Research Article

# Effect of *Clitoria ternatea* Extract Supplementation in Semen Extender on Sperm Quality of Bali Bulls

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**Abstract** | Cryopreservation imposes significant stress on spermatozoa due to the excessive production of reactive oxygen species (ROS), leading to reduced post-thaw quality. While *Clitoria ternatea* (Butterfly Pea) is known for its high antioxidant potential, its application in preserving the quality of frozen Bali bull semen remains underexplored. This study aims to evaluate the quality and kinematics of Bali bull frozen semen using Tris-egg yolk extender supplemented with *Clitoria ternatea* extract (CTE). Five independent semen collection sessions were conducted . At each session, ejaculates from two bulls were immediately pooled and then assigned to four treatment groups: T0 = Tris-egg yolk (Control), T1 = CTE 1.5%, T2 = CTE 2%, and T3 = CTE 2.5%. Parameters measured in this study were motility, viability, abnormality, membrane integrity, acrosome integrity, and kinematics. The data obtained were analyzed using one-way analysis of variance (ANOVA). The results showed that different concentrations of CTE showed a significant difference ($p < 0.05$) on motility, viability, abnormality, membrane integrity, and acrosome integrity in the post-thaw evaluation. Treatment T1 showed significantly higher ($p < 0.05$) quality compared to T0, T2, and T3, whereas T3 exhibited the lowest quality, indicating a detrimental effect likely associated with osmotic imbalance at higher concentrations. In the movement pattern (kinematics) of sperms, there was no significant difference ($p > 0.05$) across all parameters post-thawing. Based on the study's result, it can be concluded that *Clitoria ternatea* extract with a concentration of 1.5% could be used as an antioxidant in the extender and maintain characteristics of sperms effectively compared to the control and higher concentrations.

**Keywords** | *Clitoria ternatea*, Cryopreservation, Antioxidant, Bali bull, Sperm quality, Kinematics

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## INTRODUCTION

Reproductive biotechnologies, notably artificial insemination (AI) and cryopreservation, are essential for preserving genetic diversity and accelerating the genetic improvement of local livestock (Engdawork *et al.*, 2024). In Indonesia, these strategies primarily focus on Bali cattle (*Bos javanicus*), which constitute a strategic national germplasm asset and serve as a primary pillar of the domestic meat supply. Characterized by remarkable adaptability to tropical climates and high fertility rates, Bali cattle represent a critical genetic resource for the advancement of livestock breeding programs (Mansur *et al.*, 2024). Conversely, field realities demonstrate a substantial

disparity between the genetic potential of the livestock and their reproductive efficiency. Current AI application has proven to be suboptimal, as indicated by low conception rates ranging from 23% to 39%. This percentage remains significantly below the minimum standard of 60%, which is a prerequisite for the sustainability of breeding programs (Yusuf *et al*., 2026). Ultimately, the success of fertilization in field AI programs is fundamentally dictated by the quality of the frozen semen utilized (Hasbi *et al*., 2024).

Although frozen semen undergoes rigorous quality assessments adhering to the standard operating procedures of the Artificial Insemination Center, post-thaw quality degradation remains a critical determinant limiting fertilization success in the field (Pardede *et al*., 2020). This decline is an inevitable consequence of cryopreservation, which induces significant cryodamage, including cold shock and intracellular ice formation thereby compromising plasma membrane integrity and reducing sperm motility and viability by up to 50% (Tamburrino *et al*., 2023). Such cellular impairments are compounded by oxidative stress mechanisms. Specifically, the freeze-thawing process triggers a surge in reactive oxygen species (ROS) production that creates an imbalance with the cell's intrinsic antioxidant defense capacity (Iqbal *et al*., 2022). The selection of an appropriate extender is a critical factor in minimizing cellular damage during cryopreservation. Conventionally, Tris-egg yolk (TEY) has been widely utilized as a standard extender due to the protective ability of lecithin on the sperm membrane (Bustani and Baiee, 2021). Nevertheless, recent studies indicate that the endogenous antioxidant content within TEY is insufficient to counteract the excessive ROS generation during cryopreservation. This is evidenced by El-Sheshtawy and El-Nattat (2020), who demonstrated that supplementing the Tris-egg yolk extender with *Moringa oleifera* extract yielded significantly higher frozen semen quality compared to the base extender alone. Similarly, supplementation with pomegranate peel methanolic extract in Tris-egg yolk extenders has proven effective in enhancing the quality of frozen-thaw ram semen (Aboelmaaty *et al*., 2023).

Current research trends of incorporating natural antioxidants into base extenders have prompted the exploration of alternative plant sources with distinct bioactive compound profiles. *Clitoria ternatea* (Butterfly Pea), a key component of Indonesia's biodiversity, holds significant potential as a natural source of antioxidants (Filio *et al*., 2023). Extracts of *C. ternatea* are rich in bioactive compounds including alkaloids, tannins, glycosides, resins, steroids, saponins, flavonoids, and phenols that exhibit high antioxidant potential (Multisona *et al*., 2023). The efficacy of this extract has been demonstrated by Iamsaard *et al*. (2014) on male rats, showing its ability to prevent testicular damage while enhancing sperm concentration and testosterone levels. Furthermore, Mariana *et al*. (2024) reported that supplementing coconut water-egg yolk extenders with *C. ternatea* extract yielded favorable results regarding the quality of Aceh bull spermatozoa, both pre- and post-equilibration. While various plant extracts have been previously investigated, *C. ternatea* offers a promising alternative due to its uniquely stable anthocyanin profile, which is reported to exhibit high potency against oxidative damage (Fu *et al*., 2021). This exceptional chemical stability suggests a substantial antioxidative capacity to protect spermatozoa, specifically by mitigating the severe, cold-induced oxidative stress generated during cryopreservation. Despite strong indirect evidence of its antioxidant mechanisms in other species, the direct *in vitro* application of CTE in bovine models particularly Bali bulls are currently lacking. To address this critical research gap, this study aims to evaluate the efficacy of various *Clitoria ternatea* extract (CTE) concentrations added to a Tris-egg yolk extender for the cryopreservation of Bali bull semen. The dose-dependent protection of CTE flavonoids against cryo-induced lipid peroxidation is expected to enhance post-thaw sperm quality up to an optimal threshold, while exceeding this limit would likely exert detrimental effects due to potential pro-oxidant activity or altered osmolarity.

## MATERIALS AND METHODS

### Experimental design

This study was conducted from October to December 2025 at the Animal Center and Laboratory of Animal Reproduction, Faculty of Animal Science, Hasanuddin University, Makassar, Indonesia. Semen samples were obtained from two mature healthy Bali bulls (*Bos javanicus*), aged 4 years. The bulls were maintained on a daily diet consisting of elephant grass and concentrate amounting to 10% of their body weight, with clean water provided *ad libitum*. The study employed an experimental design comparing four treatment groups. Five independent semen collection sessions were conducted. At each session, ejaculates from two bulls were immediately pooled and then assigned to four treatment groups: T0 (control, without CTE), T1 (1.5% CTE), T2 (2% CTE), and T3 (2.5% CTE). All experimental procedures were reviewed and approved by the Animal Ethics Committee of Hasanuddin University, Makassar, Indonesia.

### Semen extender

#### *Clitoria ternatea* extraction

The preparation of the *Clitoria ternatea* extract commenced with cleaning the flowers to remove impurities. The petals were separated from the sepals and subsequently dried in an oven at 40°C for 48 hour. The dried material was then ground into a fine powder using a mechanical blender. Extraction was performed using the maceration method with 96%

ethanol as the solvent. The powdered material was soaked at a 1:10 (w/v) ratio for four days, followed by filtration using filter paper. The remaining residue was re-macerated for an additional 24 h and filtered. The combined filtrates were concentrated using a rotary vacuum evaporator (40 °C; 60 rpm) to obtain a paste-shaped extract. The extraction process followed the standardized methodology described by Nurhayati *et al.* (2024), which is established to yield high flavonoid and anthocyanin contents with a quantifiable antioxidant capacity (IC50 of 113.31 ppm via DPPH assay and 32.005 mgQE/g via FRAP assay).

### Preparation of dilution

The basic Tris-egg yolk extender used in this study was prepared by dissolving citric acid monohydrate (1.675 g), Tris (3.028 g), and fructose (1.25 g) in double-distilled water (up to 100 mL), supplemented with 20% (v/v) egg yolk. Additionally, antibiotics consisting of penicillin (100,000 IU/100 mL) and streptomycin (0.1 g/100 mL) were incorporated into the solution. Semen dilution was performed using a modified two-step procedure according to Arif *et al.* (2020), this protocol utilized Extender A (comprising the base extender and CTE without glycerol) and Extender B (comprising the base extender, CTE, and 14% glycerol). Initially, fresh semen was diluted with Extender A at 37 °C to achieve an initial concentration of 200 × $10^6$ sperm/mL. The partially diluted semen was then placed in a cool-top and gradually cooled to 4-5°C over 45 minutes. Upon reaching the target temperature, an equal volume (1:1 v/v) of Extender B was added gradually. This two-step 1:1 addition mechanism resulted in a final glycerol concentration of 7% and standardized the final pre-freeze sperm concentration at 100 × $10^6$ sperm/mL across all treatment groups (equating exactly to 25 × $10^6$ sperm per 0.25 mL straw). The resulting solution was then equilibrated at 4-5°C for 4 hours prior to the freezing process.

### Semen collection and evaluation

Semen collection was carried out for twice a week using an artificial vagina. Semen evaluation was done macroscopically and microscopically. The volume, pH, colour, odour, and consistency of the sperm were evaluated macroscopically. Semen quality was evaluated in the laboratory, with sperm motility (total motility and progressive motility) assessed using Computer Assisted Sperm Analysis (CASA) (Amaliah *et al.*, 2023; Alfian *et al.*, 2025). The sperm concentration was measured using a Minitube SDM 6 (Germany) photometer, following Diansyah *et al.* (2022), sperm viability and abnormality was evaluated using an eosin-nigrosin staining procedure, following the protocol of (Diansyah *et al.*, 2020). Membrane integrity was assessed using the hypo-osmotic swelling test (HOST). The composition of HOST solution was described in the study of Bebas *et al.* (2023). Acrosome integrity was assessed using formol saline solution following the method of (Bebas *et al.*, 2025). Individual ejaculates were strictly evaluated against standard inclusion criteria (total motility and viability > 70%, concentration > 400 × $10^6$ sperm/mL). Two ejaculates failed these thresholds and were excluded. Collections were continued until five high-quality pooled replicates were successfully obtained. To systematically control for the substantial natural variability in fresh sperm concentration, the paired ejaculates were pooled and standardized to a final pre-freeze concentration of 100 × $10^6$ sperm/mL.

### Semen cryopreservation

Semen cryopreservation was carried out following the procedure of Elvania *et al.* (2024). The diluted semen was packaged into 0.25 ml straws and sealed. Subsequently, the straws were equilibrated at 4–5°C for 4 hours. The freezing process was initiated by exposing the straws to liquid nitrogen vapor (approximately 4 cm above the liquid surface) for 10–15 minutes. Finally, the straws were plunged into liquid nitrogen (-196°C) and stored in a nitrogen container for at least 4 days. For post-thaw evaluation, three straws from each treatment were thawed in a water bath at 37°C for 30 seconds.

### Statistical analysis

Data compilation was carried out using Microsoft Excel. Fresh semen characteristics were analyzed using descriptive statistics and reported as mean ± standard deviation (SD) with CI 95%. For the experimental treatments, data normality and homogeneity were verified using the Shapiro–Wilk and Levene's tests, respectively. Statistical analysis was performed using Analysis of Variance (ANOVA) on IBM SPSS Statistics 26, followed by Duncan's test ($p < 0.05$) to determine significant differences between treatments.

## RESULTS

Characteristics of Bali bull fresh semen

The characteristics and kinematic profiles of fresh Bali bull semen are presented in Tables 1 and 2. The macroscopic and microscopic assessment (Table 1) established the baseline quality standards required prior to extension. Macroscopically, the ejaculates displayed normal volume, pH, and consistency, characterized by a creamy-white appearance and a distinct odor. Subsequent microscopic analysis confirmed superior sperm quality, marked by high percentages of total and progressive motility, viability as well as plasma membrane and acrosome integrity, complemented by a low incidence of morphological abnormalities, which surpassed the minimum requirements for cryopreservation (SNI 4869-1:2021; Prastiya *et al.*, 2024). Complementing these findings, the kinematic

analysis using CASA (Table 2) demonstrated active and efficient spermatozoa movement profiles. Specifically, the velocity values (VCL, VAP, and VSL) observed in this study align with the kinematic characteristics of fertile Bali bulls documented by (Diansyah *et al*., 2025). Collectively, these findings confirm that the fresh semen possessed optimal quality suitable for dilution and freezing protocols.

**Table 1:** Characteristic of Bali bull fresh semen.

| Parameters | Mean (±SD) | 95% Confidence Interval |
|---|---|---|
| **Macroscopic** | | |
| Volume (ml) | 4.8±0.57 | 4.09-5.05 |
| pH | 6.55±0.11 | 6.41-6.68 |
| Colour | Creamy | |
| Smell | Typical | |
| Consistency | Medium | |
| **Microscopic** | | |
| Consentration (million/ml) | 1,343±332.95 | 929.77-1756.62 |
| Total Motility | 89.02±1.32 | 87.37-90.67 |
| Progresif Motility | 85.07±2.00 | 82.58-87.55 |
| Viability | 90.34±1.79 | 88.11-92.56 |
| Abnormality | 10.92±2.07 | 8.34-13.49 |
| Membrane Integrity | 89.02±0.98 | 87.79-90.24 |
| Acrosome Integrity | 85.2±0.83 | 84.16-86.23 |

**Table 2:** Kinematic of Bali bull fresh semen.

| Parameters | Mean (±SD) | 95% Confidence interval |
|---|---|---|
| DCL (µm) | 63.95±8.68 | 53.16 – 74.73 |
| DAP (µm) | 38.80±4.14 | 33.66 – 43.95 |
| DCL (µm) | 29.40±4.32 | 24.03 – 34.77 |
| VCL (µm/s) | 150.26±22.87 | 121.85 – 178.66 |
| VAP (µm/s) | 91.33±10.43 | 78.37 – 104.29 |
| VSL (µm/s) | 69.50±10.01 | 57.06 – 81.93 |
| LIN (%) | 46.83±7.31 | 37.74 – 55.92 |
| STR (%) | 76.02±5.49 | 69.19 – 82.84 |
| WOB (%) | 61.30±5.72 | 54.19 – 68.40 |

## Effect of *Clitoria ternatea* extract on post-dilution semen quality

The effects of *Clitoria ternatea* extract supplementation in Tris-egg yolk extender on post-dilution sperm quality of Bali bulls are presented in Tables 3 and 4. Specifically, Table 3 details the impact of the extract on post-dilution sperm parameters. Statistical analysis revealed that the extract supplementation significantly influenced sperm motility, viability, membrane integrity, and acrosome integrity ($p < 0.001$). In contrast, no significant differences were observed in sperm abnormality rates among the treatment groups ($p= 0.197$), suggesting that short-term CTE exposure post-dilution does not alter pre-existing morphological structures. The 1.5% concentration (T1) yielded the optimal results, exhibiting significantly higher values for motility, viability, membrane integrity, and acrosome integrity compared to the other treatment groups ($p < 0.001$). Conversely, the T2 treatment (2%) demonstrated no statistically significant differences compared to the control group (T0) across all evaluated parameters ($p > 0.05$). The values for viability, membrane integrity, and acrosome integrity in the T3 group were significantly lower than those in the T0 and T1 groups. Furthermore, while sperm motility in the T3 group did not differ significantly from the control (T0) ($p > 0.05$), it was significantly lower compared to the T1 group ($p < 0.001$).

The kinematic analysis of spermatozoa using Computer-Assisted Sperm Analysis (CASA), presented in Table 4, demonstrates that a substantial proportion of motion parameters were significantly affected ($p < 0.05$) by the variation in *Clitoria ternatea* extract supplementation. Statistically significant differences were observed in vigor and velocity parameters, specifically DCL, DAP, DSL, VCL, VAP, and VSL. For these parameters, the T1 treatment yielded the highest values which were significantly different compared to the Control (T0), T2, and T3 groups ($p < 0.05$). No significant differences were observed in path linearity parameters (LIN, STR, and WOB) across the treatment groups ($p > 0.05$).

## Effect of *Clitoria ternatea* extract on post-thawing semen quality

The evaluation of post-thaw sperm quality, presented in Table 5, demonstrates that all observed parameters were significantly affected by the varying concentrations of

**Table 3:** Effect of *Clitoria ternatea* extract on sperm quality post dilution.

| Treatment | Parameters (Mean±SD) | | | | |
|---|---|---|---|---|---|
| | Motility (%) | Viability (%) | Abnormality (%) | Membran integrity (%) | Acrosome integrity (%) |
| T0 | 83.68±1.57$^{b}$ | 84.63±1.52$^{b}$ | 13.67±1.55 | 84±2.18$^{b}$ | 81.39±1.53$^{b}$ |
| T1 | 86.78±2.17$^{a}$ | 88±1.52$^{a}$ | 12.40±1.59 | 86.84±0.77$^{a}$ | 83.44±1.52$^{a}$ |
| T2 | 83±0.91$^{b}$ | 83.23±1.85$^{bc}$ | 12.72±1.68 | 82.7±0.78$^{bc}$ | 80.15±0.57$^{b}$ |
| T3 | 81.68±1.66$^{b}$ | 81.87±2.48$^{c}$ | 14.32±1.01 | 81.1±2.38$^{c}$ | 78.41±0.49$^{c}$ |
| p-Value | <0.001 | <0.001 | 0.197 | <0.001 | <0.001 |

$^{a,b,c}$ Means in a column with different superscripts differ significantly at $p < 0.001$.

**Table 4:** Effect of *Clitoria ternatea* extract on kinematic sperm post-dilution.

| Parameters | Treatment (Mean±SD) | | | | p-value |
|---|---|---|---|---|---|
| | T0 | T1 | T2 | T3 | |
| DCL (µm) | 40.24±2.34[b] | 48.01±6.56[a] | 39.65±4.52[b] | 37.74±3.79[b] | 0.013 |
| DAP (µm) | 23.89±1.05[b] | 29.19±1.89[a] | 23.94±1.62[b] | 22.79±2.04[b] | <0.001 |
| DSL (µm) | 17.67±1.45[b] | 21.54±1.33[a] | 19.29±0.94[ab] | 17.15±3.16[b] | 0.010 |
| VCL (µm/s) | 92.51±5.84[b] | 113.22±16.65[a] | 91.96±11.26[b] | 85.85±8.08[b] | 0.008 |
| VAP (µm/s) | 55.02±2.52[b] | 68.76±5.28[a] | 55.68±4.20[b] | 51.92±4.22[b] | <0.001 |
| VSL (µm/s) | 40.74±3.44[b] | 51.81±2.46[a] | 42.48±2.51[b] | 39.05±4.02[b] | <0.001 |
| LIN (%) | 44.08±3.28 | 46.41±5.71 | 46.54±3.73 | 45.53±3.01 | 0.765 |
| STR (%) | 73.99±4.26 | 75.58±4.60 | 76.39±2.33 | 75.14±3.16 | 0.780 |
| WOB (%) | 59.54±1.95 | 61.24±4.47 | 60.86±3.32 | 60.56±2.95 | 0.848 |

[a,b] Means in a row with different superscripts differ significantly at $p < 0.05$; $p < 0.001$

**Table 5:** Effect of *Clitoria ternatea* extract on sperm quality post-thawing.

| Treatment | Parameters (Mean±SD) | | | | |
|---|---|---|---|---|---|
| | Motility (%) | Viability (%) | Abnormality (%) | Membran integrity (%) | Acrosome integrity (%) |
| T0 | 46.12±1.65[b] | 46.12±0.86[b] | 15.70±0.86[b] | 46.32±1.10[b] | 43.48±1.98[b] |
| T1 | 51.96±1.86[a] | 53.70±1.34[a] | 14.32±1.05[a] | 52.90±2.04[a] | 48.80±2.04[a] |
| T2 | 43.72±1.52[c] | 45.44±1.69[b] | 15.13±0.76[ab] | 44.55±1.38[b] | 42.82±1.63[b] |
| T3 | 39.69±1.64[d] | 40.40±1.44[c] | 15.88±0.37[b] | 41.14±1.03[c] | 38.02±1.54[c] |
| p-Value | <0.001 | <0.001 | 0.03 | <0.001 | <0.001 |

[a,b,c,d] Means in a column with different superscripts differ significantly at $p < 0.05$;$p < 0.001$

**Table 6:** Effect of *Clitoria ternatea* extract on kinematic sperm post-thawing.

| Parameter | Treatment (Mean±SD) | | | | p value |
|---|---|---|---|---|---|
| | T0 | T1 | T2 | T3 | |
| DCL (µm) | 28.87±2.75 | 31.36±3.13 | 27.12±3.50 | 26.01±2.66 | 0.065 |
| DAP (µm) | 17.63±1.32 | 18.44±1.87 | 17.71±2.41 | 16.09±1.49 | 0.265 |
| DSL (µm) | 13.50±1.42 | 14.19±1.74 | 13.32±1.94 | 12.79±1.35 | 0.612 |
| VCL (µm/s) | 66.21±7.59 | 72.83±8.50 | 64.39±9.35 | 60.62±5.47 | 0.139 |
| VAP (µm/s) | 40.46±3.23 | 42.65±4.89 | 42.13±4.84 | 37.87±3.32 | 0.355 |
| VSL (µm/s) | 31.02±3.48 | 32.10±4.99 | 32.15±4.24 | 28.94±3.01 | 0.566 |
| LIN (%) | 46.97±3.45 | 43.98±3.52 | 50.06±3.49 | 48.00±6.15 | 0.203 |
| STR (%) | 76.51±2.86 | 74.98±3.61 | 76.36±1.07 | 76.40±3.22 | 0.812 |
| WOB (%) | 61.39±3.77 | 58.62±2.88 | 65.53±4.00 | 62.66±5.47 | 0.106 |

*Clitoria ternatea* extract added to the Tris-egg yolk extender ($p < 0.001$). The 1.5% concentration (T1) consistently yielded the best results, maintaining significantly higher values for motility, viability, membrane integrity and acrosome integrity compared to the other treatment groups ($p < 0.001$). Regarding sperm abnormality, T1 recorded the lowest rate, which was significantly different from T3 and the control (T0), though not significantly different from T2. Conversely, the T2 treatment resulted in a significant decrease in motility compared to the control, while other parameters remained comparable to the control. Furthermore, supplementation with the highest concentration (T3) recorded the lowest values for motility, viability, membrane integrity, and acrosome integrity relative to all other treatment groups ($p < 0.001$).

The kinematic analysis of spermatozoa using Computer-Assisted Sperm Analysis (CASA), presented in Table 6, indicates that all motion parameters were not significantly affected ($p > 0.05$) by the variation in *Clitoria ternatea* extract supplementation. Parameters including DCL, DAP, DSL, VCL, VAP, VSL, LIN, STR, and WOB displayed comparable values across all treatment groups. This suggests that the efficiency and vigor of sperm movement remained relatively consistent among the groups post-thawing, regardless of the extract concentration.

## DISCUSSION

The present study demonstrates that the supplementation of *Clitoria ternatea* extract (CTE) into Tris-egg yolk extender significantly preserves the quality of Bali bull spermatozoa during both post-dilution and post-thawing stages. Specifically, the treatment group with a concentration of 1.5% (T1) yielded the highest percentages of motility, viability, membrane integrity, and intact acrosome compared to both the control and other concentration groups. Cryopreservation typically subjects spermatozoa to severe oxidative stress, as highlighted by Khan *et al.* (2021), this condition induces mitochondrial dysfunction, which critically impairs the energy metabolism required for motility. The superior motility observed in the T1 group suggests that CTE supplementation provides a protective environment against such cryo-induced damage. This interpretation aligns with the findings of Ferramosca and Zara (2022), which suggest that bioactive compounds in *Clitoria ternatea* possess strong ROS scavenging capabilities that reduce lipid peroxidation. Consequently, the beneficial effects of 1.5% CTE on post-thaw semen quality may be attributed to its potential role in mitigating oxidative stress, thereby helping to maintain the structural and functional characteristics of the spermatozoa.

During cryopreservation, spermatozoa are subjected to cold shock and ice crystal formation, which triggers a significant surge in reactive oxygen species (ROS) production (Hai *et al.*, 2024). The improved viability and plasma membrane integrity observed in the T1 group are closely attributed to the bioactive properties of flavonoids, specifically anthocyanins, found in *Clitoria ternatea*. The plasma membrane of bull spermatozoa, characterized by high levels of polyunsaturated fatty acids (PUFAs), is exceptionally vulnerable to such oxidative attacks (Wang *et al.*, 2025). Therefore, the addition of 1.5% *Clitoria ternatea* extract (CTE) is suggested to provide an essential exogenous defense mechanism. This protective capacity is likely mediated by the multiple hydroxyl groups of anthocyanins, which are known to effectively scavenge free radicals (Christian and Setiawansyah, 2025). This scavenging activity likely prevents lipid peroxidation, which would otherwise compromise the fluidity and structural integrity of the cell membrane. These results corroborate the findings of previous studies suggesting that appropriate antioxidant supplementation minimizes cryogenic membrane damage, directly correlating with superior post-thaw sperm viability (Chianese and Pierantoni, 2021).

In addition to plasma membrane protection, the supplementation of *Clitoria ternatea* extract at a concentration of 1.5% (T1) proved effective in maintaining the structural stability of the acrosomal cap spermatozoa during both the post-dilution and post-thawing stages. This observation suggests a protective role against oxidative stress during cryopreservation. Maintaining an intact acrosome is a critical prerequisite for spermatozoa to retain the enzymes essential for subsequent oocyte fertilization. These findings are consistent with the research of Nirmala *et al.* (2025), who reported that natural antioxidant supplementation significantly preserves the physical quality of intact acrosomes compared to the control group. Furthermore, CTE supplementation at 1.5% significantly suppressed sperm abnormality rates. Interestingly, this protective effect on sperm morphology is highly stage specific. While, CTE supplementation significantly improved post-dilution motility and viability, it did not significantly alter the initial sperm abnormality rates ($p = 0.197$) prior to freezing. This lack of significance is biologically expected. During the short post-dilution period, the initial processing stress is insufficient to induce new morphological damage, thereby leaving the pre-existing baseline abnormalities stable across all treatment groups. However, the extreme oxidative stress generated during the subsequent cryopreservation process dramatically alters this phenomenon. ROS induced mitochondrial damage during freezing is known to trigger energy production dysfunction, which severely impairs the structural integrity and morphology of spermatozoa (Kaltsas, 2023). Therefore, the significantly lower percentage of abnormalities observed in the T1 treatment post-thawing demonstrates the efficacy of the extract's antioxidants in actively mitigating severe, cryo-induced oxidative damage to cellular organelles. This underscores the crucial role of bioactive compounds in preserving sperm morphology against the detrimental conditions of the cryopreservation process, which is in agreement with previous reports utilizing antioxidants in extenders (Bintara *et al.*, 2023).

The optimal concentration of 1.5% CTE is directly supported by the peak post-thaw sperm quality observed at this dosage. This specific threshold reflects an essential balance between the extract's bioactive properties and the physiological tolerance of Bali bull spermatozoa. A key factor contributing to sperm plasma membrane damage during cryopreservation is its inherent vulnerability to osmotic stress and lipid peroxidation. While bovine spermatozoa are generally susceptible to these stressors due to their low cholesterol proportion (Castro *et al.*, 2025), the exact tolerance limits vary among breeds. Due to their specific membrane lipid composition, Bali bull spermatozoa exhibit a distinct sensitivity to external solutes and osmotic changes. Consequently, a relatively low inclusion (1.5%) of this highly bioactive extract is sufficient to mitigate oxidative damage and provide maximum cryoprotection. Increasing the concentration further negates these benefits by exceeding the sperm's natural osmotic tolerance, leading

to the significant decline in quality observed at higher doses.

The beneficial effects of *Clitoria ternatea* (butterfly pea) extract supplementation in the TEY extender were also observed in the post-dilution kinematic parameters (Table 4). Specifically, the T1 treatment (1.5%) yielded the highest values for velocity and vigor metrics including DCL, DAP, DSL, VCL, VAP, and VSL exhibiting significant differences compared to the control and other treatment groups ($p < 0.05$). These results indicate that the extract effectively preserved spermatozoal activity prior to the freezing process. In contrast, statistical analysis at the post-thaw stage (Table 6) revealed no significant differences ($p > 0.05$) across all evaluated kinematic parameters, indicating a uniform decline in motion characteristics across treatments following cryopreservation. The overall decline in kinematic metrics post-thaw reflects the multifaceted impact of cryodamage induced during the freeze-thaw process likely due to formation of ice crystals and osmotic stress. Such cryoinjury is reported to cause significant structural and functional impairments, with previous studies indicating that the freezing process can downregulate the expression of proteins crucial for energy metabolism and motility (Gusdinal *et al*., 2025). Despite this general decline, the T1 group exhibited numerically higher values in certain vigor, such as Distance Curved Line (DCL) and Curvilinear Velocity (VCL), these variations did not reach statistical significance ($p > 0.05$). This indicates that while CTE provides robust protection to the general structural integrity of spermatozoa, its capacity to significantly preserve highly specific kinematic tracking patterns is limited. This selective influence aligns with the findings of Yangngam *et al*. (2021), who observed that antioxidant supplementation significantly enhances general motility while exerting limited effects on specific kinematic parameters.

In contrast to the beneficial effects observed at a concentration of 1.5%, increasing the concentration of *Clitoria ternatea* extract to 2.5% (T3) resulted in a significant decline in sperm quality parameters, with values falling below those of the control group. The substantial reduction in motility and viability observed in the T3 group (Tables 3 and 5) could be attributed to several potential mechanisms. While the saturation of antioxidant activity and a subsequent shift in the cellular redox balance toward a pro-oxidant state is a documented possibility at excessive antioxidant dosages (Henkel *et al*., 2018; Speisky *et al*., 2022), other practical factors must be considered. Specifically, based on the principle of dose-dependent cytotoxicity, the accumulation of extract components at the 2.5% dosage may simply surpass the physiological tolerance of the spermatozoa, thereby exerting a direct toxic effect. Furthermore, the addition of higher extract concentrations might alter the optimal physical properties of the extender, such as osmolarity, leading to osmotic stress that compromises cell membrane integrity. Cytotoxic impacts resulting from high concentrations have also been reported in sperm cells, particularly during the cryopreservation process (Maulida *et al*., 2024). These findings are consistent with research in bulls, where high doses of ascorbic acid and *Kaempferia parviflora* have been shown to exert detrimental effects on sperm parameters (Priyanto *et al*., 2024; Loetjettanarom *et al*., 2025).

While the current findings highlight the efficacy of *Clitoria ternatea* extract (CTE) in preserving the post-thaw quality of Bali bull spermatozoa, this research was designed as a preliminary *in vitro* screening phase, and therefore, several methodological limitations must be acknowledged. First, to establish baseline efficacy and optimal dosing, this study did not empirically quantify the specific antioxidant composition or the exact Total Antioxidant Capacity (TAC) of the administered extract batch. Therefore, the absence of a batch specific preliminary screening limits our ability to precisely correlate exact phenolic concentrations with the observed cryoprotective effects. Consequently, the direct assessment of oxidative stress mitigation is constrained, as specific biochemical markers such as reactive oxygen species (ROS) production and malondialdehyde (MDA) levels were not evaluated in the cryopreserved samples, which CTE exerts its cryoprotective effects on sperm cellular structures remain incompletely elucidated. Another notable limitation inherent to this initial proof of concept phase is the absence of *in vivo* fertility evaluations following cryopreservation.

Despite these limitations, the findings of this study hold significant implications for the advancement of reproductive biotechnologies, particularly concerning the genetic preservation and breeding efficiency of Bali cattle. The demonstration that 1.5% CTE significantly enhances post-thaw sperm parameters underscores its potential as a viable, sustainable, and natural antioxidant alternative in commercial semen extenders. Optimizing such cryopreservation protocols could directly translate to higher quality frozen semen for artificial insemination (AI) programs, fundamentally addressing the low field conception rates currently observed. Furthermore, the detrimental effects observed at higher concentrations (2.5% CTE) highlight the critical necessity of precise antioxidant dosing in cryopreservation to minimize potential damage.

To fully harness the potential of *Clitoria ternatea* extract (CTE) and account for the inherent variability of natural plant extracts, future studies should establish standardized optimal concentrations based on quantifiable bioactive markers. Furthermore, investigations using molecular and

biochemical analyses, specifically measuring oxidative stress markers such as reactive oxygen species (ROS), malondialdehyde (MDA), and total antioxidant capacity (TAC), are necessary to elucidate the precise mechanisms underlying CTE's protective role. Moreover, future studies should incorporate comprehensive fertility trials to validate the practical benefits of CTE supplementation. These must include functional assays, such as induced acrosome reactions and zona binding tests, as well as *in vivo* trials like artificial insemination (AI) or *in vitro* fertilization (IVF) experiments. Integrating these mechanistic studies and fertility assessments will provide a more comprehensive understanding of CTE's role in cryopreservation, enabling its optimized application in reproductive technologies and livestock breeding programs.

## CONCLUSION

This study explored the use of *Clitoria ternatea* extract to improve the quality of freeze-thawed semen from Bali bulls. Supplementation of semen extender with 1.5% CTE significantly improved the post-thaw quality of Bali bull semen. In contrast, a higher concentration (2.5%) had adverse effects on sperm quality parameters. These findings highlight the critical role of precise antioxidant dosing in cryopreservation protocols to maximize sperm quality while minimizing potential damage**.** However, it is imperative to acknowledge that this optimal concentration is not absolute, it may vary depending on the inherent phytochemical variability of the extract batch, individual bull differences, and the specific base extender composition.

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## NOVELTY STATEMENT

This study found the optimal concentration of *Clitoria ternatea* extract as a natural antioxidant supplement in semen extenders to improve the post-thaw quality of Bali bull spermatozoa.

## AUTHOR'S CONTRIBUTION

MY, LR, and DF conceived and designed the experiment. MY, LR supervised and coordinated the research and provided clinical data. Statistical analysis was conducted by DF and AMD. The initial draft of the manuscript was prepared by DF and MJ. All authors critically reviewed and approved the final version of the manuscript.

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### Generative AI and AI-assisted technology statement

No generative AI tools were employed for data analysis, interpretation, or the generation of original scientific content. AI assistance was strictly limited to refining English grammar and enhancing language clarity.

### Conflict of interest

The authors have declared no conflict of interest.

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