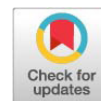


Research Article



Effect of Glycerol and Beta-Carotene Supplementation in Tris Egg Yolk Extender on the Kinetic Parameters of Frozen-Thawed Swamp Buffalo Semen Evaluated by CASA

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Abstract | Swamp buffalo is a strategic local genetic resource in Indonesia that plays an important role in supporting food security. However, its reproductive efficiency remains relatively low, making semen processing quality crucial for the success of artificial insemination (AI) programs in tropical regions. This study aimed to evaluate the effects of glycerol (2-8%) and β -carotene (0.1-0.3%) supplementation in a Tris egg yolk extender on sperm motility parameters of swamp buffalo analyzed using computer-assisted semen analysis (CASA). A completely randomized design with eight treatments and five replications was applied. Semen was diluted in 0.25 mL straws, equilibrated at 3–5 °C for 3 hours, frozen using nitrogen vapor (approximately 10 cm above liquid nitrogen for 15 minutes), stored in liquid nitrogen for seven days, and thawed at 37 °C for 30 seconds prior to CASA evaluation. The observed parameters included progressive motility, average path distance (DAP), curvilinear distance (DCL), straight-line distance (DSL), average path velocity (VAP), curvilinear velocity (VCL), straight-line velocity (VSL), straightness (STR), linearity (LIN), wobble (WOB), amplitude of lateral head displacement (ALH), and beat cross frequency (BCF). The results indicated that glycerol and β -carotene supplementation significantly ($P < 0.05$) affected several kinetic parameters of frozen-thawed swamp buffalo semen. Optimal concentrations of glycerol (2–4%) and β -carotene (0.1–0.3%) improved sperm motility characteristics, as evidenced by increased progressive motility, DAP, DCL, DSL, and velocity parameters (VAP, VCL, and VSL). Based on the findings of this study, the use of glycerol at concentrations of 2–4% combined with β -carotene at 0.1–0.3% in a Tris egg yolk extender is recommended to improve the quality of frozen-thawed swamp buffalo semen.

Keywords | β -carotene, CASA, Glycerol, Swamp buffalo, Tris egg yolk extender, Semen

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Swamp buffalo in Indonesia (*Bubalus bubalis*) possess high adaptability and play an important socio-economic role, particularly in marginal environments with limited agroecosystem resources. Despite their considerable potential, population development is still constrained by low reproductive efficiency, necessitating improved semen processing strategies to support artificial insemination (AI) programs in tropical production systems (Chiariotti *et al.*, 2025). The success of AI programs largely depends on semen quality, particularly sperm viability and motility, which require effective extenders and protective agents during low-temperature storage (Permana *et al.*, 2024).

Tris functions as a buffering agent that maintains semen pH stability during the freezing process, thereby preserving metabolism, motility, viability, and plasma membrane integrity (Kamal *et al.*, 2023). However, cryoinjury and oxidative stress remain major challenges, often leading to reduced motility and altered sperm movement characteristics during cooling, freezing, and thawing processes (Harsa *et al.*, 2023). To overcome these issues, the incorporation of cryoprotective agents and antioxidants into the extender is a widely applied strategy.

Glycerol is one of the most widely used cryoprotectants in semen freezing, functioning to reduce intracellular ice crystal formation and stabilize membrane structure (Ma *et al.*, 2022). However, its use has limitations, as it may induce dose-dependent toxic effects, osmotic imbalance, and metabolic disturbances in spermatozoa at high concentrations (Nalley *et al.*, 2011). This indicates that the use of glycerol alone is not sufficient to provide optimal protection for spermatozoa during the freezing process.

On the other hand, β -carotene, a lipid-soluble antioxidant and a precursor of vitamin A, has been reported to improve semen quality by scavenging reactive oxygen species (ROS), protecting lipid membranes from peroxidation, and enhancing sperm motility and viability in various livestock species (Tufail *et al.*, 2024; Eriani *et al.*, 2018). However, the use of antioxidants alone is not entirely effective in preventing physical damage caused by ice crystal formation during freezing.

Previous studies have evaluated various approaches to improve the quality of buffalo semen during cryopreservation. Reddy *et al.* (2010) reported that the addition of taurine and trehalose could enhance sperm quality; however, the study was limited to the use of single additives and did not incorporate CASA-based kinetic analysis. Similarly, Chaudhari *et al.* (2015) compared the effectiveness of different semen extenders, but their work focused primarily on product comparison without exploring

Kumar and Atreja (2012) investigated molecular changes such as tyrosine phosphorylation during cryopreservation, yet did not directly relate these findings to sperm kinetic parameters associated with fertilizing potential. Sakr *et al.* (2021) examined extender substitution using dried egg yolk and taurine supplementation, but the evaluation remained limited to basic semen quality parameters without in-depth kinetic analysis. Furthermore, Eriani *et al.* (2018) demonstrated that β -carotene supplementation improved semen quality and pregnancy rates; however, the study only assessed a single antioxidant.

These previous findings indicate that both cryoprotectants and antioxidants play important roles in maintaining semen quality. However, studies comparing their combined use within a single extender system, particularly those evaluated using CASA-based kinetic parameters, remain very limited. Computer-assisted semen analysis (CASA) enables objective and quantitative evaluation of sperm motion characteristics, including VCL, VSL, VAP, LIN, STR, ALH, and BCF, which are closely associated with fertilizing potential (Tanga *et al.*, 2021).

Therefore, this study proposes a more comprehensive approach by evaluating the combined use of glycerol as a permeating cryoprotectant and β -carotene as an antioxidant in a Tris egg yolk extender, and their effects on the kinetic parameters of frozen-thawed swamp buffalo spermatozoa. This approach is expected to provide a more optimal and practical extender formulation to improve the success of artificial insemination programs under tropical conditions. Therefore, this study aimed to evaluate the effect of the combination of glycerol and β -carotene in a Tris egg yolk extender on the kinetic parameters of frozen-thawed swamp buffalo spermatozoa analyzed using CASA.

MATERIALS AND METHODS

MATERIALS

This study was conducted at the Production Laboratory of the Artificial Insemination Center (AIC) Tuah Sakato, Payakumbuh, West Sumatra, Indonesia, from February 10 to March 10, 2025. The main materials used included fresh semen collected from swamp buffalo bulls aged 3–5 years maintained at the AIC, a Tris-citric acid-glucose-egg yolk-based extender as the basal medium, glycerol as a permeating cryoprotectant, and β -carotene as an antioxidant supplement. In addition, supporting materials included 70% ethanol, eosin-nigrosin stain, distilled water, liquid nitrogen, and 0.25 mL straws.

ANIMAL AND SEMEN COLLECTION

Fresh ejaculates were collected from swamp buffalo bulls aged 3–5 years that were maintained under standard management protocols at the Artificial Insemination Center (AIC). Pre-collection handling and hygienic procedures were routinely performed to ensure sample quality and biosafety in accordance with best practices in reproductive laboratories. Only ejaculates meeting the initial quality criteria (mass motility ≥ 3 , individual motility $\geq 70\%$, and normal morphology $\geq 80\%$) were selected and used in the experimental treatments. This approach was applied to minimize variability among individual ejaculates within a completely randomized design (CRD).

EXPERIMENTAL DESIGN

This study employed a completely randomized design (CRD) consisting of eight treatments with five replications per treatment. The treatments were as follows: A: basal extender without any supplementation (control); B: glycerol 2%; C: glycerol 4%; D: glycerol 6%; E: glycerol 8%; F: β -carotene 0.1%; G: β -carotene 0.2%; and H: β -carotene 0.3%.

EXTENDERS

A Tris–citric acid glucose egg yolk-based extender was prepared as the basal medium. The extender composition consisted of Tris (hydroxymethyl aminomethane), citric acid monohydrate, glucose, and fresh egg yolk (20% v/v). The glycerol concentrations were selected to represent a range from low to high levels of permeating cryoprotectant, reflecting different osmotic effects and cryoprotective properties. Meanwhile, β -carotene concentrations were determined within a range known to exhibit antioxidant activity based on previous reproductive studies. Individual straws were used as experimental units for CASA evaluation and statistical analysis.

PROCESSING, FREEZING, STORAGE, AND THAWING

Semen was gradually mixed with the extender according to the respective treatments, then loaded into 0.25 mL straws, sealed, and equilibrated at 3–5 °C for approximately 3 hours in a refrigerator. After equilibration, the straws were frozen by placing them approximately 10 cm above the surface of liquid nitrogen in a closed styrofoam container for 15 minutes (approximately –135 °C), and then plunged into liquid nitrogen (approximately –196 °C) for storage for seven days.

Following the storage period, the straws were thawed by immersion in a water bath at 37 °C for 30 seconds. Post-thaw handling was performed carefully to minimize thermal and osmotic shock prior to CASA evaluation, thereby ensuring consistency among replicates.

CASA EVALUATION

Sperm kinetics were analyzed using a computer-assisted semen analyzer (CASA) system with Sperm Vision™ software (version 3.7.5). The evaluated parameters included progressive motility (%), total motility (%), average path distance (DAP, μm), curvilinear distance (DCL, μm), straight-line distance (DSL, μm), average path velocity (VAP, $\mu\text{m/s}$), curvilinear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), straightness (STR), linearity (LIN), wobble (WOB), amplitude of lateral head displacement (ALH, μm), and beat cross frequency (BCF, Hz).

The analysis was conducted using standard settings and a heated stage (37 °C) to minimize temperature-related variability. CASA parameters were interpreted based on established definitions, where VCL, VSL, and VAP represent sperm velocity, while LIN and STR indicate the linearity of sperm trajectories, which are closely associated with fertilizing potential.

DATA ANALYSIS

The research data were analyzed using analysis of variance for a Completely Randomized Design. The significance of differences between treatments was tested with Duncan's Multiple Range Test (DMRT), with a significant value at $p < 0.05$ (Steel and Torrie, 1995). Statistical analyses were performed using SPSS software version 22 (IBM Corp., Armonk, NY, USA).

RESULTS AND DISCUSSION

The effects of glycerol and β -carotene supplementation on CASA parameters of frozen-thawed swamp buffalo semen are presented in Table 1.

Progressive motility is defined as the percentage of spermatozoa moving actively forward in a directed manner (straight or slightly curved) within a semen sample analyzed using the CASA system. Progressive motility ranged from 22.02 to 52.49%. Glycerol and β -carotene supplementation had a significant effect ($P < 0.05$) on the progressive motility of frozen-thawed swamp buffalo semen. Statistical analysis showed that glycerol 2% (B) was not significantly different ($P > 0.05$) from glycerol 4% (C), β -carotene 0.1% (F), and β -carotene 0.3% (H), but differed significantly ($P < 0.05$) from glycerol 6% (D), glycerol 8% (E), and β -carotene 0.2% (G).

The higher progressive motility observed in the optimal treatments indicates that glycerol and β -carotene supplementation was able to maintain plasma membrane integrity and mitochondrial function during cryopreservation. This condition supports optimal ATP production, enabling spermatozoa to move forward

Table 1: Effects of glycerol and β -carotene supplementation on CASA parameters of frozen-thawed swamp buffalo semen.

Parameter	Treatments							
	Control (A)	Glycerol 2% (B)	Glycerol 4% (C)	Glycerol 6% (D)	Glycerol 8% (E)	β - carotene 0.1% (F)	β - carotene 0.2% (G)	β - carotene 0.3% (H)
Progressive motility (%)*	45.71 ^{ab}	48.70 ^{ab}	45.66 ^{ab}	22.02 ^c	22.12 ^c	52.49 ^a	41.69 ^b	46.66 ^{ab}
DAP (μ m)*	20.32 ^a	19.90 ^a	19.70 ^a	15.68 ^b	16.65 ^b	21.44 ^a	20.33 ^a	19.53 ^a
DCL (μ m)*	30.95 ^a	29.71 ^{ab}	30.29 ^{ab}	24.42 ^c	26.26 ^{bc}	33.75 ^a	31.49 ^a	30.67 ^a
DSL (μ m)*	16.59 ^a	15.69 ^a	15.29 ^a	12.65 ^b	15.42 ^a	16.76 ^a	16.16 ^a	14.89 ^a
VAP (μ m/second)*	47.69 ^a	45.99 ^a	43.96 ^{ab}	34.99 ^c	40.36 ^{bc}	48.03 ^a	47.35 ^a	46.16 ^a
VCL (μ m/second)*	72.21 ^a	68.37 ^a	67.29 ^{ab}	53.84 ^c	59.91 ^{ab}	75.45 ^a	73.15 ^a	72.17 ^a
VSL (μ m/second)*	38.98 ^a	36.28 ^{abc}	34.07 ^{bc}	28.23 ^d	33.13 ^c	37.57 ^{ab}	37.70 ^{ab}	35.33 ^{abc}
STR*	0.81 ^{ab}	0.79 ^{abcd}	0.77 ^{bc}	0.80 ^{abc}	0.82 ^a	0.78 ^{bcd}	0.79 ^{abcd}	0.76 ^d
LIN ^{NS}	0.54	0.53	0.57	0.52	0.56	0.49	0.51	0.49
WOB ^{NS}	0.65	0.67	0.65	0.65	0.68	0.63	0.64	0.63
ALH (μ m)*	3.93 ^{ab}	3.62 ^{abc}	3.29 ^c	2.10 ^d	3.39 ^{bc}	3.68 ^{abc}	3.95 ^{ab}	4.10 ^a
BCF (Hz) ^{NS}	23.38	23.55	20.38	24.19	20.51	23.02	23.65	21.30

Note: ^{a-b}Different superscripts in the same column are significantly different ($P < 0.05$). NS: Non significant ($P > 0.05$). DAP: average path distance; DCL: curvilinear distance; DSL: straight-line distance; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; STR: straightness; LIN: linearity; (LIN); WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat cross frequency.

effectively, which is closely associated with fertilizing ability. In pigs, water-soluble β -carotene in cooling-freezing media has been reported to reduce oxidation and apoptosis; however, excessive concentrations may decrease motility and mitochondrial function (Juan *et al.*, 2025). The best progressive motility values in this study were observed in treatments B (glycerol 2%), C (glycerol 4%), F (β -carotene 0.1%), and H (β -carotene 0.3%).

Average path distance (DAP) is the average distance traveled by spermatozoa along the smoothed average trajectory during the observation period in CASA analysis. DAP ranged from 15.68 to 21.44 μ m. Glycerol and β -carotene supplementation significantly affected ($P < 0.05$) DAP of frozen-thawed swamp buffalo semen. Statistical analysis indicated that glycerol 2% (B) was not significantly different ($P > 0.05$) from glycerol 4% (C), β -carotene 0.1% (F), and β -carotene 0.3% (H), but differed significantly ($P < 0.05$) from glycerol 6% (D), glycerol 8% (E), and β -carotene 0.2% (G).

The higher DAP values observed in treatments B, C, F, and H were attributed to optimal protection of plasma membrane integrity and the sperm motility system during cryopreservation. Glycerol at low to moderate concentrations (2–4%) acts as a cryoprotectant that stabilizes membrane structure and reduces intracellular ice crystal formation, thereby preserving sperm movement along the average path. Glycerol has been reported to reduce ice crystal formation and excessive dehydration, thus protecting membrane and sperm structure (Zong *et*

al., 2022).

β -carotene, as an antioxidant, functions to neutralize reactive oxygen species generated during freezing and thawing, thereby preventing lipid peroxidation of the membrane that could impair flagellar function. In Aceh swamp buffalo, supplementation of 0.625% β -carotene in Tris egg yolk extender maintained post-thaw motility, viability, acrosome integrity, and plasma membrane integrity, associated with inhibition of lipid peroxidation by ROS (Eriani *et al.*, 2018). This protection allows spermatozoa to maintain more stable motility activity, reflected in increased DAP values.

The reduction in DAP observed at higher glycerol concentrations (6–8%) and β -carotene 0.2% is likely related to toxic effects or osmotic stress, which may disrupt ionic balance and damage membrane and mitochondrial structures. This condition reduces the efficiency of sperm movement, resulting in shorter average path distances. Heat stress in pig sperm has been shown to reduce motility, average path distance (APD), VSL, STR, and LIN without affecting VAP, VCL, and ALH, indicating disruption of movement patterns and trajectory efficiency associated with changes in metabolomics and membrane lipids (Sui *et al.*, 2022).

Curvilinear distance (DCL) is the total actual distance traveled by spermatozoa along their original curved trajectory during the observation period in CASA analysis. DCL ranged from 24.42 to 33.75 μ m. Glycerol and

β -carotene supplementation significantly affected ($P < 0.05$) DCL of frozen-thawed swamp buffalo semen. Statistical analysis showed that glycerol 2% (B) was not significantly different ($P > 0.05$) from glycerol 4% (C), β -carotene 0.1% (F), β -carotene 0.2% (G), and β -carotene 0.3% (H), but differed significantly ($P < 0.05$) from glycerol 6% (D) and glycerol 8% (E).

Higher DCL values in treatments B, C, F, G, and H indicate more intensive sperm movement along the natural trajectory. This may be attributed to the ability of glycerol at low-moderate concentrations to maintain membrane flexibility and flagellar function, allowing active tail movement and longer, more dynamic trajectories. In sheep and goats, 3–6% glycerol has been associated with better membrane integrity compared to treatments without glycerol or with disaccharides alone (Khalil *et al.*, 2024). β -carotene also plays a role in protecting membrane structure from oxidative damage, thereby maintaining optimal flagellar coordination.

The decrease in DCL at higher glycerol concentrations (6–8%) is likely due to osmotic stress and reduced membrane elasticity, which can impair flagellar activity and reduce the intensity of sperm movement.

Straight-line distance (DSL) is the shortest linear distance between the starting and ending points of sperm movement during the observation period. DSL ranged from 12.65 to 16.76 μm . Glycerol and β -carotene supplementation significantly affected ($P < 0.05$) DSL of frozen-thawed swamp buffalo semen. Statistical analysis indicated that glycerol 2% (B) was not significantly different ($P > 0.05$) from glycerol 4% (C), glycerol 8% (E), β -carotene 0.1% (F), β -carotene 0.2% (G), and β -carotene 0.3% (H), but differed significantly ($P < 0.05$) from glycerol 6% (D).

Higher DSL values observed in treatments B, C, E, F, G, and H indicate more efficient linear displacement of spermatozoa. This suggests that supplementation maintained directional stability during cryopreservation. DSL is influenced by membrane integrity, mitochondrial activity, flagellar coordination, and the ability to maintain directional movement. Optimal supplementation helps preserve these functions, enhancing linear displacement (Valverde *et al.*, 2020). The decrease in DSL at glycerol 6% indicates impaired directional efficiency, likely due to osmotic imbalance or subcellular damage affecting flagellar coordination.

Average path velocity (VAP) is the average velocity of spermatozoa along the smoothed average trajectory. VAP ranged from 34.99 to 48.03 $\mu\text{m/s}$. Glycerol and β -carotene supplementation significantly affected ($P < 0.05$) VAP. Higher VAP values in treatments B, C, F, G, and H indicate

stable movement speed, reflecting balanced activity and directional control. Glycerol at optimal concentrations maintains membrane integrity and enzymatic systems, allowing efficient energy utilization. In poultry, glycerol concentration influences CASA velocity parameters, with excessive levels reducing movement quality (Mehdipour *et al.*, 2020). β -carotene helps maintain mitochondrial function and ATP production by reducing oxidative stress. Lower VAP at higher glycerol concentrations reflects metabolic disruption and osmotic stress.

Curvilinear velocity (VCL) is the velocity of spermatozoa along their actual curved trajectory. VCL ranged from 53.84 to 75.45 $\mu\text{m/s}$. Higher VCL values indicate high motility activity, related to flagellar intensity and mitochondrial energy production (Nascimento *et al.*, 2008). However, high VCL does not necessarily reflect efficient movement, as sperm may move rapidly but without direction. Therefore, VCL should be interpreted alongside VSL and STR.

Straight-line velocity (VSL) is the velocity calculated based on the straight-line distance divided by time. VSL ranged from 28.23 to 38.98 $\mu\text{m/s}$. Higher VSL values indicate efficient and directed movement toward the oocyte. VSL is a key indicator of fertilizing potential (Donnellan *et al.*, 2022). β -carotene supports membrane integrity and mitochondrial function, enhancing directional movement, whereas high glycerol concentrations may impair movement efficiency due to structural and osmotic damage.

Straightness (STR) describes how straight the sperm movement is relative to its average path. Glycerol and β -carotene supplementation had a significant effect ($P < 0.05$) on STR of frozen-thawed swamp buffalo semen. STR ranged from 0.76 to 0.82. Statistical analysis indicated that glycerol 2% (B) was not significantly different ($P > 0.05$) from treatments D (glycerol 6%), E (glycerol 8%), and G (β -carotene 0.2%), but differed significantly ($P < 0.05$) from treatments C (glycerol 4%), F (β -carotene 0.1%), and H (β -carotene 0.3%).

Higher STR values observed in treatments B, D, E, and G indicate that spermatozoa exhibited more linear and directed trajectories. This reflects efficient coordination between flagellar activity and directional control, which is essential for successful fertilization. Membrane protection by glycerol and the antioxidant effect of β -carotene help maintain structural stability and motility systems, enabling spermatozoa to sustain consistent directional movement. High STR values are generally associated with improved fertilizing potential, as sperm are more efficient in reaching the oocyte. Motility landscape analysis in pigs has shown that sperm clusters with high STR (along with high VCL, VSL, VAP, and LIN) are positively associated with fertility (Fernández-López *et al.*, 2022).

Amplitude of lateral head displacement (ALH) represents the magnitude of lateral (side-to-side) displacement of the sperm head relative to its average trajectory during movement, as measured by CASA. ALH in this study ranged from 2.10 to 4.10 μm . Glycerol and β -carotene supplementation had a significant effect ($P < 0.05$) on ALH of frozen-thawed swamp buffalo semen. Statistical analysis showed that glycerol 2% (B) was not significantly different ($P > 0.05$) from treatments F (β -carotene 0.1%), G (β -carotene 0.2%), and H (β -carotene 0.3%), but differed significantly ($P < 0.05$) from treatments C (glycerol 4%), D (glycerol 6%), and E (glycerol 8%).

Higher ALH values observed in treatments B, F, G, and H indicate greater lateral movement of spermatozoa, reflecting increased flagellar activity. This may be interpreted as enhanced motility energy, although it should remain within an optimal range. Membrane protection by glycerol and the antioxidant activity of β -carotene help maintain active motility systems, resulting in more dynamic movement. However, excessively high ALH values may indicate a tendency toward hyperactivation, which is not always associated with efficient movement. Therefore, ALH should be interpreted together with parameters such as VSL and STR. ALH is defined as the amplitude of lateral displacement of the sperm head relative to the average path or progression line (Kinukawa *et al.*, 2003).

Linearity (LIN) is the degree of straightness of sperm movement based on the ratio between straight-line velocity (VSL) and curvilinear velocity (VCL) in CASA analysis. LIN ranged from 0.49 to 0.57. Glycerol and β -carotene supplementation had no significant effect ($P > 0.05$) on LIN of frozen-thawed swamp buffalo semen. The absence of significant differences in LIN indicates that supplementation did not significantly affect the linearity pattern of sperm movement. Although changes occurred in velocity parameters (VAP, VCL, and VSL), the proportion between straight-line and curvilinear velocity remained relatively constant. Biologically, LIN reflects the balance between movement activity and direction, making it more stable than velocity parameters. This suggests that the sperm motility system was able to maintain a relatively consistent movement pattern despite different cryopreservation treatments. LIN is widely used to describe movement efficiency and is defined as the ratio of VSL to VCL (Berlinguer *et al.*, 2017).

Wobble (WOB) is the ratio between average path velocity (VAP) and curvilinear velocity (VCL), indicating the stability of sperm movement in CASA analysis. WOB ranged from 0.63 to 0.68. Glycerol and β -carotene supplementation had no significant effect ($P > 0.05$) on WOB. The lack of significant differences in WOB suggests

that the treatments did not affect the stability of sperm trajectories. Although variations in movement velocity occurred, the consistency of movement remained relatively stable. Physiologically, WOB reflects synchronization between flagellar activity and movement control. Stable values indicate that flagellar structure and function were not significantly disrupted by the treatments. The sperm motility system was able to maintain trajectory stability despite changes during freezing and thawing (Zhang *et al.*, 2021).

Beat cross frequency (BCF) is the frequency at which the sperm head crosses the average path per unit time, reflecting the rhythmic activity of the flagellum in CASA analysis. BCF ranged from 20.51 to 23.65 Hz. Glycerol and β -carotene supplementation had no significant effect ($P > 0.05$) on BCF of frozen-thawed swamp buffalo semen. The absence of significant differences in BCF indicates that the treatments did not affect the frequency of flagellar movement. This suggests that the fundamental mechanical mechanism of sperm motility, particularly the rhythm of flagellar contraction, remained stable despite cryopreservation treatments. BCF is associated with flagellar mechanical activity but is not always directly related to movement efficiency or fertilizing ability (Barquero *et al.*, 2021).

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, glycerol and β -carotene supplementation in a Tris egg yolk extender significantly affected several kinetic parameters of frozen-thawed swamp buffalo spermatozoa analyzed using CASA. Optimal concentrations of glycerol (2–4%) and β -carotene (0.1–0.3%) improved sperm motility quality, as indicated by increased progressive motility, average path distance (DAP), curvilinear distance (DCL), straight-line distance (DSL), as well as velocity parameters (VAP, VCL, and VSL). These findings indicate enhanced sperm activity, movement stability, and directional efficiency following the cryopreservation process. Based on the findings of this study, the use of glycerol at concentrations of 2–4% combined with β -carotene at 0.1–0.3% in a Tris egg yolk extender is recommended to improve the quality of frozen-thawed swamp buffalo semen.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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This study provides a novel contribution to the development of swamp buffalo semen cryopreservation technology through the use of a combination of glycerol and β -carotene in a Tris egg yolk extender. The novelty of this research lies in the application of a comprehensive analysis approach based on computer-assisted semen analysis (CASA), encompassing multiple sperm kinetic parameters, including DAP, DCL, DSL, VAP, VCL, VSL, STR, LIN, WOB, ALH, and BCF. This approach offers a more in-depth understanding of sperm movement dynamics compared to conventional motility evaluation.

This study successfully identified the optimal concentration ranges of glycerol (2-4%) and β -carotene (0.1-0.3%) that enhance sperm activity, movement stability, and directional efficiency without disrupting the fundamental movement patterns. These findings provide an important contribution to the development of more effective semen preservation strategies, particularly for swamp buffalo under tropical conditions.

AUTHOR'S CONTRIBUTION

RE contributed to the conceptualization of the study, methodology, investigation, data curation, formal analysis, and preparation of the original draft. M contributed to supervision, conceptualization, validation, and manuscript review and editing. RK was involved in methodology, investigation, and data curation. NR contributed to formal analysis, visualization, and data interpretation. FM participated in investigation, laboratory work, and data collection. Dwi Ananta contributed to resources, validation, and manuscript review. FM was involved in statistical analysis, visualization, and manuscript editing. All authors read and approved the final manuscript.

ETHICAL APPROVAL

This research was conducted after receiving ethical approval under the Minister of Agriculture Decree No. 306/KPTS/TN.330/4/1994, which was approved by the Animal Research Ethics Committee of Universitas Negeri Padang, Padang, Indonesia, required for the use of experimental animals in research and teaching.

GENERATIVE AI AND AI-ASSISTED TECHNOLOGY STATEMENT

The authors declare that ChatGPT (OpenAI, San Francisco, CA, USA) was used to assist in the initial translation of the manuscript from Indonesian to English. All outputs were reviewed, edited, and verified by the authors to ensure accuracy and originality.

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