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# Synergistic Impact of Cholesterol-Loaded Cyclodextrin and Moringa Leaf Extract on Post-Thaw Boar Sperm Kinematics

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## ABSTRACT

**Structural and functional damage during cryopreservation usually impairs the quality of boar sperm, especially post-thaw motility and kinematic performance, which restricts its use in artificial insemination programs. Cholesterol-loaded cyclodextrin (CLC) and moringa leaf extract (MLE) are considered possible cryoprotective additives because they are membrane-stabilizing and antioxidants. The study investigated the possible synergistic effect of the Tris-egg yolk (TY) extender in combination with CLC and MLE on the motility and kinematics properties of thawed boar sperm, as identified in a Computer-Assisted Sperm Analysis (CASA) system. Ejaculates of four healthy boars were diluted with Tris-based extender without or with CLC (1 or 2 mg/mL), MLE (1 or 1.5 mg/mL), or both, and frozen with a conventional protocol. Kinematic variables alongside progressive and fast motility were included as post-thaw tests. The findings showed that combination treatment of 2 mg/mL CLC and 1 mg/mL MLE (T7) resulted in a significant increase in all sperm motility and kinematic variables compared to the control group (T0) ($p<0.05$). In particular, T7 had better progressive motility (38.04%), fast motility (22.42%), and higher kinematic variables, especially VCL (106.34 μm/s), VSL (36.28 μm/s), and VAP (45.41 μm/s), and better displacement and trajectory indices. T7 was always better than the single supplementation and other combinations. Finally, the addition of CLC (2 mg/mL) and MLE (1 mg/mL) into the Tris egg yolk extender could be a successful approach towards optimizing semen cryopreservation in boars.**

*Keywords: boar semen; cholesterol-loaded cyclodextrin; moringa leaf extract; sperm motility; sperm kinematic*

## INTRODUCTION

Artificial insemination (AI) has become a central instrument in swine production today and is used to promote the effective spread of genetic material and enhance the productivity of the herd stock all over the globe. The success of AI programs is essentially determined by the regular supply of semen of high functional integrity in its fresh and cryopreserved state. But the cryopreservation technique is linked with severe cellular damage, resulting in the loss of sperm viability and fertilizing ability after thawing. The functional traits adversely affected by freezing and thawing and highly susceptible to cryoinjury are progressive motility and sperm kinematics, which are critical indicators of fertilizing ability (Li *et al*., 2023).

The cryopreservation process causes damage to boar spermatozoa by destroying them, especially through cold shock, osmotic imbalance, and oxidative stress. The stressors interfere with the stability of plasma membranes, mitochondrial dysfunction, and the structure of axonemal. This eventually leads to a decrease in the parameters of motility and fertilization (Yeste, 2018; Zhang *et al*., 2021). Measures to reduce the effects of cryodamage are the addition of cryoprotectants, membrane stabilizers, and antioxidants to extenders. Cholesterol is crucial in controlling plasma membrane fluidity, and incorporating it into sperm membranes has been demonstrated to improve resistance to low temperatures (Rajoriya *et al*., 2020).

Cholesterol-loaded cyclodextrin (CLC) is a good delivery system for loading cholesterol into the sperm membranes before the sperm is cryopreserved. CLC promotes membrane cholesterol content and hence leads to membrane stability and decreased phase changes during cooling. The maintenance of sperm motility and viability after the thawing process has been established (Batissaco *et al*., 2020). On the same note, antioxidant use has been proposed more and more as a countermeasure against the production of reactive oxygen species (ROS) during the cryopreservation process, thus alleviating the dangers of lipid peroxidation, protein oxidation, and DNA damage (Valgimigli, 2023; Voronkova *et al*., 2018).

The Moringa leaves are considered an excellent source of flavonoids, polyphenols, and vitamins, among other bioactive compounds with high antioxidant effects (Olvera-Aguirre *et al*., 2022; Srivastava *et al*., 2023). MLE has been found to reduce oxidative stress and to maintain sperm activity during storage in various species (Authaida *et al*., 2025; Shokry *et al*., 2024). Nevertheless, there is a lack of research that assesses the combined effects of membrane cholesterol enrichment with plant-derived antioxidants in the cryopreservation of boar semen. Also, detailed analyses of sperm kinematics in such conditions, especially using Computer-Assisted Sperm Analysis (CASA), are scarce.

The researcher sought to determine the synergistic effect of the CLC and Moringa leaf extract in Tris-egg yolk diluent on the kinematic and motility parameters of Landrace boar sperm in the post-thaw condition using CASA. It was hypothesized that the combination of CLC-mediated membrane stabilization and MLE-derived antioxidant protection would improve post-thaw sperm quality more effectively than either treatment alone.

## MATERIALS AND METHODS

### Ethical Clearance

Ethical approval for this research protocol was granted by the Livestock, Marine, and Fishery Research Ethics Committee, Faculty of Animal Husbandry, Marine and Fisheries, Nusa Cendana University (Approval No. 132/1.KT/KEPPKP/V/2025).

### Experimental Animals

A total of four Landrace boars, clinically healthy and aged between two and three years, were enrolled in the present investigation. All the animals included in this study were maintained under intensive management at a commercial swine breeding facility of the Williams and Laura Foundation, with ad libitum supply of water and maintenance on a balanced diet designed to fulfill their nutrient requirements. Boars were kept separately in pens that provided adequate ventilation, with environmental temperature and humidity carefully controlled.

### Semen Collection and Initial Evaluation

Semen samples were obtained twice weekly through the gloved-hand massage method. Immediately after collection, filtration was used to separate and remove the gel fraction. The rest of the sperm-enriched fraction was then subjected to macroscopic and microscopic analysis. Macroscopic measurements included ejaculate volume, pH, and color, while microscopic measurements were performed on sperm concentration, motility, and morphology. The ejaculates with 70% progressive motility and less than 20% abnormal morphology were only selected to undergo cryopreservation (Yang *et al*., 2016).

### Semen Processing

Samples of ejaculate that passed the set quality criteria, i.e., at least 70% progressive motility and less than 20% morphological abnormalities, were diluted in a Tris-based extender containing egg yolk (TY) at a 1:1 ratio (7 mL of semen to 7 mL of TY). The Tris-egg yolk diluent was made of Tris (hydroxymethyl) aminomethane (3.03 g), citric acid (1.78 g), and fructose (1.25 g) mixed with 20 percent (v/v) egg yolk, 100 mL of distilled water, 1000 IU/mL penicillin, and 1 mg/mL streptomycin (Arif *et al*., 2022). Two hours of ambient temperature storage of the diluted samples were done to ensure extender holding time and adaptation of the sperm. After the incubation period, the samples were centrifuged at 800 × g (Nuve, NF 1200) for 10 minutes to separate the seminal plasma and residual extender (Ratchamak *et al*., 2019). The sperm pellet was diluted in 2 mL of fresh TY extender to obtain a uniform sperm suspension.

Subsequently, the resuspended sperms were diluted further with treatment-specific extenders until a final concentration of $300 \times 10^6$ sperms/mL. The experimental design consisted of nine groups, which were organized as follows: T0 (Control, TY); T1: TY + 1 mg/mL Cholesterol-loaded cyclodextrin (CLC); T2: TY + 2 mg/mL CLC; T3: TY + 1 mg/mL Moringa Leaf Extract (MLE); T4: TY + 1.5 mg/mL MLE; T5: TY + 1 mg/mL CLC + 1 mg/mL MLE; T6: TY + 1 mg/mL CLC + 1.5 mg/mL MLE; T7: TY + 2 mg/mL CLC + 1 mg/mL MLE; T8: TY + 2 mg/mL CLC + 1.5 mg/mL MLE.

CLC was synthesized following the method reported by Zhang *et al*. (2024). In brief, methyl-β-cyclodextrin (Sigma, C4555) (1 g in 2 mL methanol (Sigma, 34860)) was combined with cholesterol (Sigma, C3045) (0.45 mL of a 200 mg/mL solution in chloroform (Sigma, C2432)). The mixture was dried under nitrogen to obtain a white CLC powder. The CLC was ready for use.

Moringa leaf extract (MLE) preparation: Dried Moringa leaves were finely ground and sieved for uniformity. A total of 500 g of the powdered sample was subjected to maceration in 2 L of 95% (v/v) ethanol for 48 hours at ambient temperature with intermittent stirring. The resulting mixture was then filtered, and the filtrate was concentrated under reduced pressure at 40 °C using a rotary evaporator to obtain a viscous crude ethanolic extract. The airtight container with the extract was then kept at 4 °C until further examination (Laskar *et al*., 2025).

Processes of semen processing were performed under aseptic conditions. The pre-warmed extenders were mixed to room temperature to avoid thermal shock. To maintain the freshness and uniformity of samples, the experimental treatments had to be made right before the experiment.

### Semen Freezing

The cryopreservation and thawing conditions applied in this research were based on the procedures outlined by Ratchamak *et al*. (2019) and slightly changed to fit the laboratory environment and differences in

treatments. The semen samples in each of the treatment groups were put in a semi-automated filling system in sterile 0.5 mL medium straws (IMV Technologies, France) under aseptic conditions after the final dilution process. The straws were covered with the polyvinyl alcohol powder in order to avoid leakage during storage and freezing.

Then, the straws were put in a cooling cabinet and equilibrated at 5 °C over a period of two hours, where the extender components, especially the cryoprotectants, interacted with the sperm plasma membrane. This made the membrane more stable when freezing it. Once the equilibration was achieved, the pre-freezing procedure was carried out by placing unfolded straws on a metal rack that was 10 cm above the surface of the liquid nitrogen ($LN_2$) and allowed to remain for 10 minutes. This measure made it possible to reduce the temperature slowly and gradually to avoid the formation of ice crystals within cells. The straws were pre-frozen and then quickly transferred into liquid nitrogen (-196 °C) so that they could be preserved on a long-term basis.

**Post-thaw Procedure**

To determine the effectiveness of the cryopreservation procedure and the protective impact of the different treatment formulations, post-thaw evaluations were done 24 hours following the first freezing. Straws were thawed by putting them in the 37 °C water bath for 30 s to avoid thermal shock. After thawing, the respective semen of each straw was gently blended and examined with the CASA system (AndroScope, Minitube, Germany) to examine the parameters of kinematics and motility. All post-thaw analyses of the samples were done within five minutes of thawing to reduce time-related reproductive deterioration of sperm qualities.

**Assessment of Malondialdehyde (MDA) Levels**

The thiobarbituric acid (TBA) assay was used to measure malondialdehyde (MDA), which is an intermediate of lipid peroxidation. The samples of semen were combined with 0.25 mL of ferrous sulfate (0.2 mM) and 0.25 mL of ascorbic acid (1 mM) and incubated in a 37 °C water bath. After the incubation period, trichloroacetic acid (15% w/v) and thiobarbituric acid (0.375% w/v) of 1 mL each were added, and the mixture was boiled for 10 min. The reaction was stopped by cooling the samples to a temperature of 4 °C, followed by centrifugation of the samples at 800 x g at a temperature of 4 °C. A 2 mL sample of the resulting supernatant was taken and measured, respectively, in a UV-visible spectrophotometer (Analytik Jena, Specord 250 Plus) at 532 nm, according to the procedure of Ratchamak *et al*. (2019). The MDA concentrations have been determined according to a standard curve that was prepared with the 1,1,3,3-tetramethoxypropane and presented in nanomoles per $10^8$ spermatozoa per hour (nM/$10^8$ sperm/h).

**Quantitative Evaluation of Post-Thaw Sperm Kinematic and Motility Profiles with CASA**

Sperm kinematic and motility parameters were measured with the help of a CASA system (AndroScope 1283, Minitube, Germany). Upon thawing, the semen samples were swirled gently and then diluted with a Tris extender pre-equilibrated at 37 °C to bring the sperm concentration to a favorable level of about 20-30 × $10^6$ to the point where the samples can be easily tracked and analyzed using the CASA system. The CASA system was adjusted to the analysis according to the recommendations of the manufacturer and optimized according to previous reports (Fraser *et al*., 2025). The CASA system was tuned with the following analytical parameters: 45 frames were studied during a frame rate of 60 Hz; the minimum contrast of the cell was calibrated at 46, and the minimum size of the detectable cell was calibrated as 7 pixels. The criteria of sperm motility were a straightness (STR) value of 45%, a velocity average path (VAP) value of 45 μm/s, a lower limit of VAP of 20 μm/s, and a minimum straight-line velocity (VSL) of 5.0 μm/s.

The settings of the analysis were pre-optimized with species-specific default parameters defined by the AndroScope software to work with boar sperm. The 5 μL of diluted semen was loaded on the chamber slides (20 μm depth; Leja, Netherlands) prewarmed at 37 °C. Each specimen was assessed: motility assessments included progressive and fast motility. Progressive motility of spermatozoa refers to the percentage of sperm undergoing swift, straight, and direct movements (VSL ≥ 40 μm/s and STR > 45%) (Li *et al*., 2023). The subpopulation of sperm that displayed high velocity classes at the highest percentile of the CASA-defined thresholds was referred to as fast motility.

The following measures of the kinematic parameters were taken: 1) Velocity curvilinear (VCL, um/s): speed of sperm movement averaged by the course actually covered; 2) Velocity straight-line (VSL, mms/s): the speed of the sperm at the first and the final point where it passes; 3) Velocity average path (VAP, um/s): the speed of a reasonably computed smooth path; 4) Distance curvilinear (DCL, um): the total length of the path that the sperm follows in reality; 5) Distance straight-line (DSL, m 5): the linear distance of the endpoint of the sperm path to the origin point; 6) Distance average path (DAP, um): the sum of the distance of movement in the average (calculated) path; 7) Amplitude of lateral head displacement (ALH, um): is the amplitude of the over-side movement of the sperm head in motion; 8) Beat cross frequency (BCF, Hz): the frequency at which the sperm head crosses its mean path, which is the dynamics of flagellar motion; 9) Head area circularity (HAC, percent): This is another geometric ratio of sperm head circularity; 10) Linearity (LIN, percent): the value indicates the straightness of the path in relation to the curvilinear path; and 11) Straightness (STR, percent): an indicator of the extent to which the movement mean path is linear (Tomás-Almenar & de Mercado, 2022). At least five randomly chosen fields in the microscope were noted in each and

every sample, and at least 200 motile spermatozoa in any one particular sample were assessed to achieve a good statistical analysis. All the treatment groups were triplicated to make sure that the data were reproducible.

### Sperm Plasma Membrane Integrity

A hypoosmotic swelling (HOS) test of sperm plasma membrane integrity was performed based on the one presented by Kang *et al*. (2019) and slightly modified. Semen samples were then thawed following cryopreservation and moved into 1.5 M microcentrifuge tubes. A 30 μL of the thawed semen was combined with 300 μL of hypoosmotic swelling (HOS) solution and incubated at 37 °C for 40 minutes. Following incubation, 5 uL of the mixture was dropped onto a clean glass slide, and it was covered with a coverslip, and it was viewed under a light microscope at 400x magnification. At least 200 sperm cells on a slide were counted and labeled as swollen and non-swollen. Spermatozoa that contained typical tail swellings were regarded as having intact plasma membranes, whereas non-swelling spermatozoa were detected as affected by membrane integrity.

### Statistical Analysis

One-way ANOVA was used to compare the effects of treatments, and the multiple range test was used to do the post hoc analysis on the results in pairs, as given by Duncan. The statistical analysis was done with SPSS (version 20; IBM Corp., Armonk, NY, USA) and presented as mean ± standard deviation (SD). The level of statistical significance was established as $p<0.05$.

## RESULTS

### Fresh Semen Quality

Fresh semen of the Landrace boars showed excellent reproductive performance with an average ejaculate volume of 225 mL and a sperm concentration of 467 x $10^6$ cells/mL. The percentage of progressive sperm motility was 95.77, with the viability of 96.84, and morphological defects were insignificant (2.0%). Kinematic analysis revealed vigorous and well-coordinated movement (VCL = 205.5 μm/s; VSL = 87.86 μm/s; VAP = 104.21 μm/s), supported by strong flagellar activity (ALH = 4.13 μm; BCF = 15.60 Hz) and balanced trajectory control (LIN = 0.43; STR = 0.82). Altogether, these parameters prove the high-quality motility, morphology, and viability of Landrace boar semen, which are the key aspects of high fertility potential and the applicability of this semen in the context of artificial insemination.

### Sperm Motility

There was a significant difference between the treatment groups on post-thaw progressive sperm motility, with the lowest value of the control T0 (11.00%) and the highest of the treatment T7 (38.04%). Fast sperm motility was similar and grew from 4.79% in T0 to 22.42% in T7. These findings indicate clearly that supplementation by cholesterol-loaded cyclodextrin (CLC) and Moringa leaf extract (MLE) improved the parameters of motility, and the combination was better than the individual supplementation. The effect of the treatment was verified as strong, with a statistically significant ($p<0.05$; Table 1). The progressive sperm motility in the treatments T5, T6, T7, and T8 was more than the minimum percentage of 30 as stated by the Indonesian National Standard (SNI), which means that the treatments could prevent the sperm activity outside the range of an acceptable level to be used in artificial insemination.

### Sperm Kinematics

Further evidence for these findings was provided by the kinematic analysis given. Computer-Assisted Sperm Analysis (CASA) revealed that boar sperm that were cryopreserved with the joint supplementation of CLC and MLE, especially in treatment T7, had a significant increase in the velocity index. Specifically, the VCL, VSL, and VAP values were 106.34 μm/s, 36.28 μm/s, and 45.41 μm/s, respectively, compared to the control group values (46.72 μm/s, 13.32 μm/s, and 19.95 μm/s; $p<0.05$; Table 2). There were also consistent improvements on the distance-related parameters, such as DCL, DSL, and DAP, in the combined treatments ($p<0.05$; Table 3). Also, the indices of flagellar activity, including ALH, BCF, and HAC, showed a significant increase, especially in treatments T7 and T8 ($p<0.05$; Table 4). Equally, the linearity (LIN) and straightness (STR) measures were greater in the extenders containing CLC and MLE than in the control group ($p<0.05$; Table 5). The sperm kinematic values achieved in the current experiment (T7) were within the range being reported in the previous studies; the values of VCL were 50-135 μm/s, the values of VSL were 28-68

Table 1. Motility variables of cryopreserved Landrace boar sperm in Tris–egg yolk extender supplemented with CLC and/or MLE

| Treatments | Motility variables | |
|---|---|---|
| | Progressive motility (%) | Fast motility (%) |
| T0 | 11.00±1.86$^{f}$ | 4.79±1.05$^{g}$ |
| T1 | 17.55±3.19$^{e}$ | 10.89±1.44$^{f}$ |
| T2 | 24.51±4.86$^{cd}$ | 13.26±1.65$^{de}$ |
| T3 | 22.87±4.23$^{d}$ | 11.60±0.90$^{ef}$ |
| T4 | 29.42±5.22$^{bc}$ | 14.30±0.84$^{d}$ |
| T5 | 31.89±4.58$^{b}$ | 17.43±1.13$^{c}$ |
| T6 | 33.50±3.33$^{ab}$ | 18.78±1.70$^{bc}$ |
| T7 | 38.04±2.70$^{a}$ | 22.42±2.06$^{a}$ |
| T8 | 31.53±3.33$^{b}$ | 20.51±2.80$^{ab}$ |
| p-value | 0.00 | 0.00 |

Note: All data are expressed as mean ± standard deviation (SD) based on five independent replicates. Different superscripts within the same column indicate significant differences ($p<0.05$). T0 (TY, control), T1 (TY + CLC 1 mg/mL), T2 (TY + CLC 2 mg/mL), T3 (TY + MLE 1 mg/mL), T4 (TY + MLE 1.5 mg/mL), T5 (TY + CLC 1 mg/mL + MLE 1 mg/mL), T6 (TY + CLC 1 mg/mL + MLE 1.5 mg/mL), T7(TY + CLC 2 mg/mL + MLE 1 mg/mL), T8 (TY + CLC 2 mg/mL + MLE 1.5 mg/mL). TY= Tris-egg yolk, CLC= Cholesterol-loaded cyclodextrin, MLE= Moringa leaf extract.

Table 2. Velocity variables of cryopreserved Landrace boar sperm in Tris–egg yolk extender supplemented with CLC and/or MLE

| Treatments | Velocity variables | | |
|---|---|---|---|
| | VCL (μm/s) | VSL (μm/s) | VAP (μm/s) |
| T0 | 46.72±4.80$^{e}$ | 13.32±1.57$^{e}$ | 19.95±2.05$^{e}$ |
| T1 | 76.21±13.32$^{d}$ | 23.80±3.06$^{d}$ | 32.54±5.69$^{d}$ |
| T2 | 83.29±4.39$^{cd}$ | 26.12±2.22$^{cd}$ | 35.56±1.88$^{cd}$ |
| T3 | 84.58±7.92$^{cd}$ | 26.54±2.19$^{cd}$ | 36.12±3.38$^{cd}$ |
| T4 | 95.33±17.33$^{b}$ | 32.47±3.96$^{b}$ | 40.70±7.40$^{bc}$ |
| T5 | 82.10±11.97$^{cd}$ | 26.73±4.16$^{cd}$ | 35.06±5.11$^{cd}$ |
| T6 | 86.16±6.55$^{cd}$ | 29.46±3.23$^{bc}$ | 36.79±2.80$^{cd}$ |
| T7 | 106.34±4.35$^{ab}$ | 36.28±1.97$^{a}$ | 45.41±1.86$^{ab}$ |
| T8 | 108.30±5.23$^{a}$ | 36.72±2.02$^{a}$ | 46.24±2.23$^{a}$ |
| p-value | 0.00 | 0.00 | 0.00 |

Note: All data are expressed as mean ± standard deviation (SD) based on five independent replicates. Different superscripts within the same column indicate significant differences (p<0.05). T0 (TY, control), T1 (TY + CLC 1 mg/mL), T2 (TY + CLC 2 mg/mL), T3 (TY + MLE 1 mg/mL), T4 (TY + MLE 1.5 mg/mL), T5 (TY + CLC 1 mg/mL + MLE 1 mg/mL), T6 (TY + CLC 1 mg/mL + MLE 1.5 mg/mL), T7 (TY + CLC 2 mg/mL + MLE 1 mg/mL), T8 (TY + CLC 2 mg/mL + MLE 1.5 mg/mL). TY= Tris-egg yolk, CLC= Cholesterol-loaded cyclodextrin, MLE= Moringa leaf extract. VCL= Curvilinear Velocity, VSL= Straight-Line Velocity, VAP= Average Path Velocity.

Table 3. Distance variables of cryopreserved Landrace boar sperm in Tris–egg yolk extender supplemented with CLC and/or MLE

| Treatments | Distance variables | | |
|---|---|---|---|
| | DCL (μm) | DSL (μm) | DAP (μm) |
| T0 | 17.01±2.83$^{e}$ | 7.14±1.38$^{d}$ | 9.23±1.77$^{c}$ |
| T1 | 25.74±3.27$^{d}$ | 8.79±1.40$^{c}$ | 9.91±1.05$^{bc}$ |
| T2 | 26.15±3.26$^{cd}$ | 10.25±1.42$^{b}$ | 12.26±1.60$^{abc}$ |
| T3 | 28.66±2.79$^{bcd}$ | 10.50±0.75$^{ab}$ | 11.73±0.78$^{bc}$ |
| T4 | 31.24±6.45$^{abc}$ | 10.95±1.06$^{ab}$ | 12.66±1.54$^{abc}$ |
| T5 | 26.19±3.88$^{cd}$ | 10.30±0.63$^{b}$ | 16.76±9.41$^{a}$ |
| T6 | 28.19±4.15$^{bcd}$ | 11.51±1.06$^{ab}$ | 13.98±1.32$^{abc}$ |
| T7 | 32.63±2.75$^{ab}$ | 11.96±1.02$^{a}$ | 14.28±1.00$^{ab}$ |
| T8 | 33.98±1.29$^{a}$ | 10.62±0.81$^{ab}$ | 12.94±0.41$^{abc}$ |
| p-value | 0.00 | 0.00 | 0.04 |

Note: All data are expressed as mean ± standard deviation (SD) based on five independent replicates. Different superscripts within the same column indicate significant differences (p<0.05). T0 (TY, control), T1 (TY + CLC 1 mg/mL), T2 (TY + CLC 2 mg/mL), T3 (TY + MLE 1 mg/mL), T4 (TY + MLE 1.5 mg/mL), T5 (TY + CLC 1 mg/mL + MLE 1 mg/mL), T6 (TY + CLC 1 mg/mL + MLE 1.5 mg/mL), T7(TY + CLC 2 mg/mL + MLE 1 mg/mL), T8 (TY + CLC 2 mg/mL + MLE 1.5 mg/mL). TY= Tris-egg yolk, CLC= Cholesterol-loaded cyclodextrin, MLE= Moringa leaf extract. DCL= Distance Curvilinear, DSL= Distance Straight Line, DAP= Distance Average Path.

Table 4. Head movement variables of cryopreserved Landrace boar sperm in Tris–egg yolk extender supplemented with CLC and/or MLE

| Treatments | Head movement variables | | |
|---|---|---|---|
| | ALH (μm) | BCF (Hz) | HAC rad) |
| T0 | 1.23±0.16$^{c}$ | 7.09±1.84$^{d}$ | 0.21±0.02$^{c}$ |
| T1 | 1.95±0.35$^{b}$ | 8.33±1.21$^{abcd}$ | 0.28±0.04$^{b}$ |
| T2 | 2.08±0.37$^{b}$ | 7.60±0.69$^{cd}$ | 0.26±0.03$^{b}$ |
| T3 | 1.98±0.22$^{b}$ | 8.46±1.63$^{abcd}$ | 0.28±0.02$^{b}$ |
| T4 | 2.01±0.32$^{b}$ | 9.23±1.88$^{abc}$ | 0.27±0.05$^{b}$ |
| T5 | 1.85±0.13$^{b}$ | 8.09±1.50$^{bcd}$ | 0.25±0.02$^{b}$ |
| T6 | 2.07±0.26$^{b}$ | 9.00±1.83$^{abcd}$ | 0.29±0.06$^{b}$ |
| T7 | 2.62±0.15$^{a}$ | 10.36±1.37$^{a}$ | 0.37±0.02$^{a}$ |
| T8 | 2.80±0.09$^{a}$ | 9.92±0.29$^{ab}$ | 0.37±0.01$^{a}$ |
| p-value | 0.00 | 0.02 | 0.00 |

Note: All data are expressed as mean ± standard deviation (SD) based on five independent replicates. Different superscripts within the same column indicate significant differences (p<0.05). T0 (TY, control), T1 (TY + CLC 1 mg/mL), T2 (TY + CLC 2 mg/mL), T3 (TY + MLE 1 mg/mL), T4 (TY + MLE 1.5 mg/mL), T5 (TY + CLC 1 mg/mL + MLE 1 mg/mL), T6 (TY + CLC 1 mg/mL + MLE 1.5 mg/mL), T7(TY + CLC 2 mg/mL + MLE 1 mg/mL), T8 (TY + CLC 2 mg/mL + MLE 1.5 mg/mL). TY= Tris-egg yolk, CLC= Cholesterol-loaded cyclodextrin, MLE= Moringa leaf extract. ALH= Amplitude of Lateral Head Displacement, BCF= Beat Cross Frequency, HAC= Hyperactivation Ratio.

Table 5. Trajectory indices variables of cryopreserved Landrace boar sperm in Tris–egg yolk extender supplemented with CLC and/or MLE

| Treatments | Trajectory indices variables | |
|---|---|---|
| | LIN | STR |
| T0 | 0.29±0.01$^{c}$ | 0.67±0.01$^{c}$ |
| T1 | 0.32±0.03$^{b}$ | 0.74±0.06$^{b}$ |
| T2 | 0.31±0.02$^{b}$ | 0.74±0.04$^{b}$ |
| T3 | 0.32±0.01$^{b}$ | 0.74±0.02$^{b}$ |
| T4 | 0.34±0.02$^{a}$ | 0.80±0.05$^{a}$ |
| T5 | 0.33±0.02$^{ab}$ | 0.76±0.05$^{ab}$ |
| T6 | 0.34±0.01$^{a}$ | 0.80±0.04$^{a}$ |
| T7 | 0.34±0.02$^{a}$ | 0.80±0.03$^{a}$ |
| T8 | 0.34±0.01$^{a}$ | 0.79±0.02$^{a}$ |
| p-value | 0.00 | 0.00 |

Note: All data are expressed as mean ± standard deviation (SD) based on five independent replicates. Different superscripts within the same column indicate significant differences (p<0.05). T0 (TY, control), T1 (TY + CLC 1 mg/mL), T2 (TY + CLC 2 mg/mL), T3 (TY + MLE 1 mg/mL), T4 (TY + MLE 1.5 mg/mL), T5 (TY + CLC 1 mg/mL + MLE 1 mg/mL), T6 (TY + CLC 1 mg/mL + MLE 1.5 mg/mL), T7(TY + CLC 2 mg/mL + MLE 1 mg/mL), T8 (TY + CLC 2 mg/mL + MLE 1.5 mg/mL). TY= Tris-egg yolk, CLC= Cholesterol-loaded cyclodextrin, MLE= Moringa leaf extract. LIN= Linearity, STR= Straightness.

μm/s, and the values of VAP were 37.7-82 μm/s (Fraser *et al*., 2025; Peña *et al*., 2022).

### Sperm Plasma Membrane Integrity and Malondialdehyde Concentration

The supplementation with CLC and/or MLE had a significant effect (p<0.05) on the plasma membrane integrity and malondialdehyde (MDA) concentration of cryopreserved landrace boar sperm (Table 6). Integrity of plasma membranes also rose as the CLC and MLE were added to reach the maximum level of 58.19% intact membrane in T7 (2 mg/mL CLC + 1 mg/mL MLE). On the other hand, MDA levels, which are an indicator of lipid peroxidation, declined from 36.60 nM/$10^{8}$ sperm/h in the control group (T0) to 26.40 nM/$10^{8}$ sperm/h in T7, followed by a slight increase to 27.40 nM/$10^{8}$ sperm/h in T8. These results indicate that 2 mg/mL CLC and 1 mg/mL MLE should be used as the best combination to improve the stability of the sperm plasma membrane and successfully decrease the level of oxidative damage on sperm in the process of cryopreservation.

Table 6. Plasma membrane integrity and malondialdehyde concentration of cryopreserved landrace boar sperm in tris–egg yolk extender supplemented with CLC and/or MLE

| Treatments | Variables | |
|---|---|---|
| | Plasma membrane integrity (%) | MDA (nM/108 sperm/h) |
| T0 | 22.10±2.28$^{f}$ | 36.60±0.55$^{e}$ |
| T1 | 28.00±2.55$^{e}$ | 31.40±0.54$^{d}$ |
| T2 | 35.21±4.97$^{d}$ | 29.80±0.84$^{c}$ |
| T3 | 33.85±4.23$^{d}$ | 32.20±0.83$^{d}$ |
| T4 | 38.26±4.34$^{d}$ | 29.60±0.89$^{c}$ |
| T5 | 45.19±3.96$^{c}$ | 28.20±0.84$^{b}$ |
| T6 | 52.15±5.16$^{b}$ | 27.80±0.83$^{b}$ |
| T7 | 58.19±3.77$^{a}$ | 26.40±0.55$^{a}$ |
| T8 | 45.23±2.34$^{c}$ | 27.40±0.54$^{b}$ |
| p-value | 0.00 | 0.00 |

Note: All data are expressed as mean ± standard deviation (SD) based on five independent replicates. Different superscripts within the same column indicate significant differences ($p<0.05$). T0 (TY, control), T1 (TY + CLC 1 mg/mL), T2 (TY + CLC 2 mg/mL), T3 (TY + MLE 1 mg/mL), T4 (TY + MLE 1.5 mg/mL), T5 (TY + CLC 1 mg/mL + MLE 1 mg/mL), T6 (TY + CLC 1 mg/mL + MLE 1.5 mg/mL), T7(TY + CLC 2 mg/mL + MLE 1 mg/mL), T8 (TY + CLC 2 mg/mL + MLE 1.5 mg/mL). TY= Tris-egg yolk, CLC= Cholesterol-loaded cyclodextrin, MLE= Moringa leaf extract. MDA= malondialdehyde

## DISCUSSION

The current research revealed that the addition of a mixture of CLC and MLE to the boar semen diluent had a significant effect in enhancing post-thaw sperm motility and other kinematic parameters in comparison to when either of the components was added individually. The evident increased performance of T7 shows the significance of considering both structural and biochemical properties of cryoinjury. Cryopreservation exposes sperm cells to immense stress because of membrane phase changes, osmotic variations, and oxidative damage. It was discovered that the addition of CLC boosted the physical stability of sperm membranes by boosting the cholesterol level in them, thus leading to reduced cold shock vulnerability. This observation is also evidenced by the high levels of sperm plasma membrane integrity recorded in the samples that were supplemented with CLC over those that were not supplemented (Table 6). MLE is also oxidative stress counteracting since it is rich in antioxidants (El-Seadawy *et al*., 2022). The two mechanisms work together in a synergistic manner to maintain sperm functionality following the thawing process.

The positive changes in T7, over T6 and T5, indicate that potentially the best ratio of cholesterol incorporation and antioxidant protection is 2 mg/mL of CLC and 1 mg/mL of MLE. In this level, the incorporation of cholesterol is adequate to enhance the rigidity of the membrane and minimize the changes in permeability without affecting capacitation (Rajoriya *et al*., 2020). In the meantime, MLE of 1 mg/mL offers strong antioxidant protection (Iqbal *et al*., 2022) without leveling off or having a pro-oxidant effect. It is essential to optimize the dose optimization since oversupplementation may change the surface membrane fluidity or disrupt the metabolic pathways.

In line with these results, malondialdehyde (MDA) levels, which are the main indicators of lipid peroxidation, showed a significant decrease from 36.60 nM in the control group (T0) to 26.40 nM at T7 and slightly increased to 27.40 nM at T8 when more MLE was added to the solution, namely 1.5 mg/mL (Table 6). This trend indicates that 1.0 mg/mL MLE is adequate to reduce the oxidative damage of lipids, and additional antioxidant effects are not evident in higher concentrations. The incremental rise of MDA at the increased level of MLE might be a sign of a threshold above which the benefit of antioxidant supplementation is no longer possible, possibly because of redox imbalance or the emergence of mild pro-oxidant effects.

Regarding the functional aspect, better progressive and fast sperm motility would be especially useful in the case of artificial insemination programs since the two parameters are directly related to the fertilization potential. The increase of VCL, VSL, and VAP values suggests more efficient and linear tracks, which means that the sperm uses reduced energies and, thus, has high chances of reaching the oocyte (Barquero *et al*., 2021). The T7 high VCL values are an indication of intact axonemal and mitochondrial functioning. Higher VSL and VAP mean that there are intentional movement mechanisms that are necessary to move through the female reproductive tract. These enhancements are in line with the reports that relate mitochondrial integrity to high CASA parameters and fertility performance (Ahmed *et al.,* 2019).

Mechanistically, CLC facilitates ion gradient stability in the plasma membrane, which is vital in the operation of the flagellum (Zhang *et al*., 2024). In the meantime, MLE can inhibit oxidative changes in mitochondrial enzymes and DNA that will maintain the generation of adenosine triphosphate (ATP) necessary to maintain motility (Hirao *et al*., 2024). MLE enhanced motility in the absence of supplementation with additional antioxidants as compared to the control, which highlights the importance of antioxidant supplementation in reducing cryodamage. However, the combined treatment was evidently more effective in boar sperm, which need interventions that address the stability of the membranes and the balance of the oxidative processes.

The practical implications of the observed improvements in swine breeding are practical. The boar spermatozoa are particularly sensitive to cryopreservation, which makes them more susceptible to cold shock and oxidative stress in comparison with sperm in other species of livestock because of their comparatively low cholesterol-to-phospholipid ratio and abundance of polyunsaturated fatty acids in their membrane (Castro *et al*., 2025). The low protective efficiency limits of the conventional extenders have curtailed the effective application of frozen boar semen in artificial insemination. The CLC–MLE formulation tested in this study addresses a longstanding limitation by demonstrating substantial gains in motility and kinematics.

Highly VCL, VSL, and VAP contribute to the progression of sperm and increase the probability of successful sperm penetration to the oocyte at fertilization. These parameters of kinematics have quantitative measures of functional competence, which provide more information than percentages of motility. An increase in T7 values does show that the sperm is not just surviving the freeze-thaw procedure but that it also still has the physiological properties to go through with a fertilization process. The result of this observation conforms to the results of previous studies that have also established that there is a correlation between the enriched CASA-derived parameters and increased conception rates (Li *et al*., 2025). Therefore, current findings are not just laboratory advances, but they also have well-informed translational opportunities in enhancing fertility outcomes in the discipline.

The beneficial effects of cholesterol supplementation on sperm freezing have been highly examined in different species and clearly reported (Zhang *et al*., 2024). The presence of CLC compared to controls in the current research has continuously favored the use of the treatment in stabilizing the membrane, which is regarded as the essence of cryosurvival. The use of cholesterol must have reduced the loss of membrane proteins required to recognize the sperm-egg and inhibited premature acrosome reactions, which are vital to fertilization (Rajoriya *et al*., 2020). The most effective concentrations were noted as 2 mg/mL, which correlates with prior results in the case of bovine semen (Aly *et al*., 2025) and implies a dose-responsive reaction. Nevertheless, overloading of cholesterol may prevent capacitation, which emphasizes the significance of the correct concentration level (LaVelle *et al*., 2023).

The input of MLE as an antioxidant source is also extensive. The outcome of cryopreservation is the excess production of ROS and, as a result, oxidative stress, which disrupts the integrity of lipids, proteins, and DNA (Shi *et al*., 2024). MLE is rich in flavonoids, polyphenols, and vitamin C, can survive ROS, and disperse oxidative chain reactions (Nuapia *et al*., 2020). Interestingly, a significant difference was not found at 1-to 1.5 mg/mL, suggesting a plateau in the efficacy. This finding is aligned with the fact that excessive antioxidant supplementation can be neutral or pro-oxidant, and the need to carefully control the amount of the supplements (Andrés *et al*., 2023).

The fact that CLC and MLE supplementation have a synergetic effect is a strong indication that it is better to target multiple pathways of cryoinjury rather than single pathways. Cryodamage is compound: fragility of the membrane predisposes the membrane to oxidative stress, and oxidative alteration contributes to decreasing the membrane stability (Castellini *et al*., 2024). This strategy is achieved by stabilizing the membrane while at the same time minimizing oxidative damage, interrupting the cycle, and greatly improving the survival of the sperm in cryopreservation.

The findings are consistent with the large-scale studies of cryobiology, which indicate that combined approaches are usually more effective. It has already been demonstrated that equine, ram, rabbit, and bovine semen possess researched ways of combining cholesterol analogues with antioxidants to improve post-thaw motility as compared to single treatments (Ahmet *et al*., 2025; Contreras *et al*., 2022). The current paper elaborates on the idea, utilizing it with swine reproduction, and proposing the use of Moringa oleifera as a new source of natural, efficient, and relatively inexpensive antioxidants. The availability of moringa in most swine-producing areas makes this method more viable, especially in developing nations where low-cost interventions are needed to boost livestock production.

The artificial insemination programs have serious implications. Traditionally, frozen boar semen is less fertile than fresh semen, and this fact limits its application in the business environment (Waberski *et al*., 2019). The use of the CLC-MLE extender leads to high-quality post-thaw sperm and can potentially improve the conception rates, litter sizes, and the efficiency of AI programs in general. Besides boosting production, the development contributes to biosecurity, the dissemination of genetic materials, and germ conservation, as frozen semen can be easily stored and moved between regions.

Lastly, the motility and kinematic characteristics of boar semen that had undergone thawing were hugely enhanced when the extender was supplemented by a cholesterol-loaded cyclodextrin/moringa leaf extract complex. This innovation has a massive potential of improving artificial insemination of pigs in production as well as accepting conservation in case of maintaining genetic resources.

## CONCLUSION

The experiment shows that the addition of cholesterol-loaded cyclodextrin (CLC) and Moringa oleifera leaf extract (MLE) to the Tris-egg yolk extender provides boar sperm synergist protection during the process of cryopreservation. Among all the treatments, a mixture of 2 mg/mL CLC and 1 mg/mL MLE (T7) brought out the best results of the progressive motility, fast motility, and kinematic parameters (VCL, VSL, VAP, DCL). This confirms its superior capacity to preserve both structural and functional sperm integrity after thawing. These results suggest that dual supplementation with CLC and MLE is more effective than either additive alone and provides an optimized strategy to enhance post-thaw semen quality in boars.

## CONFLICT OF INTEREST

The authors declare that they have no competing interests, financial or otherwise, that could be perceived to have influenced the research and conclusions presented in this paper.

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

We state that throughout the production of this work, language was refined using generative AI and AI-assisted technologies. Following their use of the service, the authors took full responsibility for the publication's content and reviewed and revised it as needed.

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