studies. In addition, Diansyah *et al*. (2025) demonstrated that albumin-based sperm separation using a tris-egg yolk diluent could maintain spermatozoa quality and kinematic characteristics, with post-sexing motility exceeding 75% and post-thaw motility exceeding 45%, while producing separation of spermatozoa bearing X and Y chromosomes, with X- and Y-bearing spermatozoa proportions of 64.61% and 27.49%, respectively, in the upper fraction and 24.16% and 73.98%, respectively, in the lower fraction. Therefore, these findings support the potential use of egg white albumin as an easily accessible medium for sperm sex determination.

Previous studies have demonstrated the potential of Trico extender to maintain post-thaw sperm quality and of albumin-based separation to preserve sperm quality and kinematic characteristics during sperm sexing. However, the interaction between albumin gradient and incubation duration when using fresh egg white albumin with Trico extender has not been comprehensively evaluated in HF bull semen, particularly in relation to sperm separation efficiency and the preservation of sperm functionality after cryopreservation. Therefore, this study evaluates X- and Y-bearing spermatozoa enrichment, conventional sperm quality variables, total motile spermatozoa (TMS), and post-thaw computer-assisted sperm analysis (CASA)-derived kinematic characteristics to assess the effects of gradient and incubation duration on sperm separation and functional preservation from sexing through cryopreservation. The novelty of this study lies in the comprehensive evaluation of egg white albumin gradients and incubation durations using Trico extender in HF bull semen.

This study aimed to evaluate the effects of egg white albumin gradients (two and three gradients) and incubation durations (20 and 40 minutes) using Trico extender on X- and Y-bearing spermatozoa enrichment, post-sexing semen quality, and post-thaw sperm kinematic characteristics in HF bulls. The evaluated variables included sperm motility, viability, abnormality, concentration, plasma membrane integrity (PMI), TMS, and CASA-derived kinematic variables. These findings are expected to identify the optimal combination of albumin gradient number and incubation duration for a simpler, more practical, and potentially more economical sperm sex-selection protocol for HF bulls.

MATERIALS AND METHODS

Ethical Approval

This study was exempted from ethical review under institutional guidelines for animal research, as determined by the Research and Innovation Ethics Committee of Universitas Brawijaya (certificate number: 149/EC/KEPK/04/2026). Standard procedures were followed at the Singosari National Artificial Insemination Center (SNAIC), Malang, Indonesia, certified to ISO 9001:2015, and supervised by SNAIC veterinarians.

Animal and Semen Collection

Fresh semen was obtained from two 5-year-old HF bulls, each weighing approximately 800 kg, maintained at SNAIC under standard operating procedures. Ten ejaculates were collected twice weekly using an artificial vagina by trained SNAIC staff, comprising eight ejaculates from one bull and two from the other. Ejaculates met the minimum criteria of mass motility ++, individual motility ≥70%, viability ≥80%, abnormality ≤20%, and sperm concentration ≥1,000 million/mL.

Experimental Design and Treatments

Ten ejaculates obtained from semen collections served as biological replicates. Each ejaculate was equally divided into four portions, which were randomly assigned to combinations of two albumin gradient numbers, consisting of two gradients (G2) and three gradients (G3), and two incubation durations of 20 minutes (I20) and 40 minutes (I40). The four treatment combinations were G2I20: two-gradient for 20 minutes; G3I20: three-gradient for 20 minutes; G2I40: two-gradient for 40 minutes; and G3I40: three-gradient for 40 minutes, using egg white albumin sedimentation. Each ejaculate contributed one replicate to each treatment combination, resulting in 10 replicates per combination and 40 experimental units. Following sexing, the upper and lower fractions from each experimental unit were collected separately and evaluated for sperm quality and spermatozoa characteristics.

Sexing Procedure Using Egg White Albumin Sedimentation

Spermatozoa separation was performed using a modified egg white albumin gradient sedimentation method based on Diansyah *et al*. (2025) and Maulana *et al*. (2022). Fresh semen that met the quality criteria was diluted 1:1 with Trico egg-yolk extender, a modified Tris-based extender for semen cryopreservation, prepared according to the formulation described by Pramudhita *et al.* (2026). Egg white albumin was prepared as the sexing medium and added to sterile test tubes. The albumin gradients were then prepared according to the treatment protocol, specifically two-layer gradients (30% and 50%) and three-layer gradients (30%, 40%, and 50%). The gradients were carefully prepared using a micropipette, starting with the highest concentration at the bottom and the lowest concentration at the top to form distinct barriers between layers. Subsequently, a 1-mL aliquot of the diluted semen was carefully layered onto the top of the gradient column and incubated for 20 or 40 minutes, depending on the treatment. After incubation, 2 mL of the upper fraction was collected as the X-enriched fraction, and 2 mL of the lower fraction adjacent to the interlayer boundary was collected as the Y-enriched fraction. The fractions were centrifuged at 277 × *g* for 10 minutes, after which the supernatant was discarded and the spermatozoa pellets were collected for quality and proportion analyses. The separated fractions were cryopreserved according to Wahyudi *et al.* (2025), stored for 24 h, and then thawed at 37 °C for 30 s before CASA analysis to evaluate post-thaw motility and kinematic variables.

Variables Observed

Semen evaluation was conducted in three stages: fresh semen, post-sexing semen, and frozen-thawed semen. Fresh and post-sexing semen were evaluated using conventional macroscopic and microscopic assessments and sperm quality variables, whereas frozen-thawed semen was evaluated using CASA to assess sperm motility and kinematic characteristics. CASA was applied to frozen-thawed semen because this stage reflects the cumulative effects of sexing, cryopreservation, and thawing on sperm quality and therefore represents the functional condition of semen intended for AI.

Macroscopic evaluation. Macroscopic evaluation was performed on fresh semen to assess semen quality through direct visual inspection, which includes volume, color, odor, pH (universal pH indicator paper, Merck, Germany), and consistency. Semen volume is evaluated using a graduated cylinder placed vertically on a flat surface. Color and odor are observed directly in the semen sample within the cylinder. Semen consistency was evaluated by slowly tilting the tube and observing the flow of semen along the tube wall as an indicator of viscosity, or it can be estimated by calculating sperm concentration.

Microscopic evaluation. Microscopic evaluation included spermatozoa mass motility, individual motility, concentration, viability, and morphology.

Mass motility. Mass motility was evaluated in fresh semen by placing 10 µL of semen on a slide, covering it with a cover glass, and observing it under a microscope at 100× magnification at 37 °C. Mass motility was assessed subjectively based on the pattern and intensity of spermatozoa movement, following the assessment approach described by Firmawati *et al*. (2025) based on Indonesian national standards, in which the scoring criteria were +++ (very good, with strong swirling movement), ++ (good, with less distinct wave patterns and slower movement), and + (very low, with no wave formation and only individual progressive movement).

Individual motility (%). Individual sperm motility was evaluated by placing 10 µL of the sample on an object glass, covering it with a cover glass, and then observing five visual fields using a 400× microscope at a constant temperature (37 °C). The assessment was based on the proportion of spermatozoa exhibiting progressive, backward, or circular movement (Maulana *et al*., 2022).

Sperm viability (%). Sperm viability was evaluated using eosin–nigrosin staining, which was mixed 1:1 with the sample, smeared on a glass slide, and observed under a 400× microscope by counting at least 200 spermatozoa. Live spermatozoa are characterized by heads that remain white/translucent (do not absorb the stain). In contrast, dead spermatozoa have red-purple heads (absorbing the stain) due to damage to the sperm membrane, allowing the stain to penetrate.

Sperm abnormality (%). The preparation for sperm abnormality assessment was the same as that used for viability assessment and involved eosin–nigrosin staining. Spermatozoa abnormality is classified into three categories: primary, secondary, and tertiary.

Sperm concentration (million/mL). Sperm concentration was determined using a hemocytometer following Yekti *et al*. (2024). Briefly, 990 µL of 3% NaCl solution was placed in a microtube, followed by the addition of 10 µL of semen to obtain a 1:100 dilution. The sample was gently homogenized, and 10 µL of the diluted semen was loaded into the hemocytometer counting chamber and covered with a cover glass. Spermatozoa were counted in five squares (upper right, upper left, lower right, lower left, and central squares) using an Olympus CX23 binocular light microscope. Spermatozoa concentration is calculated using the following formula:

$$\text{Spermatozoa total/mL} = N \times 5 \times DF \times 10.000$$

where N is the average number of spermatozoa counted in the chamber, DF is the dilution factor (100), 5 is the correction factor for counting 5 of 25 squares (25/5), and 10,000 is the volume conversion factor based on the chamber depth of 0.0001 mL.

Plasma membrane integrity (%). Plasma membrane evaluation was performed using a hypo-osmotic swelling test (HOST) solution at a 1:10 ratio. A total of 20 µL of semen is added to a microtube containing 200 µL of HOST solution (1.351 g of fructose and 0.735 g of sodium citrate in 100 mL of distilled water). The PMI percentage can be calculated using the formula:
PMI (%)= [Number of swollen (coiled-tail sperm) / Total number of sperm counted] × 100%

Total motile spermatozoa (million/mL). TMS concentration was calculated by multiplying sperm concentration by sperm motility percentage, following Kaka *et al.* (2025), using the formula:
TMS (Million/mL)= individual motility (%) × sperm concentration

Identification of X and Y spermatozoa (%). Spermatozoa morphometry was assessed using a micrometer under a phase-contrast microscope at 400× magnification, with head length and width measured (Figure 1). The X- and Y-bearing sperm proportions were determined based on sperm head area. For fresh semen, the mean head area of 1,000 spermatozoa from each bull was calculated as the individual control value, and the values from both bulls were averaged to obtain the overall control mean. For sexed semen, 100 spermatozoa from each treatment sample in the upper and lower layers were evaluated across 10 replicates and classified according to their head area relative to the fresh-semen control mean. Spermatozoa with a relatively larger nuclear area tended to be associated with X-bearing spermatozoa, whereas Y-bearing spermatozoa exhibited a relatively smaller nuclear area (Santolaria *et al.*, 2016). The proportions of X- and Y-bearing spermatozoa were calculated using the following formula:

Percentage of X/Y spermatozoa: (Number of X/Y spermatozoa / Total X and Y spermatozoa) × 100%

Evaluation of sperm motility after freezing using computer-assisted sperm analysis. Post-thaw sperm motility and kinematic characteristics were assessed using CASA with the IVOS II computer system (Hamilton Thorne, USA), equipped with a microscope stage maintained at 37 °C. The system detects sperm head movement patterns and analyzes individual sperm trajectories. Samples were diluted 1:2 with Trico to obtain an appropriate sperm concentration for CASA analysis, and 10 µL of the diluted sample was placed on a pre-warmed glass slide (Microscope Slide, China) and covered with a coverslip (Herma). Analysis was performed at 10× objective magnification in five selected fields of view adjusted using the instrument controls (Utami *et al.*, 2025). The CASA system automatically generated sperm-level, field-level, and sample-level data. The evaluated variables included total motility, progressive motility, average path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), and wobble (WOB).

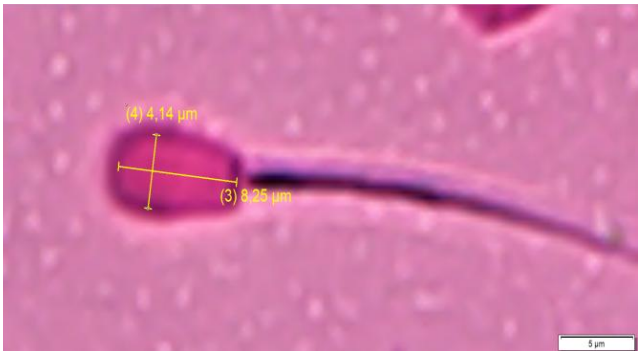

Figure 1. Morphometric measurements of Holstein–Friesian bull sperm head. Sperm head length is indicated by (L) and sperm head width is indicated by (W). Measurements were performed using LCmicro software.

Statistical Analysis

Data on semen quality were analyzed using analysis of variance (ANOVA) for a 2 × 2 factorial arrangement in a randomized complete block design (RCBD), using RStudio version 2026.04.0 Build 526. The factors were albumin gradient number (two and three gradients) and incubation duration (20 and 40 min). Significant effects ($p<0.05$) were further evaluated using pairwise comparisons. The statistical model was:

$$Yijk = \mu + G_i + I_j + (GI)_{ij} + B_k + \varepsilon_{ijk}$$

where $Yijk$ was the observed value of the dependent variable for G_i and I_j in the -th block; μ was the grand mean; G_i was the effect of the first factor at the -th level; I_j was the effect of the second factor at the -th level; $(GI)_{ij}$ was the interaction effect between the first and second factors; B_k was the effect of the -th block; ε_{ijk} was the experimental error term, assumed to be normally distributed, independent, and homogeneous (with constant variance). The proportions of X- and Y-bearing spermatozoa in the upper and lower fractions were analyzed separately using a beta-binomial generalized linear mixed model (GLMM), with gradient number, incubation duration, and their interaction as fixed effects and ejaculate as a random effect. Additionally, chi-square goodness-of-fit tests were performed against an expected 70:30 distribution, with 70% X-bearing spermatozoa as the target in the upper fraction and 70% Y-bearing spermatozoa in the lower fraction. Statistical significance was set at $p<0.05$.

RESULTS

Fresh Semen Quality in Holstein-Friesian Bulls

Fresh semen was evaluated macroscopically and microscopically to assess its physical and biological characteristics and confirm its suitability for subsequent processing and analysis. Samples meeting the quality requirements of the Indonesian National Standard were used in the subsequent experimental stages.

The results of the fresh semen evaluation in Table 1 show that fresh semen from HF bulls had a volume of 8.77 ± 1.23 mL, milky-white color, characteristic odor,

and a mean pH of 6.5 ± 0.35. Mass motility was classified as ++, with individual motility of 70.97%, viability of 86.72%, abnormality of 6.02%, sperm concentration of 1,529 × 10^6 sperm/mL, and PMI of 83.1%, indicating good semen quality.

Quality of Holstein-Friesian Semen After the Sexing Process

Post-sexing semen quality following egg white albumin sedimentation is presented in Table 2. In the upper fraction, neither the number of albumin gradients nor incubation duration significantly affected motility, viability, abnormality, concentration, PMI, or TMS ($p>0.05$). Numerically, G2I40 resulted in the highest motility (54.00 ± 9.07%), G3I20 showed the highest viability (80.00 ± 7.93%), and G3I40 produced the lowest abnormality (6.15 ± 3.13%). Meanwhile, the highest concentration and PMI were observed in G2I20, at 93.88 ± 32.87 million/mL and 80.80 ± 6.16%, respectively. The highest TMS was also recorded in G2I20 (49.56 ± 3.05 million/mL).

In the lower fraction, gradient number significantly affected concentration and TMS ($p<0.05$). The mean concentration was higher in G2 (77.94 ± 53.62 million/mL) than in G3 (48.06 ± 18.36 million/mL). Similarly, TMS was higher in G2 (40.53 ± 3.71 million/mL) than in G3 (26.11 ± 1.65 million/mL). Among the treatment combinations, G2I20 showed the highest TMS (41.58 ± 2.96 million/mL). Numerically, G3I40 resulted in the highest motility (54.50 ± 6.85%), whereas G3I20 produced the highest viability (80.50 ± 7.71%). The lowest abnormality was observed in G2I40 (5.85 ± 2.94%), while the highest concentration and PMI were recorded in G2I20, at 81.38 ± 55.69 million/mL and 75.20 ± 10.16%, respectively. No significant effects of incubation duration were observed on the measured semen quality variables in the lower fraction ($p>0.05$).

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

Variables	Mean ± SD
Macroscopic	
Volume (mL)	8.77 ± 1.23
Color	Milky white
Odor	Specific
pH	6.5 ± 0.35
Consistency	Thick/Viscous
Microscopic	
Mass motility	++
Individual motility (%)	70.97 ± 0.40
Viability (%)	86.72 ± 2.00
Abnormality (%)	6.02 ± 0.31
Concentration (million/mL)	1529 ± 32.87
Plasma membrane integrity (%)	83.1 ± 8.68
Total motile spermatozoa (million/mL)	1085.13 ± 16.34

Note: Quantitative data are presented as mean ± standard deviation (SD). Mass motility was scored as +++ (very good, strong swirling movement), ++ (good, less distinct wave patterns and slower movement), and + (very low, no wave formation and only individual progressive movement).

Proportion of X-and Y-bearing Spermatozoa of Holstein-Friesian Bulls

The results presented in Table 3 showed that the proportion of X-bearing spermatozoa was higher in the upper fraction across all treatment combinations. The proportion of X-bearing spermatozoa ranged from 70.70% to 74.00%, whereas the proportion of Y-bearing

Table 2. Post-sexing semen quality of Holstein-Friesian bulls following egg white albumin sedimentation

Variables	Gradient number	Upper fraction			Lower fraction		
		Incubation duration		Average	Incubation duration		Average
		I20	I40		I20	I40	
Motility (%)	G2	52.80 ± 9.28	54.00 ± 9.07	53.40 ± 8.95	51.10 ± 9.00	53.00 ± 8.23	52.05 ± 8.45
	G3	51.10 ± 9.00	52.50 ± 5.89	51.80 ± 7.44	53.00 ± 7.62	54.50 ± 6.85	53.75 ± 7.09
	Average	51.95 ± 8.94	53.25 ± 7.48	—	52.05 ± 8.17	53.75 ± 7.41	—
Viability (%)	G2	78.10 ± 7.69	79.37 ± 3.76	78.74 ± 5.93	77.80 ± 5.79	77.49 ± 6.41	77.65 ± 5.95
	G3	80.00 ± 7.93	74.80 ± 8.02	77.40 ± 8.21	80.50 ± 7.71	76.67 ± 6.93	78.58 ± 7.40
	Average	79.05 ± 7.67	77.09 ± 6.53	—	79.15 ± 6.78	77.08 ± 6.51	—
Abnormality (%)	G2	8.20 ± 3.39	8.34 ± 4.04	8.27 ± 3.63	6.80 ± 3.79	5.85 ± 2.94	6.33 ± 3.34
	G3	7.40 ± 3.20	6.15 ± 3.13	6.77 ± 3.15	7.50 ± 4.62	9.06 ± 3.25	8.28 ± 3.97
	Average	7.80 ± 3.24	7.24 ± 3.69	—	7.15 ± 4.13	7.46 ± 3.44	—
Concentration (million/mL)	G2	93.88 ± 32.87	83.50 ± 21.62	88.69 ± 27.60	81.38 ± 55.69	74.50 ± 54.23	77.94 ± 53.62^x
	G3	82.12 ± 28.93	79.12 ± 14.99	80.62 ± 22.48	47.88 ± 19.90	48.25 ± 17.76	48.06 ± 18.36^y
	Average	88.00 ± 30.73	81.31 ± 18.25	—	64.62 ± 44.18	61.38 ± 41.52	—
Plasma membrane integrity (%)	G2	80.80 ± 6.16	79.95 ± 8.62	80.37 ± 7.31	75.20 ± 10.16	74.68 ± 7.38	74.94 ± 8.65
	G3	79.70 ± 11.44	79.76 ± 8.41	79.73 ± 9.77	68.20 ± 11.81	74.85 ± 11.89	71.52 ± 12.03
	Average	80.25 ± 8.96	79.86 ± 8.29	—	71.70 ± 11.31	74.77 ± 9.63	—
Total motile spermatozoa (million/mL)	G2	49.56 ± 3.05	45.09 ± 1.96	47.32 ± 2.50	41.58 ± 2.96	39.49 ± 4.46	40.53 ± 3.71^x
	G3	41.96 ± 2.60	41.53 ± 0.88	41.74 ± 1.74	25.38 ± 2.20	26.84 ± 1.10	26.11 ± 1.65^y
	Average	45.76 ± 2.82	45.66 ± 1.42	—	33.48 ± 2.58	33.16 ± 2.78	—

Note: Data are presented as mean ± standard deviation (SD). Superscript (a, b) = significant differences between means within the same row ($p<0.05$); superscript (x, y) = significant differences between means within the same column ($p<0.05$). I20 = incubation duration of 20 min; I40 = incubation duration of 40 min; G2 = two-gradient number; and G3 = three-gradient number. Upper and lower fractions indicate sperm fractions collected from the respective layers after albumin sedimentation.

Table 3. Proportions of X- and Y-bearing spermatozoa following egg white albumin sedimentation in Holstein-Friesian bulls

Variables	Gradient number	Upper fraction			Lower fraction		
		Incubation duration		Average	Incubation duration		Average
		I20	I40		I20	I40	
Spermatozoa X (%)	G2	71.10 ± 11.70	74.00 ± 5.56	73.13 ± 2.70	25.80 ± 13.01	19.90 ± 11.12	21.54 ± 3.18
	G3	71.30 ± 11.80	70.70 ± 8.19	71.65 ± 2.77	20.30 ± 8.06	20.80 ± 11.17	19.51 ± 3.02
	Average	72.03 ± 2.75	72.76 ± 2.73	—	21.80 ± 3.22	19.28 ± 2.98	—
Spermatozoa Y (%)	G2	28.90 ± 11.70	26.00 ± 5.56	26.87 ± 2.70	74.20 ± 13.01	80.10 ± 11.12	78.46 ± 3.18
	G3	28.70 ± 11.80	29.30 ± 8.19	28.35 ± 2.77	79.70 ± 8.06	79.20 ± 11.17	80.49 ± 3.02
	Average	27.97 ± 2.75	27.24 ± 2.73	—	78.20 ± 3.22	80.72 ± 2.98	—

Note: Treatment values are presented as mean ± standard deviation (SD), whereas marginal means are presented as estimated marginal means ± standard error (SE). G2 = two-gradient number; G3 = three-gradient number; I20 = incubation duration of 20 min; and I40 = incubation duration of 40 min. Upper and lower fractions indicate sperm fractions collected from the respective layers after albumin sedimentation.

spermatozoa ranged from 26.00% to 29.30%. The highest proportion of X-bearing spermatozoa was observed in G2I40 (74.00%), while the highest proportion of Y-bearing spermatozoa was found in G3I40 (29.30%). The factorial analysis showed that gradient number and incubation duration had no significant effects on the proportions of X- and Y-bearing spermatozoa in the upper fraction ($p>0.05$).

In the lower fraction, the proportion of Y-bearing spermatozoa was higher than that of X-bearing spermatozoa across all treatment combinations. The proportion of Y-bearing spermatozoa ranged from 74.20% to 80.10%, while the proportion of X-bearing spermatozoa ranged from 19.90% to 25.80%. The highest proportion of Y-bearing spermatozoa was observed in G2I40 (80.10%), accompanied by the lowest proportion of X-bearing spermatozoa (19.90%). However, the factorial analysis showed no significant effects of gradient number or incubation duration on the proportions of X- and Y-bearing spermatozoa in the lower fraction ($p>0.05$).

The chi-square goodness-of-fit test showed that, in the upper fraction, G2I20, G3I20, and G3I40 did not differ significantly from the expected 70:30 ratio ($p>0.05$), whereas G2I40 showed a significant deviation ($\chi^2 = 7.619$; $p<0.05$). In the lower fraction, all treatment combinations differed significantly from the expected 30:70 ratio ($p<0.05$), namely G2I20 ($\chi^2 = 8.400$; $p<0.05$), G2I40 ($\chi^2 = 48.576$; $p<0.05$), G3I20 ($\chi^2 = 44.805$; $p<0.05$), and G3I40 ($\chi^2 = 40.305$; $p<0.05$).

Kinematic Analysis of Spermatozoa after Freezing using Computer-Assisted Sperm Analysis

The kinematic characteristics of spermatozoa in the upper and lower fractions following egg white albumin sedimentation are presented in Table 4. In the upper fraction, total motility ranged from 47.78% to 72.34%, whereas progressive motility ranged from 29.26% to 38.28%, with the highest values recorded in G2I40 for both variables. Among the velocity-related variables, VCL, VSL, and VAP ranged from 100.89 to 123.35, 57.84 to 66.51, and 65.11 to 74.67 μm/s, respectively. The highest values for all three variables were observed in G2I20. The highest STR (88.71%), LIN (61.75%), BCF (26.16 beats/s), and WOB (68.13%) were recorded in G2I40, whereas the highest ALH (6.10 μm) was observed in G3I40. Statistical comparisons revealed a significant effect of incubation duration on the overall mean VSL, with higher values at I20 (64.14 μm/s) than at I40 (59.30 μm/s). Simple effect comparisons further indicated a significant difference between incubation durations for VCL within G2. For BCF, significant differences were observed between gradient numbers at I40 and between incubation durations within G3 ($p<0.05$).

In the lower fraction, total motility ranged from 49.34% to 60.20%, while progressive motility ranged from 29.42% to 33.02%. The highest values were observed in G3I20 and G2I20, respectively. For the velocity variables, VCL, VSL, and VAP ranged from 99.13 to 113.76, 55.62 to 63.44, and 61.92 to 70.40 μm/s, respectively. The highest VCL (113.76 μm/s) was recorded in G3I40, whereas the highest VSL (63.44 μm/s) and VAP (70.40 μm/s) were observed in G2I20. Regarding the remaining kinematic variables, the highest STR (90.64%), LIN (61.30%), and BCF (26.19 beats/s) were recorded in G3I20. Meanwhile, the highest ALH (5.70 μm) and WOB (67.45%) were observed in G3I40 and G2I20, respectively. However, neither gradient number, incubation duration, nor their interaction significantly affected any of the measured kinematic variables in the lower fraction ($p>0.05$).

DISCUSSION

The fresh semen characteristics were within the physiological range reported for HF bulls. Semen volume was comparable to that reported by Shah *et al.* (2019) and may vary with ejaculation frequency, bull age, health status, breed, and collection time (Ngoda *et al.*, 2023). The normal milky-white color and characteristic odor further indicated acceptable semen quality, whereas deviations may suggest contamination or reproductive tract disorders (Almadaly *et al.*, 2019; Prastiya *et al.*, 2023). Although individual motility was lower than that reported by Indriastuti et al. (2020), it exceeded the Indonesian National Standard threshold of >70%, indicating that the semen met the minimum motility criterion for subsequent processing. Such variation may be influenced by environmental conditions, including ambient temperature, relative humidity, and temperature–humidity index, which can affect spermatogenesis, sperm kinematics, mitochondrial activity, and cryotolerance (Landaeta-Hernández *et al.*, 2020; Llamas-Luceño *et al.*, 2020; Sabés-Alsina *et al.*, 2019).

Table 4. Kinematic characteristics of spermatozoa in frozen semen following egg white albumin sedimentation in Holstein-Friesian bull

Variables	Gradient	Upper Fraction			Lower Fraction		
		Incubation duration		Average	Incubation duration		Average
		I20	I40		I20	I40	
Total motility (%)	G2	55.74 ± 15.00	72.34 ± 26.80	64.04 ± 5.33	53.12 ± 18.61	52.18 ± 14.44	52.65 ± 4.60
	G3	55.80 ± 8.81	47.78 ± 10.65	51.79 ± 5.33	60.20 ± 12.52	49.34 ± 11.59	54.77 ± 4.60
	Average	55.77 ± 5.33	60.06 ± 5.33	—	56.66 ± 4.60	50.76 ± 4.60	—
Progressive motility (%)	G2	37.16 ± 7.13	38.28 ± 13.57	37.72 ± 2.56	33.02 ± 15.55	29.42 ± 3.22	31.22 ± 2.97
	G3	32.92 ± 2.08	29.26 ± 4.77	31.09 ± 2.56	30.24 ± 3.04	31.06 ± 9.58	30.65 ± 2.97
	Average	35.04 ± 2.56	33.77 ± 2.56	—	31.63 ± 2.97	30.24 ± 2.97	—
VAP (μm/s)	G2	74.67 ± 7.98	65.11 ± 4.99	69.89 ± 1.99	70.40 ± 10.01	67.58 ± 7.78	68.99 ± 2.75
	G3	70.35 ± 6.73	70.26 ± 4.96	70.31 ± 1.99	61.92 ± 8.77	69.64 ± 8.06	65.78 ± 2.75
	Average	72.51 ± 1.99	67.69 ± 1.99	—	66.16 ± 2.75	68.61 ± 2.75	—
VSL (μm/s)	G2	66.51 ± 7.53	57.84 ± 3.34	62.17 ± 1.67	63.44 ± 9.94	59.37 ± 6.99	61.41 ± 2.69
	G3	61.77 ± 6.30	60.77 ± 2.01	61.27 ± 1.67	55.62 ± 9.46	61.45 ± 7.25	58.54 ± 2.69
	Average	64.14 ± 1.67^{a}	59.30 ± 1.67^{b}	—	59.53 ± 2.69	60.41 ± 2.69	—
VCL (μm/s)	G2	123.35 ± 14.36^{a}	100.89 ± 13.15^{b}	112.12 ± 4.14	109.00 ± 17.85	108.64 ± 12.66	108.82 ± 4.58
	G3	115.23 ± 11.72^{a}	116.69 ± 12.96^{a}	115.96 ± 4.14	99.13 ± 13.74	113.76 ± 13.12	106.45 ± 4.58
	Average	119.29 ± 4.14	108.79 ± 4.14	—	104.06 ± 4.58	111.20 ± 4.58	—
STR (%)	G2	87.94 ± 1.11	88.71 ± 4.77	88.32 ± 1.63	89.60 ± 1.77	88.02 ± 2.95	88.81 ± 0.88
	G3	86.93 ± 1.98	87.04 ± 8.85	86.98 ± 1.63	90.64 ± 4.28	87.73 ± 0.73	89.18 ± 0.88
	Average	87.43 ± 1.63	87.87 ± 1.63	—	90.12 ± 0.88	87.88 ± 0.88	—
LIN (%)	G2	55.02 ± 1.95	61.75 ± 9.20	58.39 ± 1.78	61.30 ± 6.70	59.46 ± 6.00	60.38 ± 1.67
	G3	55.15 ± 3.76	52.97 ± 4.88	54.06 ± 1.78	61.28 ± 4.96	56.97 ± 2.40	59.13 ± 1.67
	Average	55.09 ± 1.78	57.36 ± 1.78	—	61.29 ± 1.67	58.22 ± 1.67	—
ALH (μm)	G2	5.99 ± 0.58	4.69 ± 0.95	5.34 ± 0.25	4.97 ± 1.41	5.15 ± 0.61	5.06 ± 0.28
	G3	5.75 ± 0.73	6.10 ± 0.87	5.93 ± 0.25	4.73 ± 0.67	5.70 ± 0.56	5.21 ± 0.28
	Average	5.87 ± 0.25	5.40 ± 0.25	—	4.85 ± 0.28	5.42 ± 0.28	—
BCF (beats/s)	G2	24.41 ± 2.04^{a}	26.16 ± 1.02^{ax}	25.29 ± 0.45	26.03 ± 2.57	25.57 ± 0.90	25.80 ± 0.52
	G3	24.94 ± 1.09^{a}	23.55 ± 1.34^{by}	24.24 ± 0.45	26.19 ± 1.15	25.65 ± 1.50	25.92 ± 0.52
	Average	24.68 ± 0.45	24.86 ± 0.45	—	26.11 ± 0.52	25.61 ± 0.52	—
WOB (%)	G2	61.87 ± 1.65	68.13 ± 6.78	65.00 ± 1.25	67.45 ± 6.03	66.15 ± 4.63	66.80 ± 1.38
	G3	62.51 ± 2.89	61.07 ± 2.42	61.79 ± 1.25	66.79 ± 3.73	63.74 ± 2.22	65.27 ± 1.38
	Average	62.19 ± 1.25	64.60 ± 1.25	—	67.12 ± 1.38	64.94 ± 1.38	—

Note: Treatment values are presented as mean ± standard deviation (SD), whereas marginal means are presented as estimated marginal means ± standard error (SE). Superscripts (a, b) indicate significant differences between incubation durations within the same row, while superscripts (x, y) indicate significant differences between gradient numbers within the same incubation-duration column ($p<0.05$). Simple-effect comparisons were adjusted using the Holm method. I20 = incubation duration of 20 min; I40 = incubation duration of 40 min; G2 = two-gradient number; and G3 = three-gradient number. Upper and lower fractions indicate sperm fractions collected from the respective layers after albumin sedimentation. VAP: Average Path Velocity; VCL: Curvilinear Velocity; VSL: Straight Line Velocity; LIN: Linearity; STR: Straightness; ALH: Amplitude of Lateral Head; BCF: Beat Cross Frequency; and WOB: Wobble.

The decrease in sperm motility observed after sperm separation may be associated with the incubation period and processing procedures, both of which can affect sperm quality. Kapitonova *et al.* (2023) reported no significant difference in motility between sexed and non-sexed semen, although the lowest motility observed in sexed semen was 32%, with a higher proportion of immotile spermatozoa. Similarly, Safa *et al.* (2025) reported a progressive decline in motility from fresh (77.9%) to sexed (65%) and frozen semen (44%–51%), suggesting cumulative effects of separation and cryopreservation. Because sperm motility is an important indicator of sperm functional status, the preservation of motility after sexing suggests that egg white albumin sedimentation maintained relatively good sperm quality despite processing-related stress.

Sperm viability in fresh semen was comparable to that reported by Prajapati *et al.* (2022), which ranged from 85% to 90%, indicating that the semen was within the normal range for further processing. The high viability indicates that most spermatozoa remained alive and had intact plasma membranes. During staining, viable spermatozoa remain transparent because their intact plasma membranes prevent dye uptake (Figure 2) (Susilawati *et al.*, 2022). However, viability may decline with improper semen handling, prolonged incubation during separation, and cryopreservation. Priyanto *et al.* (2023) reported that viability of BSA-sexed semen decreased from 85.30% to 80.40% and 72.10% as incubation duration increased from 40 to 50 and 60 min, respectively. This decline may result from prolonged cellular metabolism and lactate accumulation, which can promote oxidative stress and membrane damage.

The sperm abnormality rate in this study was relatively low, compared with that reported by Witri *et al*. (2025), which ranged from 9.44% to 21.38% in Bali bulls of different ages. Differences in sperm morphology may be related to the physiological condition of spermatozoa and their susceptibility to cellular damage. Sperm abnormality reflects both the

physiological condition of spermatozoa and post-collection handling and may involve structural defects of the head, midpiece, or tail. According to Dursunoglu (2025), tail defects can impair progressive motility, head abnormalities may be associated with acrosomal defects and reduced fertilization capacity, and midpiece defects can compromise sperm movement. Such abnormalities may originate from disturbances during spermatogenesis, genetic mutations, mitochondrial damage, oxidative stress, or handling procedures such as sexing and incubation (Beurois *et al*., 2020; Marzouni *et al*., 2022; Kusumawati *et al*., 2019). The relatively low abnormality rates observed in this study suggest that the egg white albumin sedimentation conditions did not cause substantial morphological damage to spermatozoa.

Sperm concentration is influenced by genetic factors, environmental adaptation, and bull reproductive efficiency and is an important determinant of frozen semen production and AI success because insufficient sperm numbers may reduce the likelihood of spermatozoa reaching the female reproductive tract (Gogol & Trzcińska, 2022). In the present study, sperm concentration remained sufficiently high and exceeded that reported by Saranholi *et al*. (2021), indicating that the recovered fractions contained sufficient spermatozoa for subsequent cryopreservation. However, higher concentrations have been reported for PDGC, with Yekti *et al.* (2024) reporting >200 million/mL and Meles *et al.* (2022) reporting 1,507 and 1,610 million/mL using three and five gradients, respectively. The higher recovery with PDGC may be attributed to the combination of sperm sedimentation and centrifugation, which can enhance sperm recovery compared with albumin sedimentation based primarily on passive sperm migration.

PMI was evaluated using the HOST, which measures functional sperm membrane integrity based on the ability of spermatozoa to maintain osmotic balance. Figure 3 shows the condition of spermatozoa in the HOST medium.

Spermatozoa with intact plasma membranes respond to the HOST with tail swelling and coiling, whereas straight tails generally indicate membrane damage or an impaired osmotic response (Chang *et al*., 2025). The PMI percentage in this study was lower than that reported by Iskandar *et al.* (2022), which may reflect differences in breed, individual bull characteristics, semen handling, or evaluation methods. Nevertheless, preserved membrane integrity is important for maintaining essential sperm functions, including motility and acrosomal activity, which are required for fertilization and AI (Utami *et al*., 2026). The findings of the present study are consistent with previous reports indicating that the duration of sperm separation may influence membrane integrity. Yendraliza *et al*. (2026) reported better post-thaw functional integrity following a 30-min BSA separation compared with longer separation periods, although the response of PMI varied with bull age and sperm fraction. Such variation may reflect differences in the intrinsic resilience of sperm membranes and their susceptibility to osmotic and oxidative stress during prolonged exposure to the separation medium. Similarly, Damayanti *et al*. (2022) reported plasma membrane integrity of 87.00% and 80.64% in the upper and lower fractions, respectively, after 30 min of incubation, which decreased with longer incubation. Membrane damage during sperm processing may arise from mechanical and oxidative stress, osmotic imbalance, and temperature fluctuations, with centrifugation potentially exacerbating damage through sperm–sperm and sperm–tube collisions. Y-bearing spermatozoa may differ in their susceptibility to processing-related stress because of differences in their physical and biological characteristics (Safa *et al*., 2025). As the plasma membrane is essential for maintaining cellular homeostasis and supporting mitochondrial energy metabolism, its integrity is critical for preserving sperm functionality.

The proportions of X- and Y-bearing spermatozoa in the present study differed from those reported for other sperm sexing methods, with Magnetic-Activated Cell Sorting (MACS) yielding 79.50% X-bearing spermatozoa in the X-enriched fraction and 78.56% Y-bearing spermatozoa in the Y-chromosome-enriched fraction (Paitoon *et al*., 2024). Furthermore, PDGC produced 77.1% X-bearing spermatozoa in the X-enriched fraction and 80.6% Y-bearing spermatozoa in the Y-enriched fraction in HF bull semen (Yekti *et*

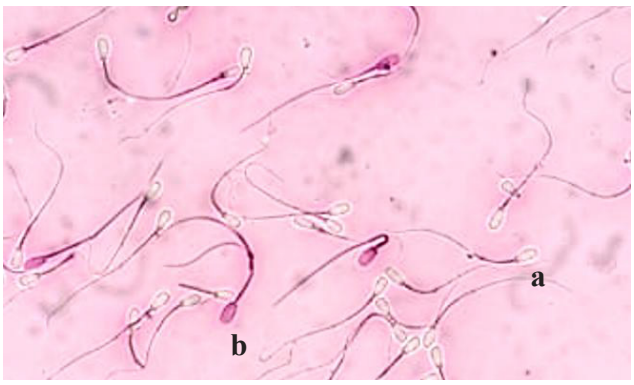

Figure 2. Viability of Holstein-Friesian bull spermatozoa assessed using eosin–nigrosin staining: (a) live spermatozoa; (b) dead spermatozoa.

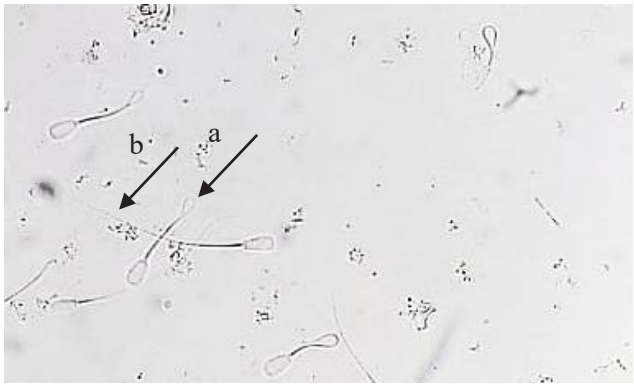

Figure 3. Plasma membrane integrity of Holstein-Friesian bull spermatozoa assessed using the hypo-osmotic swelling test: (a) intact membrane with a coiled tail; (b) damaged membrane with a straight tail.

al., 2023). In contrast, albumin sedimentation in Bali cattle resulted in 64.61% X-bearing spermatozoa and 27.49% Y-bearing spermatozoa in the upper fraction, whereas the lower fraction contained 24.16% X-bearing spermatozoa and 73.98% Y-bearing spermatozoa (Diansyah *et al.*, 2025). These variations in X:Y spermatozoa proportions may reflect differences in the separation mechanisms and experimental conditions among sexing methods and cattle breeds.

The higher proportion of Y-bearing spermatozoa in the lower fraction indicates Y-sperm enrichment following separation, consistent with Yekti *et al.* (2025), who reported Y-bearing sperm enrichment in the lower fraction and X-bearing sperm enrichment in the upper fraction. Albumin sedimentation is thought to separate spermatozoa according to differences in motility and biophysical properties. Differences in the motility and biophysical properties of X- and Y-bearing spermatozoa may contribute to their differential migration through the albumin medium, resulting in greater Y-sperm enrichment in the lower fraction and X-sperm enrichment in the upper fraction (Diansyah *et al.*, 2025).

Spermatozoa distribution in this study may be influenced by incubation duration, the gradient number, and initial semen quality. Yendraliza *et al.* (2023) similarly reported that variation in initial semen quality, including differences among species, individual animals, and collection methods, can affect sperm motility and viability after sexing, with buffalo semen showing lower post-sexing quality than that reported in other livestock. Accordingly, treatment selection was based not only on X- and Y-sperm enrichment but also on post-sexing sperm quality, assessed using motility, concentration, and TMS. Treatment performance was therefore evaluated based on both the separation of X- and Y-bearing spermatozoa and the preservation of sperm quality after sexing. Therefore, a comprehensive evaluation was conducted using sperm motility, concentration, viability, abnormality, PMI, TMS, and kinematic variables.

CASA-based analysis of sperm kinematic variables was employed to provide objective and quantitative measurements of sperm movement. By providing quantitative measurements of sperm movement, CASA enables detection of treatment-related changes in specific kinematic characteristics. The significant differences observed in VCL, VSL, and BCF in the upper fraction indicate that the gradient number and incubation duration influenced specific aspects of sperm movement, particularly sperm velocity and flagellar activity. These changes may reflect differences in the physiological status of sperm and the ability of spermatozoa to maintain regular movement following treatment. VSL is an important indicator of sperm linear motility, whereas higher values of VCL, VSL, and VAP generally reflect higher swimming capacity and may be associated with fertilization potential (İnanç *et al*., 2018). The values observed in this study exceeded those reported by Hasbi *et al*. (2024), which showed VCL, VAP, and VSL values of 67.45, 94.98, and 46.43 μm/s, respectively. Variations in kinematic responses among treatments indicate that the albumin gradient number and incubation duration can influence specific sperm motility characteristics, although the effects were not consistent across all CASA variables.

The efficiency of sperm movement is further described by STR, LIN, and WOB, which characterize the directionality and stability of sperm trajectories. STR is calculated from the VSL/VAP ratio, LIN from the VSL/VCL ratio, whereas WOB is derived from the VAP/VCL ratio (Susilawati *et al*., 2022). Safa *et al*. (2025) reported that sperm motility progressively declined during the sexing and freezing processes, resulting in less linear and less stable movement patterns that were reflected by reductions in STR, LIN, and WOB values. These alterations are likely associated with plasma membrane damage, mitochondrial dysfunction, and oxidative stress induced during semen processing.

The ALH and BCF variables provide information on sperm head movement and flagellar activity and may be associated with fertilization potential. ALH and BCF represent the amplitude of lateral head movement and the frequency of flagellar beat, respectively, and their values may be associated with sperm movement characteristics and fertilization potential (Utami *et al*., 2025). Belala *et al*. (2019) reported that spermatozoa with ALH values of 2.5–6.5 μm and BCF values above 20 Hz were associated with favorable motility characteristics and fertilization potential. In comparison, Wahyudi *et al*. (2025) reported higher ALH values (6.80–8.41 μm) with comparable BCF values (23.87–26.85 beats/s). These differences may reflect variation in semen characteristics, experimental conditions, or CASA settings among studies.

In terms of target spermatozoa enrichment, the highest observed proportion was obtained in G2I40. However, G2I20 exhibited higher mean values for sperm concentration, PMI, and TMS in both fractions. Although the observed differences in most variables were not statistically significant, the numerical variation between treatments highlights the potential influence of incubation duration on the balance between sperm separation and quality preservation. From a practical standpoint, the shorter incubation period in G2I20 offers an advantage in processing time while maintaining comparable sperm enrichment outcomes.

CONCLUSION

Egg white albumin sedimentation using different gradients and incubation durations resulted in limited differences in post-sexing sperm quality and X/Y-bearing spermatozoa enrichment. The treatment using two gradient numbers with 20 min of incubation showed higher mean values for sperm concentration, PMI, and TMS concentration in both fractions, while maintaining comparable sperm motility and X/Y-bearing spermatozoa proportions across treatments. The upper fractions generally approached the predefined target of 70% X-bearing spermatozoa, whereas the lower fractions generally exceeded 70% Y-bearing spermatozoa. Given its comparable enrichment outcomes, higher values for several sperm quality variables, and shorter processing time, the

treatment using two gradient numbers with 20 min of incubation may offer a more practical and time-efficient approach for egg white albumin-based sperm sexing under the conditions evaluated. Further optimization and validation are needed to improve the method's consistency and separation efficiency and to assess its effects on post-thaw sperm quality and subsequent fertility.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest with any party regarding the subject matter discussed in this manuscript.

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DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the author(s) used an AI tool to improve the language and readability of the manuscript. After using this tool, the authors carefully reviewed and edited the content as needed and take full responsibility for the manuscript's content.

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