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Full Length Research

Kinematic responses of cold-preserved rote ram sperm to supplementation with bovine serum albumin and moringa leaf extract

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ABSTRACT: The decrease in the sperm kinematic quality during cold storage is still a significant limitation in the artificial insemination programs, especially on local breeds like the Rote sheep. This experiment was an attempt to determine the impacts of adding Bovine Serum Albumin (BSA), a protein derived from bovine blood plasma that functions as an antioxidant and stabilizer in biological media, and Moringa leaf extract (MLE), a natural extract from Moringa oleifera leaves rich in antioxidants, vitamins, and bioactive compounds, to Tris-based extender on the kinematic properties of Rote ram sperms at 5ºC in 48 hours. Two healthy Rote rams (2-3 years old) were collected with an electroejaculator, and semen was evaluated macroscopically and microscopically. Processing was done on ejaculates whose progressive motility was ≥70%, and sperm abnormalities were less than 20%. In the control (P0), the semen samples were diluted with Tris-egg yolk extender; supplementation was made with 0.3% BSA (P1), 0.125% MLE (P2), 0.3% BSA + 0.125% MLE (P3), 0.3% BSA + 0.25% MLE (P4), 0.45% BSA + 0.125% MLE (P5), and 0.45% BSA + 0.25% MLE (P6). Diluted semen samples were stored in Eppendorf tubes and maintained at 5°C in a refrigerator for up to 48 hours. The Computer-Assisted Sperm Analysis (CASA; an automated and objective system used to evaluate sperm motility and kinematic characteristics through digital image processing) was used to determine sperm motility and kinematic parameters. It was established that treatment P3 had the highest and significantly different ($p<0.05$) results in the majority of the parameters, such as progressive motility (52.80%), fast motility (27.31%), VCL (curvilinear velocity, 105.85 µm/s), VSL (straight-line velocity, 41.55 µm/s), VAP (average path velocity, 49.55 µm/s), and STR (straightness ratio, 0.36). Finally, it was found that Tris extender supplemented with 0.3% BSA and 0.125% MLE was effective in preserving sperm kinematic quality of Rote ram semen throughout 48 hours of cold storage.

Keywords: Computer-assisted sperm analysis, extender supplementation, oxidative stress, semen preservation, sperm kinematics.

INTRODUCTION

Artificial insemination (AI) is a key factor in the genetic enhancement of improving reproduction efficiency in small ruminant production systems all over the world. AI can be used specifically in the tropical and developing areas, where it helps in preserving and distributing the best gametophytes of native breeds that are well adapted to the local environment. Nonetheless, the effectiveness of AI programs highly depends on the quality of the semen (Zuidema *et al*., 2021) that has good functional integrity during storage. Short-term liquid preservation at 4-5°C has been widely used in sheep, particularly in the field environment, because it is less expensive than cryopreservation (Alragubi, 2020; Dziekońska & Partyka, 2022). However, all semen storage methods are often

believed to cause a progressive reduction in sperm motility and kinematic properties and eventually reduce fertilising capacity (García-Molina *et al*., 2023; Mofadel *et al*., 2024).

Cold shock and oxidative stress are believed to be the main causes of the decrease in sperm quality during cold preservation. Reduction of temperature changes the lipid phase of the sperm plasma membrane and causes greater membrane permeability, ion imbalance, and dysfunction of the mitochondria. Moreover, reactive oxygen species (ROS) are built up in the course of storage, leading to lipid peroxidation, protein denaturation, and the destruction of DNA. These mechanisms are especially harmful to ram sperm, which have a high percentage of polyunsaturated fatty acids in their plasma membranes and therefore are very vulnerable to oxidative damage (Rizkallah *et al*., 2022; Zhang *et al*., 2024). There is thus a need to maximise the semen extenders with protective additives to diminish structural and functional harm when stored at low temperatures.

Among all the widely used extender supplements, Bovine Serum Albumin (BSA) has found much attraction due to its stabilising and antioxidant properties of the membrane (Fonseca *et al*., 2017). BSA functions as a macromolecular shield since it binds toxin metabolites and radicals, suppresses osmotic changes and anticipates premature capacitation-like changes. The presence of the property that it has to interact with membrane phospholipids assists in maintaining the sperm's structural integrity (Álvarez-Rodríguez *et al*., 2024) along with the motility patterns. Previous studies in other livestock species found that supplementation with BSA in extender can be used to improve chilled or frozen storage motility and viability (Álvarez-Rodríguez *et al*., 2024; Rahman *et al*., 2015; Rayan *et al*., 2023). However, the optimum amounts appear to be species-specific, and the quantity of excessive levels will not proportionately equal the benefits in any way.

At the same time, more focus has been given to the plant-derived antioxidants, as they are natural and sustainable in the preservation of semen. It is not a secret that Moringa oleifera is highly endowed in terms of bioactive compounds, including flavonoids, phenolic acids, vitamins, and essential amino acids. These elements are very strong antioxidants that can be applied to neutralise the ROS to inhibit lipid peroxidation (Abo El-Fadl *et al*., 2020; Park *et al*., 2022). The possible potential of Moringa leaf extract (MLE) in preserving ram semen and the use of standard protein supplements, such as BSA in particular, has limited information on the topic.

The Rote sheep, a native breed of the Rote Island, is a valuable genetic material that has adapted to extreme environmental conditions. This breed has enormous socio-economic implications for the local farmers, yet there is a lack of scientific information regarding its reproductive biotechnology, particularly in relation to semen preservation methods. Moreover, the use of advanced sperm motion testing with Computer-Assisted Sperm Analysis (CASA) is seldom used in the research on local Indonesian breeds. Kinematic data derived using CASA, e.g., curvilinear (VCL) and straight-line (VSL) velocity, average path (VAP) and straightness (STR), are more objective and complete descriptors of sperm functionality than traditional motility measurements (Van Der Horst, 2020).

So far, no researcher has conducted any study in a systematic investigation of the joint supplementation of BSA with Moringa leaf extract in a Tris based extender on the short-term cold storage of Rote ram semen or any research that has been conducted to examine the effects of this intervention through a comprehensive CASA kinematic profiling. A more effective approach in preserving the functionality of the sperm during chilled storage may be the potential synergistic effect between the protein-based membrane stabilisers and the plant-derived antioxidants.

Thus, this paper was meant to test the impact of varying levels of BSA and Moringa leaf extract on the motility and kinematic properties of Rote ram semen stored at 5°C after 48 hours of storage. The study hypothesised that the synergistic effects of the BSA and MLE supplementation would be beneficial in improving sperm kinematic quality more than either single supplementation or the control on sperm, therefore, offering an optimised formulation in short-term liquid semen storage in this native breed.

MATERIALS AND METHODS

Ethical clearance

The Livestock, Marine, and Fishery Research Ethics Committee (Approval No. 133/1.KT/KEPPKP/IX/2025) granted the ethics clearance of this research protocol.

Experimental animals

The samples used were fresh semen collected from two adult Rote rams, aged two to three years, raised on a small scale by local farmers on Timor Island. A total of five ejaculates from each ram were collected and subsequently pooled into a single composite sample prior to further processing. The rams were kept in individual pens and fed fresh grass and concentrates.

Experimental design

This experiment was designed in a completely randomised design with seven treatments and five replications, where each replication consisted of pooled semen collected from two Rote rams. Semen that met the standards was diluted with Tris-egg yolk diluent (control, P0) or supplemented with 0.3% BSA (P1), 0.125% MLE (P2), 0.3% BSA+0.125%

MLE (P3), 0.3% BSA + 0.25% MLE (P4), 0.45% BSA + 0.125% MLE (P5), and/or 0.45% BSA + 0.25% MLE (P6).

Moringa oleifera leaf extract (MLE) preparation

The Moringa leaf extract (MLE) was prepared by using the methods outlined by El-Seadawy et al. (2022), with slight changes. Fresh leaves of Moringa oleifera were washed in running water, emptied, and dried in a hot-air oven at 50°C until a constant weight was achieved. The dried leaves were blended in a blender to a fine powder, and sieved through a 40–60 mesh sieve to obtain a uniform particle size. The powder obtained was kept in air-tight containers until further extraction. The maceration method was used in the extraction. In a word, 100 g of the Moringa leaf powder was soaked with 500 mL of 96 per cent ethanol at room temperature. The mixture was stirred and shaken manually after every 12 h. After the maceration period, the solution was filtered using the Whatman filter paper and a glass funnel after maceration. Maceration was repeated twice in order to maximise the extraction effectiveness. The evaporated version of the mixed filtrates was again concentrated by rotary evaporator at 40-50°C till a viscous extract was achieved. To avoid degradation and maintain stability of bioactive compounds, the extract was kept in dark glass bottles at 4°C to prevent degradation and maintain the stability of bioactive compounds until further use.

Preparation of Tris-based extender

The extender was mixed in three portions: 3.03 g of Tris(hydroxymethyl)aminomethylsulfone (Sigma, 1.08307), 1.78 g of citric acid monohydrate (Sigma, 1.00244), and 1.25 g of fructose (Sigma, F3510) in 100 mL of the double-distilled water and homogenised with the help of magnetic stirring. This solution was combined with 20 per cent (v/v) of egg yolk, penicillin (1000 IU/mL), and streptomycin (1000 µg/mL) and stirred (Hine *et al*., 2026). Extender was also added to Bovine Serum Albumin (BSA) and MLE in the corresponding levels of the treatment groups.

Semen collection and evaluation

Electroejaculator was used to collect sample semen from two healthy and fertile Rote rams, with collections performed once a week for five weeks. The ejaculates were then immediately evaluated for both macroscopic and microscopic parameters to be in a position to test whether they were fit to be diluted. The parameters were measured as semen volume, semen colour, semen consistency, semen pH, semen concentration, total motility, and progressive motility (Blaga Petrean *et al*., 2023).

Semen dilution and preservation

Semen with progressive sperm motility exceeding 70 per cent and sperm abnormality less than 20 per cent was diluted in one of seven diluent formulations to 1 x 10^7 sperm/mL. The preserved samples of the diluted semen were kept at 5°C, and sperm motility and kinematics were subsequently determined after incubating the samples for 48 hours in a refrigerator.

Computer-Assisted Sperm Analysis (CASA)

The Computer-Assisted Sperm Analysis (CASA) system was used to assess sperm motility, and the parameters of sperm kinematics were applied according to the protocol by Hine and Nalley (2025). The samples of semen belonging to each treatment group were diluted with the corresponding extender to a final concentration of 6 × 10^6 cells/mL. Diluted samples were pipette transferred to 2 mL capped tubes and equilibrated at 37 ^oC in a mobile warming unit (Minitube) within 2-3 minutes. A 3 µL aliquot of each sample was loaded into a counting chamber and then inserted into the Androscope CASA system (Androscope CASA System 12500/3000, Minitube), to which a computer was attached to analyse. Sperm motility and kinematic parameters were determined after appropriate focusing. At least eight randomly chosen microscopic fields were examined on each sample, with a minimum of 1000 spermatozoa per sample being examined. The recorded motility parameters were progressive motility and fast motility. Kinematic parameters that were measured included curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), curvilinear distance (DCL), straight-line distance (DSL), average path distance (DAP), amplitude of lateral head movement (ALH), beat-cross frequency (BCF), head activity (HAC), linearity (LIN), and straightness (STR).

Research parameters

The progressive motility is a measure of the percentage of spermatozoa moving forward with a velocity value above the minimum value set in the CASA settings. Fast motility is defined as the ratio of spermatozoa that are classified as the rapid movers based on velocity limits that are set in the CASA system. Kinematic parameters: VCL (Curvilinear Velocity, µm/s): Velocity of real curved line. VSL (Straight-Line Velocity, µm/s): Straight-line velocity between the endpoint and the start. VAP (Average Path Velocity, -1mm/s): This is an average of a smooth path. DCL (Distance Curvilinear, µm): Distance which is covered by the curvilinear path. DSL (Distance Straight Line, µm): This is the straight line distance between the point of origin and the destination. DAP (Distance Average Path, µm):

The mean distance in the mean path. ALH (Amplitude of Lateral Head Displacement µm): This is the amplitude of the lateral movement of the sperm head. BCF (Beat Cross Frequency, Hz): The frequency of the traverse movement of the head. HAC (Hyperactivation Ratio, per cent): Per cent of hyperactivated motility sperms. LIN (Linearity, per cent): This is a ratio of VSL/ VCL, which is the measure of the straightness of movement. STR (Straightness, percentage): that is the Ratio of VSL to VAP, which is the efficiency of movement.

Statistical analysis

All data were processed by means of tabulations and the one-way analysis of variance (ANOVA) to show differences between treatment groups. Post-hoc comparisons between groups were done in case significant differences were found through the use of Honest Significant Difference (HSD) test by Tukey. The statistical analyses were performed with the help of the IBM SPSS Statistics version 25 (IBM Corp., Armonk, NY, USA), and the results were defined as statistically significant at $p<0.05$.

RESULTS

Sperm motility and kinematic parameters of fresh semen

Fresh ram semen exhibited quality parameters within the acceptable range according to standard ovine semen criteria, with progressive motility reaching 84.78% and rapid motility recorded at 39.37%, which indicated that a high percentage of actively motile spermatozoa that had sufficient fertilising potential existed. Kinematic analysis indicated high velocity parameters, such as curvilinear velocity (VCL; 135.16 µm/s), straight-line velocity (VSL; 45.97 µm/s), and average path velocity (VAP; 53.49 µm/s), which are indicators of vigorous flagellar activity and effective sperm pathways. All these values are signs of the optimal physiological status of fresh semen and establish its validity in dilution and short-term preservation of liquids.

Effect of treatments on sperm motility

Sperm motility parameters varied greatly ($P = 0.00$) after 48 h of storage at 5°C across treatments. The highest percentage of progressive and rapid motility was in treatment P3 (Tris + 0.3% BSA + 0.125% MLE), which was 52.80 and 27.31, respectively. The values were much more than those of all other treatments. Conversely, the control group (P0; Tris alone) and P6 (Tris + 0.45% BSA + 0.25% MLE) had the lowest progressive motility values (40.06% and 41.18%, respectively), and fast motility values (8.75%10.30%), which revealed reduced preservation efficacy at elevated additive concentrations (Figure 1).

All the treatments recorded reduced motility after being subjected to cold storage, but the extent to which they were reduced was significantly different compared with fresh semen. A combination of the balanced implementation of BSA and MLE offered effective protection against cold-induced motility loss, which was indicated by higher motility retention in P3. This, biologically, means that the moderate levels of membrane-stabilising and antioxidant constituents retain the functional integrity of sperm, but in excess amounts, they can reduce sperm motility, probably because they elevate cellular extender viscosity or disrupt cellular redox balance.

Effect of treatments on sperm kinematics

It was found that there were significant effects of the treatment on the sperm velocity and on the sperm trajectory parameters after 48 h of storage. Treatment P3 had the highest values of VCL (110.85 µm/s; $P = 0.019$) and VSL (41.55 µm/s; $P = 0.017$) and VAP (49.55 µm/s; $P = 0.016$) which were very high compared to those in P0, P2 and P6. Importantly, spermatozoa in P3 maintained over 80 per cent of the relevant fresh semen VCL and VSL values, which means that flagellar propulsion and forward movement have been preserved successfully. P6, on the other hand, exhibited the highest deterioration with very low values of velocity (VCL: 93.72 µm/s; VSL: 29.35 µm/s; VAP: 37.53 µm/s) (Figure 2).

Parameters of trajectory distances took a similar fashion. P3 showed the maximum distances of curvilinear (DCL; 36.48 µm), straight-line (DSL; 13.59 µm) and average path movement (DAP; 16.32 µm), which were significantly above the ones of the control and P6 treatments ($P < 0.05$). These results suggest that motility of the flagella and effective sperm movement in P3, and the decreased distances of DCL, DSL, and DAP in P6 (28.89 µm, 8.78 µm and 11.47 µm, respectively) point to a loss of flagellar integrity and motility mechanics, respectively (Figure 3).

P3 (2.54 µm) lateral head displacement (ALH) was within an optimum physiological bandwidth, which facilitated regulated flagellar beating. The beat cross frequency (BCF) had maximum values in P3 (14.34 Hz) and much higher than in P0, P2, and P6 ($P < 0.05$) which showed an improvement in flagellar activity (Figure 4a). In the same way, hyperactivation coefficient (HAC) was at the peak in P3 (0.40 rad) (Figure 4b), which indicated sperm at higher energetic state without excessive lateral displacement.

In addition, linearity (LIN; 0.36) and straightness (STR; 0.79) were much greater than in P3 than in P0 and P2 ($P < 0.05$) (Figure 5). These parameters indicate more targeted and effective sperm courses which are biologically related to enhanced ability of approaching

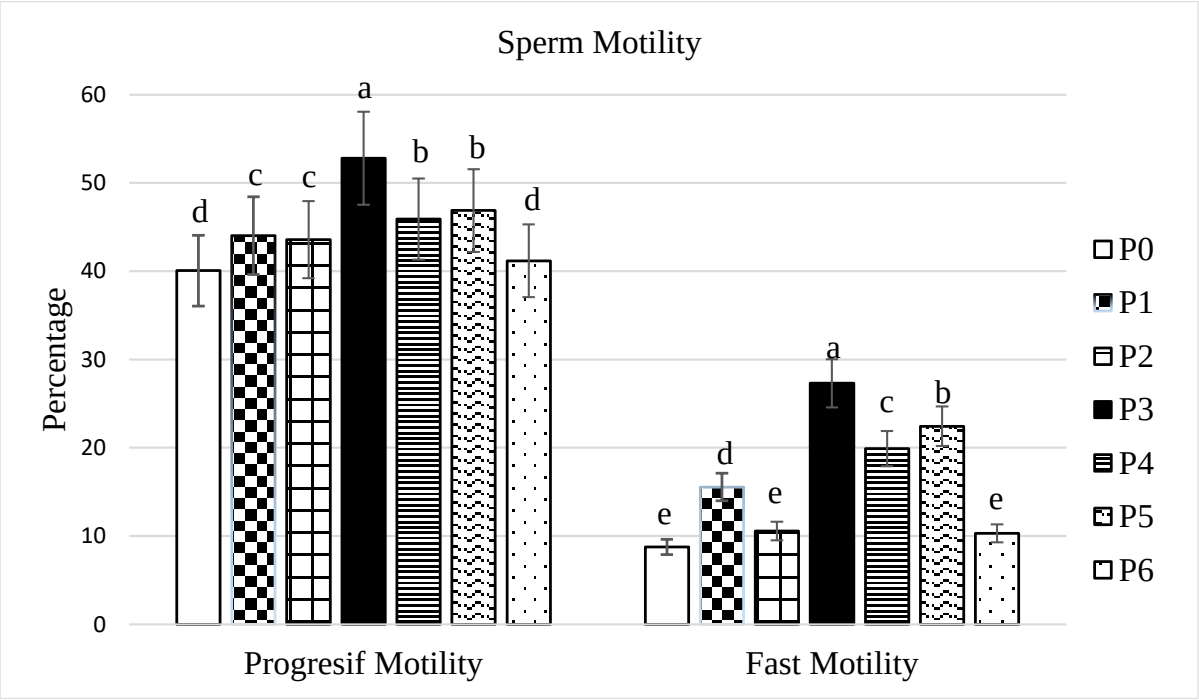

Figure 1. Rote ram sperm motility (percentage). Bars with different letters denote significantly different values ($p < 0.05$). P0 (Tris – egg yolk), P1 (P0 + 0.3% BSA) P2 (P0 + 0.125% MLE), P3 (P0 + 0.3% BSA + 0.125% MLE), P4 (P0 + 0.3% BSA + 0.25% MLE), P5 (P0 + 0.45% BSA + 0.125% MLE), P6 (P0 + 0.45% BSA + 0.25% MLE). BSA (bovine serum albumin), MLE (moringa leaf extract).

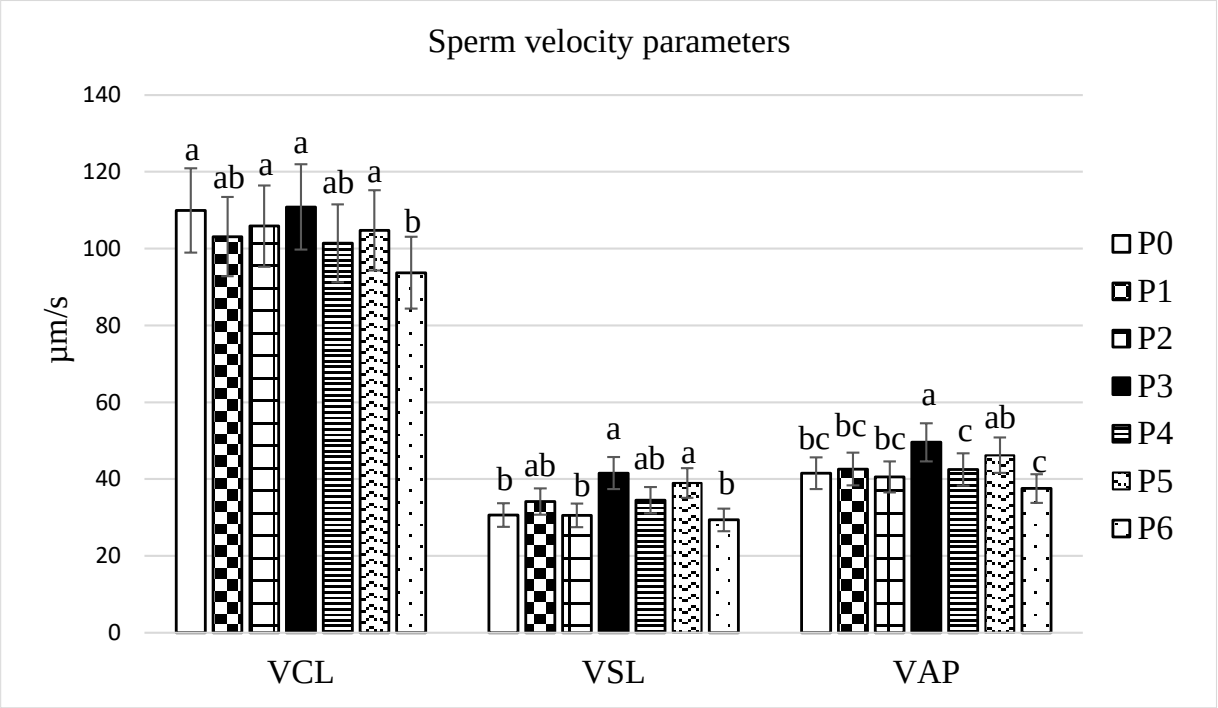

Figure 2. Rote ram sperm velocity parameters (µm/s). Bars with different letters denote significantly different values ($p < 0.05$). P0 (Tris – egg yolk), P1 (P0 + 0.3% BSA) P2 (P0 + 0.125% MLE), P3 (P0 + 0.3% BSA + 0.125% MLE), P4 (P0 + 0.3% BSA + 0.25% MLE), P5 (P0 + 0.45% BSA + 0.125% MLE), P6 (P0 + 0.45% BSA + 0.25% MLE). BSA (bovine serum albumin), MLE (moringa leaf extract).

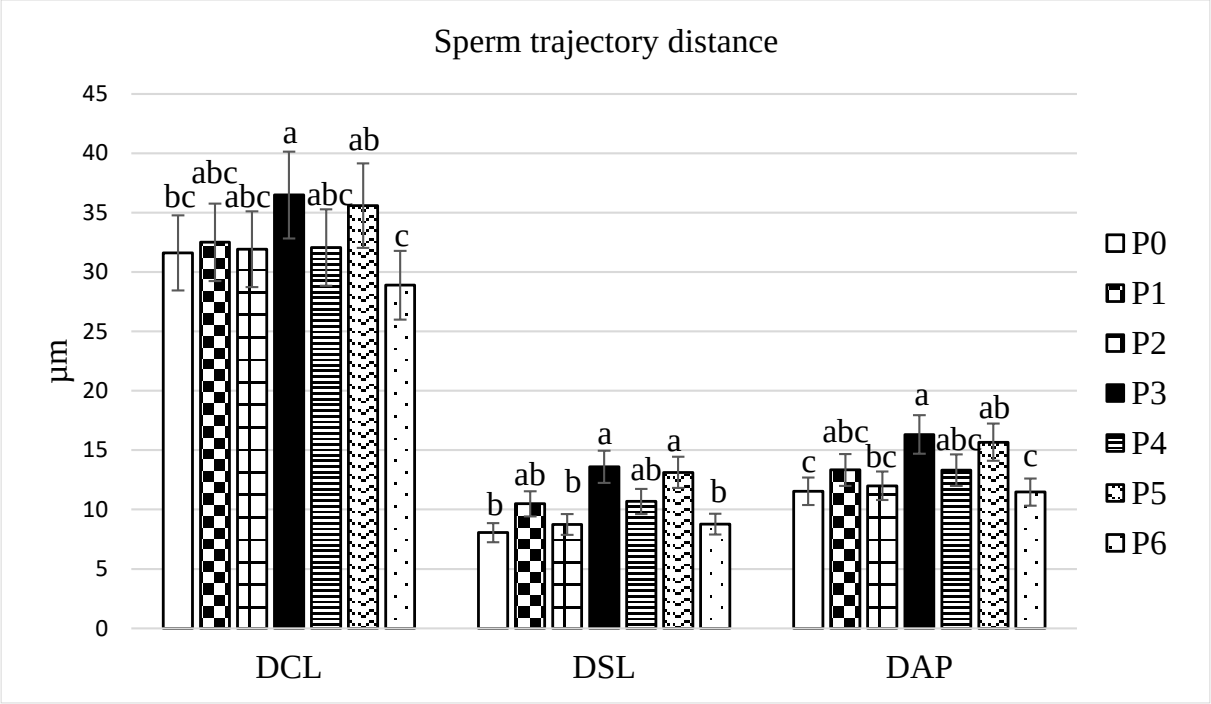

Figure 3. Rote ram sperm trajectory distance parameters (µm). Bars with different letters denote significantly different values ($p < 0.05$). P0 (Tris – egg yolk), P1 (P0 + 0.3% BSA) P2 (P0 + 0.125% MLE), P3 (P0 + 0.3% BSA + 0.125% MLE), P4 (P0 + 0.3% BSA + 0.25% MLE), P5 (P0 + 0.45% BSA + 0.125% MLE), P6 (P0 + 0.45% BSA + 0.25% MLE). BSA (bovine serum albumin), MLE (moringa leaf extract).

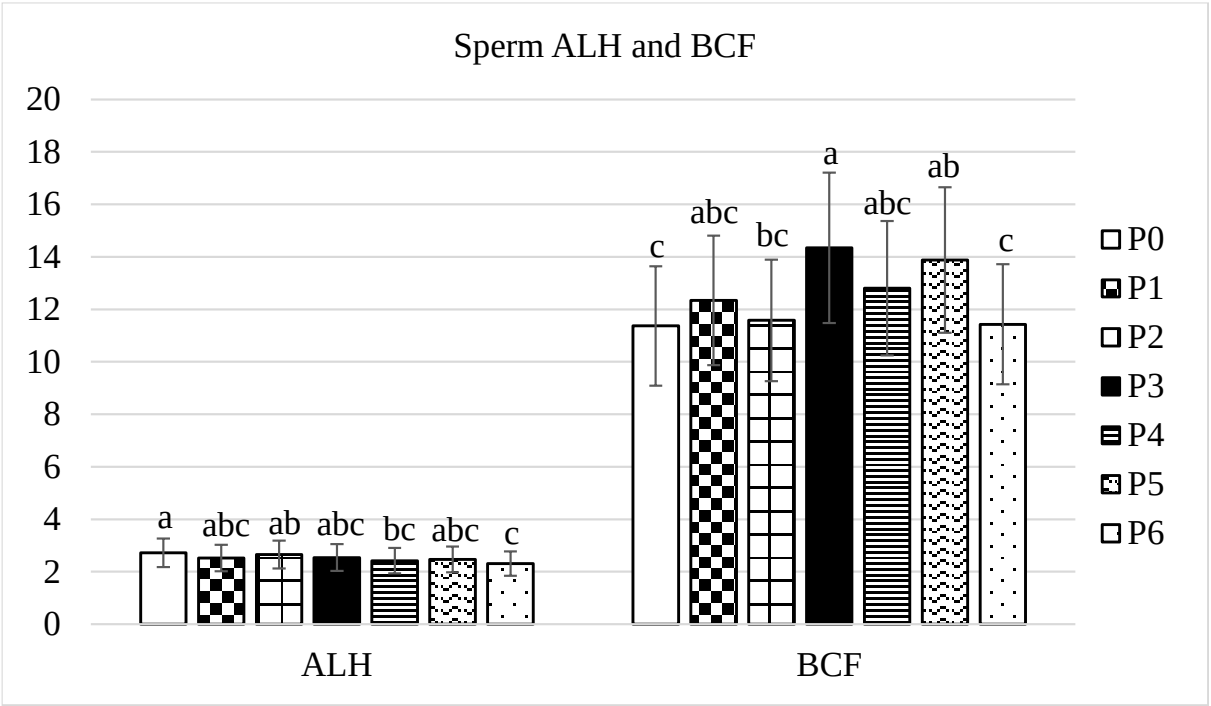

Figure 4a. Rote ram sperm ALH (µm) and BCF (Hz) parameters. Bars with different letters denote significantly different values ($p < 0.05$). P0 (Tris – egg yolk), P1 (P0 + 0.3% BSA) P2 (P0 + 0.125% MLE), P3 (P0 + 0.3% BSA + 0.125% MLE), P4 (P0 + 0.3% BSA + 0.25% MLE), P5 (P0 + 0.45% BSA + 0.125% MLE), P6 (P0 + 0.45% BSA + 0.25% MLE). BSA (bovine serum albumin), MLE (moringa leaf extract).

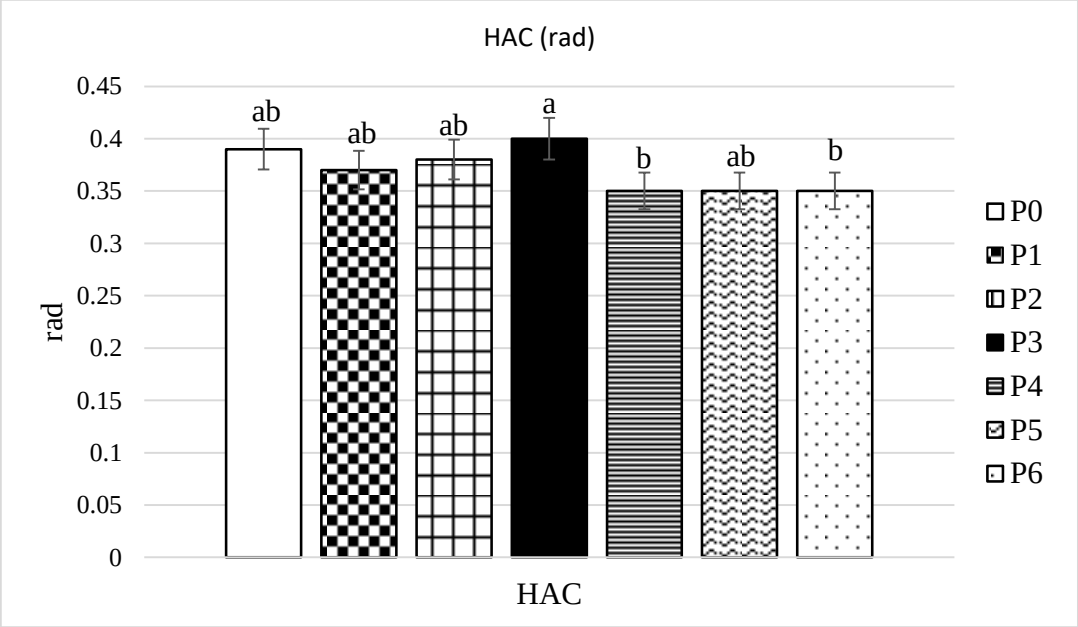

Figure 4b. Rote ram sperm HAC (rad) parameters. Bars with different letters denote significantly different values ($p < 0.05$). P0 (Tris – egg yolk), P1 (P0 + 0.3% BSA) P2 (P0 + 0.125% MLE), P3 (P0 + 0.3% BSA + 0.125% MLE), P4 (P0 + 0.3% BSA + 0.25% MLE), P5 (P0 + 0.45% BSA + 0.125% MLE), P6 (P0 + 0.45% BSA + 0.25% MLE). BSA (bovine serum albumin), MLE (moringa leaf extract).

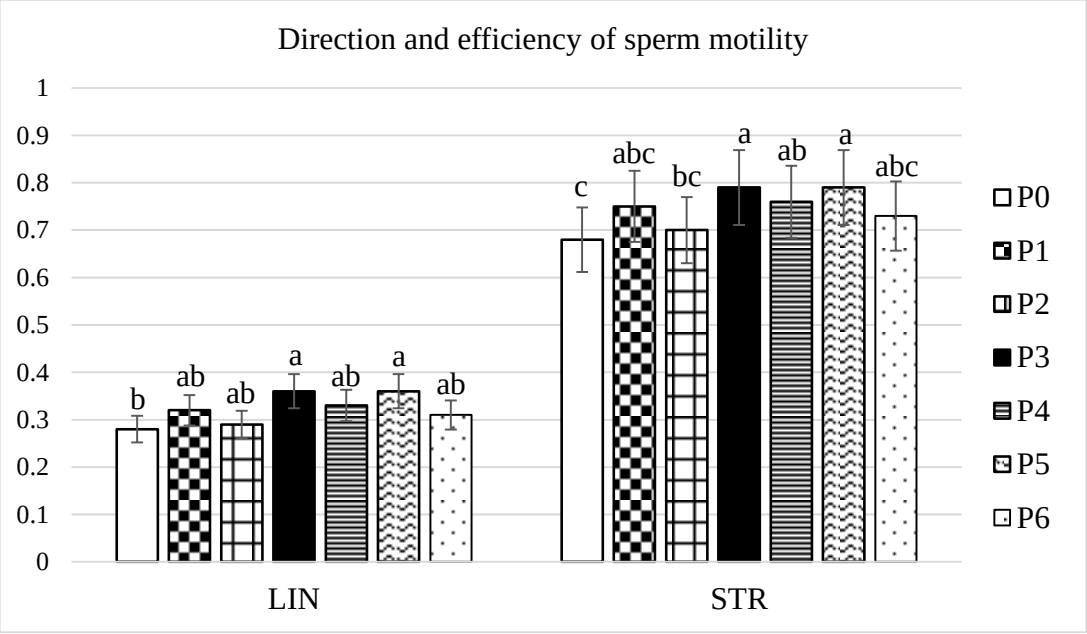

Figure 5. Rote ram sperm linearity (LIN) and straightness (STR) parameters. Bars with different letters denote significantly different values ($p < 0.05$). P0 (Tris – egg yolk), P1 (P0 + 0.3% BSA) P2 (P0 + 0.125% MLE), P3 (P0 + 0.3% BSA + 0.125% MLE), P4 (P0 + 0.3% BSA + 0.25% MLE), P5 (P0 + 0.45% BSA + 0.125% MLE), P6 (P0 + 0.45% BSA + 0.25% MLE). BSA (bovine serum albumin), MLE (moringa leaf extract).

oocytes and penetrating zona pellucida. All in all, the Tris-based extender supplemented with 0.3% BSA and 0.125% MLE (P3) had the best capacity to maintain sperm motility and kinematic performance when subjected to 48 h of cold storage.

DISCUSSIONS

The current research is a strong indication that the efficacy of liquid semen preservation of Rote ram strongly relies on the accurate ratio of membrane-protective and antioxidant factors in the extender. In this regard, the Tris-based extender supplemented with 0.3% bovine serum albumin (BSA) and 0.125% Moringa oleifera leaf extract (MLE) showed superior preservation of sperm motility and kinematic integrity after 48 h of storage at 5°C compared to all other formulations tested, highlighting that this concentration achieves the best functional performance of both additives in the present study. These functional outcomes provide the foundation for understanding the mechanistic basis of extender optimisation.

This process of identifying this optimum level is biologically important because it clarifies how specific concentrations of protective agents influence sperm physiology during cold storage. BSA of 0.3 per cent seems adequate to stabilise the sperm plasma membrane (Akhter *et al*., 2014; Álvarez-Rodríguez *et al*., 2024; Balan *et al*., 2020; Rahimizadeh *et al*., 2021) by balancing osmotic conditions, inhibiting the effect of low-temperature shock on lipid phase transition, and protecting the membrane-bound proteins critical to the transport of ions and flagella. At this level, BSA ensures the fluidity of membranes (Diansyah *et al*., 2025; Gökçe and Alçay, 2017; Lee and Park, 2015; Lee *et al*., 2019) without an unreasonable rise in the extender viscosity, and, therefore, allows the flagellar bending and forward propulsion to be efficient. Next, addition of additional BSA to 0.45% resulted in slowing motility and kinematic activity, presumably due to mechanical resistance of sperm motility, and due to alterations of the dynamics of diffusion within the extender medium. Thus, the positive impact of BSA seems to be concentration-related and strictly connected with membrane stability and physical characteristics of the extender.

On the same note, MLE had a definite dose-related action that complemented the membrane-stabilising role of BSA. The medium level of 0.125 per cent was found to offer the best level of antioxidant protection and was effective in inhibiting the increase of the reactive oxygen species (ROS) (Carrera-Chávez *et al*., 2020; El-Seadawy *et al*., 2022; Iqbal *et al*., 2022; Moichela *et al*., 2021; Shokry *et al*., 2020) throughout cold storage without causing an effect on physiological redox signalling, which is essential in mitochondrial respiration and ATP formation. An increase in MLE (0.25%) was linked to slowed sperm velocity and efficiency of trajectory, which proves the idea that excessive use of antioxidant supplements can interfere with intracellular redox homeostasis and cause a metabolic imbalance. This finding adds to the emerging body of evidence that the efficacy of antioxidants in preserving semen is hormetically related, as opposed to a linear dose-response relationship (Bahmyari *et al*., 2020; Hungerford *et al*., 2024). Thus, both BSA and MLE exhibit optimal functional windows beyond which their protective effects diminish.

Taken together, these data can be considered in order to suggest a mechanistic model of BSA and MLE that work in synergy and target complementary cell compartments. BSA primarily operates at the plasma membrane level, which preserves the structural integrity, osmotic pressure, and denaturation of proteins in case of cooling (Zheng *et al*., 2022). MLE, in its turn, acts at intracellular and mitochondrial levels and is engaged in scavenging of excess ROS, inhibition of lipid peroxidation of mitochondrial membranes, and facilitation of ATP production required in flagellar movement (González-Burgos *et al*., 2021; Mohlala *et al*., 2023; Moichela *et al*., 2021). A combination of these defensive processes results in conserved sperm velocity and hyperactivated and directional flagellar biomechanics (VCL, VSL, VAP), elevated beat cross frequency (BCF) and preserved hyperactivation and directional efficiency (HAC, LIN, STR), which are indicative of functional flagellar biomechanics and directional efficiency. Notably, biochemical and structural markers of oxidative stress and membrane integrity should also show such functional properties.

The extra biochemical and structural evidence also supports the conclusions of the functionality of sperm motility and kinematics and offers the objective material to prove the worked-out mechanistic model. Malondialdehyde (MDA) is an established final product of lipid peroxidation, which is a good marker of oxidative cell damage to sperm plasma membranes (Catalá and Díaz, 2016; Yekti *et al*., 2018). In our current research, P3 had the lowest MDA concentration of 16.528 nmol/ml, which is significantly lower than P0 (26.465 nmol/ml) and P6 (24.128 nmol/ml), which were treated with a high dose combination. This lipid peroxidation decrease was accompanied by the maximum progressive motility, the best velocity parameters (VCL, VSL, VAP), and the best characteristics of the trajectory. These results have a strong implication that a reduction in oxidative stress is directly linked to maintenance of flagellar biomechanics and sperm movement efficiency in 48 h cold storage.

This relationship is also strengthened by the pattern in membrane integrity. The highest plasma membrane integrity (67.11%) was seen in spermatozoa in P3, with the control and P6 groups showing much lower values (52.57% and 55.16%, respectively). The high concentration of polyunsaturated fatty acids in sperm plasma membrane and its extreme vulnerability to peroxidative destruction, the increase in MDA levels in P0 and P6 are probably results of structural destabilisation of the lipid bi-layer. This destabilisation impairs the control of ion channels, mitochondrial operation, ATP-sensitive flagellar movement and eventually decreases motility and kinematic performance (Akbarinejad *et al*., 2020; Davila *et al*., 2016; Nowicka-Bauer & Szymczak-Cendlak, 2021). On the other hand, the reduced MDA level in P3 indicates the presence of good protection against membrane lipid peroxidation and

therefore its ability to retain its fluidity as well as functional competence to coordinate the flagellar beating.

Notably, the negative correlation between the levels of MDA and the two parameters of membrane integrity and motility indicates some mechanistic interconnection of oxidative balance and sperm biomechanics. Moderate levels of antioxidant supplementation (0.125% MLE) of treatments seem to maintain redox homeostasis to the level necessary to inhibit excess generation of reactive oxygen species (ROS) without affecting physiological redox signalling. This is a very important balance, which normal sperm metabolism and the regulation of sperm motility require regulated ROS levels (Gallo *et al*., 2021; Zhu *et al*., 2019). The higher MDA level of P6, despite antioxidant addition, is a validation of the idea of a hormetic effect, where too much exposure to antioxidants can disrupt the intracellular redox status and indirectly affect the workings of mitochondria and flagellation (Fafula *et al*., 2018; Sabeti *et al*., 2016).

Together, the joint assessment of MDA concentration and membrane integrity in conjunction with the kinematic parameters gives a convergent signal that oxidative membrane damage is a key factor that determines post-storage sperm functionality (Peña and Gibb, 2022; Rizkallah *et al*., 2022). The enhanced effectiveness of the P3 proves the existence of synergistic effects of the most advantageous concentrations of membrane-stabilising (BSA) and antioxidant (MLE) compounds in reducing lipid peroxidation and maintaining the structure of the plasma membrane and flagellar motion that is powered by ATP (Álvarez-Rodríguez *et al*., 2024; Fu *et al*., 2017; Iqbal *et al*., 2022). Therefore, the biochemical data of the oxidative stress is not merely some supporting measurements, but those that are mechanistically related to predict the sperm motility retention and directional competence in the liquid semen preservation, which justifies the relevance of correct extender formulation that will conserve the reproductive efficiency.

Conclusively, this paper shows that a well-balanced mixture of 0.3% of BSA and 0.125% of Moringa oleifera leaf extract in Tris based extender is a good preservative of sperm motility and kinematic functionality of Rote ram semen in 48 h of liquid storage. The findings give a mechanistic understanding of the role of membrane stabilisation and antioxidant protection working in synergy, the significance of species-specific extender optimisation, and a viable sustainable solution to increasing the effectiveness of artificial insemination in the production system of tropical sheep. Nevertheless, it is important to acknowledge a key limitation of this study, namely that the semen samples were obtained from only two Rote rams. This limited sample size may constrain the generalizability of the findings, as it does not fully capture potential inter-individual variability in semen quality and response to extender formulations. Therefore, further studies involving a larger and more diverse population of animals are warranted to validate and strengthen the applicability of these results.

Conclusions

The current research has shown that the addition of Tris-based extender with bovine serum albumin and Moringa leaf extract helps in the preservation of sperm kinematic quality of Rote sheep during 48 hours of cold storage at 5°C. The highest results were obtained with the extender with 0.3% BSA and 0.125% Moringa leaf extracts, as evidenced by better progressive and fast motility, as well as better kinematic parameters (VCL, VSL, VAP, LIN, and STR). These findings underscore an additive protective impact of protein based and plant-based bioactive substances against the cold-induced sperm deterioration. It is a good and viable formulation of research to enhance the efficiency of semen preservation within the artificial insemination programmes, especially in the sustainable use and maintenance of the indigenous breed of sheep.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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