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Abstract

This study evaluated the effects of supplementing a cryo-extender with Ashwagandha (*Withania somnifera*) root extract (ARE) on post-thaw sperm quality, oxidative status, DNA integrity, and fertility in rams. Ejaculates were collected from four fertile Assaf rams (2–3.5 years), pooled, diluted, and divided into four aliquots supplemented with ARE at 0 (control), 10, 25, and 50 µg/mL. Samples were cooled, packaged in 0.5-mL straws, and cryopreserved in liquid nitrogen. Post-thaw sperm quality was assessed using computer-assisted sperm analysis (CASA), plasma membrane and acrosomal integrity, total antioxidant capacity (TAC), malondialdehyde (MDA), and DNA fragmentation. Fertility was evaluated following artificial insemination. ARE supplementation significantly improved several sperm parameters compared with the control (*P* < 0.05). The 10 µg/mL treatment showed the highest total motility (73.75 ± 2.81%) and progressive motility (68.33 ± 3.73%), and the lowest immotile sperm percentage (26.25 ± 2.81%), with improved kinematic parameters. Plasma membrane integrity was highest at 10 and 25 µg/mL (56.0 ± 5.87% and 55.6 ± 2.87%, respectively), while acrosomal integrity was highest

at 10 µg/mL (87.2 ± 1.43%). ARE increased TAC, with the highest value at 25 µg/mL, and reduced MDA, with the lowest value at 50 µg/mL. DNA fragmentation was lowest at 10 µg/mL, whereas 50 µg/mL was associated with increased DNA damage. In the fertility trial, 10 µg/mL ARE resulted in a higher conception rate (51.72%) than the control (30.43%). Overall, 10 µg/mL ARE supplementation improved post-thaw sperm quality, antioxidant status, and DNA integrity and was associated with improved fertility.

Keywords: Ashwagandha, *Withania somnifera*, Ram semen, Cryopreservation, CASA kinematics, Antioxidants, DNA integrity, Conception rate.

1. Introduction

Sheep are among the most economically vital livestock globally, particularly in arid and semi-arid regions, due to their adaptability and their crucial contributions to meat, milk, wool, and leather production. Consequently, enhancing reproductive efficiency and maximizing genetic potential are paramount goals in modern sheep breeding programs (FAO, 2021). Artificial insemination (AI) is one of the most powerful biotechnological tools for accelerating genetic gain in small ruminants. It facilitates the widespread dissemination of germplasm from genetically superior rams, mitigates the risk of sexually transmitted diseases, and improves overall flock productivity (Langerová *et al.,* 2026).

Semen cryopreservation is an indispensable component of successful AI protocols, enabling long-term storage, international trade, and ex situ conservation of genetic resources (Dziekońska *et al*., 2022). Nevertheless, the freeze-thaw process subject's spermatozoa to severe thermal, osmotic, and mechanical stresses. This leads to overproduction of reactive oxygen species (ROS), causing oxidative stress (Zhu *et al*., 2023; Soria-Meneses *et al*., 2025). Excessive ROS

levels trigger lipid peroxidation of plasma and organelle membranes, compromise mitochondrial membrane potential, and induce DNA fragmentation, collectively diminishing post-thaw motility, structural integrity, and fertility (Shehab-El-Deen *et al*., 2023; Zhu *et al*., 2023). Therefore, oxidative stress is recognized as a primary driver of cryoinjury in ram spermatozoa (Riha *et al*., 2026).

To counteract cryo-induced oxidative damage, supplementation of semen extenders with Exogenous antioxidants has been extensively studied (Elshamy *et al*., 2026). Synthetic and natural antioxidants—such as glutathione, ascorbic acid, curcumin, quinic acid, and plant-derived phytochemicals—have shown varying degrees of success in preserving post-thaw sperm motility, membrane integrity, mitochondrial function, and chromatin structure (Moretti & Signorini, 2024; Kaya *et al*., 2025; Denk *et al*., 2025). Naturally derived antioxidants have attracted particular interest for their biocompatibility, safety profile, and cost-effectiveness compared with synthetic analogs (Berean *et al*., 2024).

Ashwagandha (*Withania somnifera*) is a renowned medicinal herb known for its potent antioxidant, anti-inflammatory, and cytoprotective properties (Lerose *et al*., 2024). It contains a rich blend of bioactive phytochemicals, including withanolides, flavonoids, and phenolic compounds, which enhance endogenous antioxidant enzyme defenses and scavenge free radicals (Lerose *et al*., 2024; Wiciński *et al.,* 2025). Recent studies suggest that Ashwagandha supplementation preserves sperm motility, viability, membrane function, and chromatin stability across species, underscoring its utility in reproductive technologies (Dar *et al*., 2022; Kalaiselvan *et al*., 2025; Yadav & Mishra, 2025). However, information on the optimal concentration of Ashwagandha root extract in ram semen cryo-extenders and its impact on field fertility remains limited.

Therefore, this study was designed to investigate the protective effects of incorporating varying concentrations of Ashwagandha root extract into Tris-based extenders on post-thaw motility kinetics, structural and organelle integrity, oxidative stress markers, DNA integrity, and *in vivo* fertilizing capacity after transcervical artificial insemination in ewes.

2. Materials and Methods

2.1 Chemicals

The Ashwagandha root extract powder was supplied by Royal Aromatic (Alexandria, Egypt). Unless otherwise stated, all other chemicals and culture media were purchased from Sigma (St. Louis, Missouri).

2.2. Preparation of Ashwagandha Root Extract

Commercial Ashwagandha (*Withania somnifera*) root extract powder (2:1), standardized to contain ≥1.5% total withanolides, was obtained from Royal Aromatic (Alexandria, Egypt). The extract was prepared by hydroalcoholic extraction of dried roots. According to the supplier's Certificate of Analysis, the extract was a water-soluble, pale brown powder with a moisture content of 6.6%, a pH of 5.4 (1% aqueous solution), and microbiological compliance was verified. Stock solutions were prepared fresh on the day of experimentation using ultra-pure water (Abdelwahed, *et al*., 2023).

2.3. Experimental Animals and Husbandry

This study was conducted from October 2025 to May 2026 at the Animal Reproduction Research Institute (ARRI), Agricultural Research Center (ARC), Giza, Egypt. Four sexually mature Assaf rams (2.0–3.5 years of age), of proven fertility and in excellent clinical health, served as semen

donors. In addition to 52 ewes (Ossimi and Rahmani breeds) were used for insemination. Animals were housed in semi-open sheds under uniform management, hygiene, and nutritional conditions Animals had free access to clean drinking water and mineral licks.

2.4. Semen Collection and Initial Processing

Semen was collected using an artificial vagina (AV) modified for small ruminants. Prior to collection, the inner latex liner was filled with warm water at 42–45°C to maintain an internal temperature of 40–42°C. The internal pressure was regulated with air to ensure adequate stimulation. The inner liner was lightly lubricated with a sterile, non-spermicidal lubricant. A sterile, graduated glass tube was affixed to the AV cone (Cseh, *et al*., 2012).

Ejaculates were transferred to the laboratory immediately after collection and placed in a water bath at 37◦C. Individual ejaculates were assessed for volume, mass motility, progressive motility, concentration, and morphology. Only high-quality ejaculates meeting pre-established criteria (volume ≥1.0 mL, sperm concentration $\geq 3\times10^9$ spermatozoa/mL, and progressive motility ≥70%) were pooled to minimize individual ram variation (Hozyen, *et al*., 2019).

2.5. Semen Dilution and Cryopreservation

Pooled semen was diluted at 37°C using a Tris-citric acid-egg yolk-glycerol extender to achieve a final sperm concentration of 200×10^6 spermatozoa/mL. The diluted semen was then divided into four equal aliquots, each supplemented with a different concentration of Ashwagandha root extract (ARE):

1. Control: 0µg/ml ARE
2. ARE-10: 10µg/mL ARE

3. ARE-25: 25µg/mL ARE
4. ARE-50: 50µg/mL ARE

In a cold cabinet (*IMV* Technologies, L'Aigle, France), samples were gradually cooled to 4°C over 90 min and equilibrated at 4°C for 2 h. Subsequently, the extended semen was loaded into 0.5 mL French straws (*IMV* Technologies), frozen in liquid nitrogen vapor (4–5 cm above the liquid phase) for 15 min, plunged directly into liquid nitrogen (−196°C), and stored until evaluation (Hozyen, *et al*., 2019).

2.6. Post-Thaw Sperm Quality Evaluation

For evaluation, three straws per treatment group from each experimental replicate were thawed in a pre-warmed water bath at 37°C for 30 s and then pooled.

2.6.1. CASA Motility and Kinematic Parameters

Sperm motility and motion kinetics were assessed using a Computer-Assisted Sperm Analysis (CASA system, AndroVision® software, version 1.2.1; Minitube, Tiefenbach, Germany) coupled with a phase-contrast microscope (Zeiss, Germany). An aliquot of 3µL of thawed semen was placed on a pre-warmed (37°C) Leja counting chamber (20µm depth; Leja Products B.V., Nieuw-Vennep, Netherlands). Images were captured at 60 frames/s using species-specific thresholds for ovine spermatozoa. Parameters evaluated included: Total Motility (TM, %), Progressive Motility (PM, %), Curvilinear Velocity (VCL, µm/s), Straight-Line Velocity (VSL, µm/s), Average Path Velocity (VAP, µm/s), Distance Curvilinear (DCL, µm), Distance Straight-Line (DSL, µm), and Distance Average Path (DAP, µm) (Elshamy *et al*., 2026).

2.6.2. Functional Plasma Membrane Integrity (HOST)

Functional membrane integrity was determined using the Hypo-Osmotic Swelling Test (HOST) as described by Nalley *et al.* (2013). A hypo-osmotic solution (150 mOsmol/kg) was prepared by dissolving 7.35 g sodium citrate and 13.51 g fructose in 1 L of distilled water. A 10µL semen aliquot was incubated in 2.0 mL hypo-osmotic solution at 37°C for 60 min. A drop of the mixture was placed on a clean glass slide, covered with a coverslip, and examined under a phase-contrast microscope at 400× magnification. A total of 200 spermatozoa per slide were evaluated, and the percentage of spermatozoa with swollen, coiled tails was recorded.

2.6.3. Acrosomal Membrane Integrity

Acrosome integrity was assessed by Giemsa staining as described by Chowdhury *et al*. (2014). Air-dried smears of thawed semen were prepared on clean slides, fixed in 5% formaldehyde for 30 min, rinsed under running tap water, and air-dried. The working Giemsa stain was prepared by mixing 3 mL of Giemsa stock solution, 2 mL of phosphate-buffered saline (PBS), and 45 mL of Milli-Q water. Slides were immersed in the working solution at 37°C for 2 h, washed with running water, and dried. Slides were evaluated under an oil immersion light microscope (Olympus, Tokyo, Japan) at 1000× magnification. At least 200 spermatozoa per slide were classified as either acrosome-intact or acrosome-damaged.

2.6.4. Seminal Plasma Antioxidant and Lipid Peroxidation Assays

Thawed semen straws from each group were centrifuged at 500×g for 15 min at 4°C. The clear supernatant (seminal plasma) was collected to measure Total Antioxidant Capacity (TAC) and Malondialdehyde (MDA) levels using commercial colorimetric diagnostic kits (Bio-Diagnostic, Giza, Egypt) following the manufacturer's protocols (Mahfouz *et al.,* 2009).

2.6.5. Sperm DNA Integrity Assessment (Alkaline Comet Assay)

Single-cell gel electrophoresis (Comet assay) was performed to quantify sperm DNA damage following the protocol of Singh *et al.* (1988), with minor modifications. Frosted slides were pre-coated with 1% Normal Melting Agarose (NMA) and allowed to dry. Sperm cells were suspended in Hank's Balanced Salt Solution (HBSS). A 10µL aliquot of the cell suspension was mixed with 90µL of 0.5% Low Melting Point Agarose (LMPA) at 37°C, then layered onto pre-coated slides, covered with a coverslip, and placed on ice for 10 min to solidify.

Coverslips were removed, and slides were immersed in cold, freshly prepared lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, pH 10, supplemented with 1% Triton X-100 and 10% DMSO) and refrigerated at 4°C in the dark for 2 h. Slides were then placed in an alkaline electrophoresis chamber containing buffer (300 mM NaOH, 1 mM EDTA, pH >13) for 20 min to allow DNA unwinding. Electrophoresis was performed at 25 V (300 mA) for 20 min. Following electrophoresis, slides were neutralized by washing three times (5 min each) with 0.4 M Tris-HCl buffer (pH 7.5).

Slides were stained with Ethidium Bromide (20µg/mL) and evaluated under an epifluorescence microscope (Zeiss, Germany) connected to an image analysis system (Comet Assay II; Perceptive Instruments Ltd., UK). A total of 100 comets per slide (50 comets per replicate) were scored at 400× magnification. Quantitative parameters recorded included: % Tailed DNA (DNA in tail), Tail Length (µm), and Tail Moment.

2.7. Transcervical Artificial Insemination (TCAI) and Field Fertility Trial

2.7.1. Animals and Oestrus Synchronization

The *in vivo* fertilizing ability of preserved semen was evaluated in clinically healthy Egyptian ewes (Ossimi and Rahmani breeds, n=52; aged 2–5 years; weighing 40–60 kg). Ewes were kept under standardized feeding and management. Estrus was synchronized with intravaginal progesterone-releasing sponges inserted for 12 days. After sponge removal, each ewe received an intramuscular injection of 400–500 IU of equine Chorionic Gonadotropin (eCG). Estrus detection was performed at 12-hour intervals using vasectomized teaser rams.

2.7.2. Insemination Procedure

Ewes showing standing heat were randomly allocated into two treatment groups:

1. Control Group (n=23): Inseminated with frozen-thawed semen from the 0µg/mL ARE group.
2. Optimal ARE Group (n=29): Inseminated with frozen-thawed semen from the 10µg/mL ARE group (selected based on superior post-thaw *in vitro* results).

Transcervical artificial insemination was performed 48–56 h post-sponge withdrawal (Fair et al., 2005). Semen straws were thawed at 37∘C for 30 s, thoroughly dried, and loaded into a pre-warmed sheep AI gun fitted with a sterile sheath. Ewes were restrained, and the perineal area was sanitized (Campbell *et al*., 1996). A lubricated speculum with a built-in light source was inserted into the vagina to visualize the external cervical os. A specialized sheep transcervical catheter was advanced through the cervical rings, and a dose of 150×10^6 motile spermatozoa was deposited into the cervical canal or uterine body (Anel *et al*., 2005).

2.7.3. Pregnancy Diagnosis

Pregnancy was diagnosed using transabdominal real-time ultrasonography with a 3.5/5.0 MHz probe at 30–40 days post-insemination (Schrick & Inskeep, 1993). Conception rate was calculated as follows:

Conception Rate (%) = Total number of inseminated ewes/Number of pregnant ewes×100

2.8. Statistical Analysis

Data were analyzed using SPSS (Version 22.0; IBM Corp., Armonk, NY, USA). Continuous parametric variables (sperm motility, kinematics, membrane integrity, biochemical assays, and Comet parameters) were reported as mean ± standard error of the mean (SEM). Differences among concentrations were evaluated using One-Way Analysis of Variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. Nominal conception rate data were analyzed using the Chi-square ($\chi 2$) test. Statistical significance was defined as $P<0.05$.

Results

Table 1: Effect of different concentrations of Ashwagandha root extract (ARE) supplementation on post-thaw ram sperm CASA motility and kinematic parameters

Parameter	Control *Mean ± SEM*	ARE (10 µg/mL) *Mean ± SEM*	ARE (25 µg/mL) *Mean ± SEM*	ARE (50 µg/mL) *Mean ± SEM*
Total Motility (%)	36.91 ± 2.27^{c}	73.75 ± 2.81^{a}	66.36 ± 2.09^{a}	52.12 ± 3.19^{b}
Progressive Motility (%)	27.51 ± 2.15^{d}	68.33 ± 3.73^{a}	57.23 ± 3.93^{b}	45.47 ± 2.67^{c}
Fast Motility (%)	8.30 ± 1.10^{b}	23.42 ± 4.29^{a}	20.42 ± 4.40^{a}	16.26 ± 1.74^{ab}
Slow Motility (%)	19.16 ± 1.33^{d}	44.85 ± 3.69^{a}	36.79 ± 0.80^{b}	29.10 ± 1.38^{c}
Local Motility (%)	9.39 ± 0.53	5.42 ± 1.04	9.12 ± 1.86	6.65 ± 1.26

Parameter	Control *Mean ± SEM*	ARE (10 µg/mL) *Mean ± SEM*	ARE (25 µg/mL) *Mean ± SEM*	ARE (50 µg/mL) *Mean ± SEM*
Immotile (%)	63.11 ± 2.28^{c}	26.25 ± 2.81^{a}	33.64 ± 2.09^{a}	47.88 ± 3.19^{b}
DCL (µm)	12.90 ± 0.81^{d}	20.41 ± 0.86^{a}	15.59 ± 0.50^{b}	17.74 ± 0.70^{c}
DSL (µm)	4.38 ± 0.41^{b}	7.32 ± 0.26^{a}	5.27 ± 0.33^{b}	6.49 ± 0.29^{a}
DAP (µm)	5.73 ± 0.40^{d}	9.41 ± 0.32^{a}	6.96 ± 0.30^{b}	8.19 ± 0.27^{c}
VCL (µm/s)	50.05 ± 5.90^{c}	103.88 ± 3.50^{a}	75.72 ± 7.10^{b}	67.69 ± 3.50^{b}
VSL (µm/s)	19.74 ± 2.98^{c}	42.77 ± 0.79^{a}	30.21 ± 3.54^{b}	27.62 ± 0.99^{b}
VAP (µm/s)	23.94 ± 3.09^{c}	51.10 ± 1.21^{a}	36.48 ± 3.70^{b}	32.89 ± 1.20^{b}
Radius (µm)	1.28 ± 0.11^{d}	2.14 ± 0.07^{a}	1.56 ± 0.08^{b}	1.87 ± 0.07^{c}
Rotation	0.14 ± 0.02^{c}	0.31 ± 0.01^{a}	0.21 ± 0.13^{b}	0.22 ± 0.01^{b}
BCF (Hz)	9.09 ± 0.88^{b}	11.86 ± 0.63^{a}	10.56 ± 0.34^{ab}	11.91 ± 0.29^{a}
ALH (µm)	0.57 ± 0.04^{c}	1.05 ± 0.05^{a}	0.80 ± 0.05^{b}	0.72 ± 0.02^{b}
HAC (µm²)	0.12 ± 0.01^{c}	0.24 ± 0.01^{a}	0.18 ± 0.01^{b}	0.16 ± 0.01^{b}
STR	0.85 ± 0.02	0.84 ± 0.01	0.82 ± 0.01	0.84 ± 0.004
LIN	0.39 ± 0.01	0.41 ± 0.01	0.40 ± 0.01	0.41 ± 0.01
WOB	0.48 ± 0.04	0.49 ± 0.05	0.48 ± 0.05	0.49 ± 0.08

Data are presented as Mean ± SEM. Different superscript letters (a, b, c, d) within the same row indicate statistically significant differences ($P < 0.05$). Abbreviations: DCL: Distance Curved Line; DSL: Distance Straight Line; DAP: Distance Average Path; VCL: Curvilinear Velocity; VSL: Straight-Line Velocity; VAP: Average Path Velocity; BCF: Beat Cross Frequency; ALH: Amplitude of Lateral Head Displacement; HAC: Head Area Covered; STR: Straightness; LIN: Linearity; WOB: Wobble.

Post-Thaw Sperm Motility and Kinematics

Supplementation of the cryo-extender with ARE significantly improved post-thaw semen CASA motility parameters compared to the control ($P < 0.05$). The control group exhibited the lowest total motility (36.91%) and the highest proportion of immotile spermatozoa (63.11%), illustrating cryopreservation-induced damage. Among the treated groups, 10 µg/mL ARE achieved the most pronounced enhancement, with the highest total motility (73.75%), progressive motility (68.33%), and fast motility, along with the lowest proportion of immotile sperm (26.25%). Although the 25 µg/mL group significantly outperformed the control, its parameters were slightly inferior to those of the 10 µg/mL group. Increasing the concentration to 50 µg/mL resulted in a noticeable decline in motility parameters and an increase in the proportion of immotile sperm (47.88%).

Regarding kinematic parameters, ARE supplementation increased most velocity and distance metrics. The 10 µg/mL concentration yielded the highest values for DCL, DSL, DAP, VCL, VSL, and VAP ($P < 0.05$). Furthermore, ALH, BCF, Radius, Rotation, and HAC were higher in treated groups, particularly at 10 µg/mL. Conversely, trajectory ratios (STR, LIN, and WOB) showed no significant differences among groups ($P > 0.05$), indicating that ARE primarily enhances sperm vigor rather than altering its movement pattern.

Table 2: Effect of different concentrations of Ashwagandha root extract (ARE)-supplementation in the cryo-extender on post-thaw ram sperm membrane integrity

Group according to The Concentration	Plasma Membrane Integrity (%) *Mean ± SEM*
Control 0 µg/mL	34.20 ± 1.69^{b}
ARE 10 µg/mL	56.00 ± 5.87^{a}

Group according to The Concentration	Plasma Membrane Integrity (%) *Mean ± SEM*
ARE 25 µg/mL	55.60 ± 2.87[a]
ARE 50 µg/mL	48.60 ± 2.42[a]

Data are presented as Mean ± SEM. Different superscript letters (a, b) within the same column denote significant differences (P < 0.05). ARE: Ashwagandha root extract.

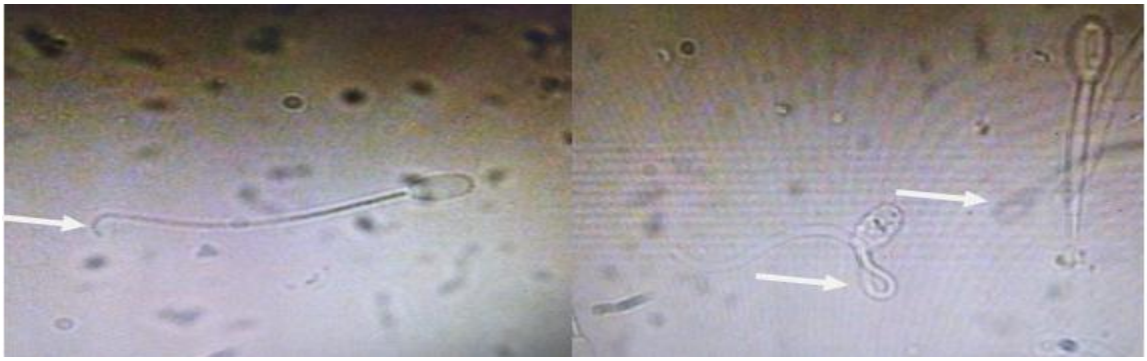


Figure 1. Representative micrograph of ram spermatozoa subjected to the hypo-osmotic swelling test (HOST). Spermatozoa with coiled or swollen tails (white arrows) have functionally intact plasma membranes, whereas cells with straight, non-reactive tails indicate compromised membrane integrity.

Plasma Membrane Integrity

As presented in Table 2, ARE supplementation significantly preserved post-thaw sperm plasma membrane integrity compared to the control ($P < 0.05$). The control group demonstrated the lowest membrane integrity (34.20%). All ARE concentrations (10, 25, and 50 µg/mL) resulted in significant protection, with values of 56.00%, 55.60%, and 48.60%, respectively. The 10 and 25 µg/mL treatments offered optimal membrane protection with no significant difference between them (Figure 1).

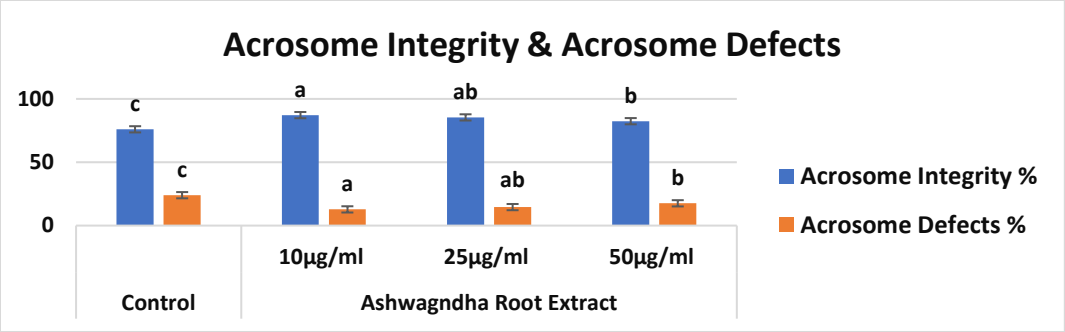


Figure 2. Effect of different concentrations of Ashwagandha root extract (ARE) supplementation in the cryo-extender on post-thaw ram sperm acrosome integrity and defects.



Figure 3. Representative micrographs of post-thaw ram spermatozoa stained with Giemsa stain demonstrating acrosomal status: (A) Intact acrosome; (B) Defective acrosome.

Acrosomal Integrity

ARE supplementation significantly maintained acrosomal integrity and reduced structural defects post-thawing compared with the control group ($P < 0.05$; Figure 2). The 10 µg/mL concentration yielded the best outcome, with the highest acrosomal integrity (87.20 ± 1.43%) and the lowest defect rate (12.80 ± 1.43%), differing significantly from all other groups ($P < 0.05$). The 25 µg/mL group followed, with 85.40 ± 0.93% integrity and 14.60 ± 0.93% defects. The 50 µg/mL group also improved acrosomal preservation relative to the control (82.40 ± 1.66% integrity and 17.60 ± 1.66% defects), though its protective efficacy was significantly lower than that of the 10 and 25 µg/mL treatments. The control group displayed the highest rate of acrosomal damage (24.00 ± 1.73%; Figure 3).

Table 3: Effect of different concentrations of Ashwagandha root extract (ARE) supplementation in the cryo-extender on post-thaw ram sperm biochemical parameters

Group	Concentration	TAC (mM/L) *Mean ± SEM*	MDA (nmol/mL) *Mean ± SEM*
Control	0 µg/mL	0.348 ± 0.001^{c}	1.54 ± 0.08^{c}
ARE	10 µg/mL	0.352 ± 0.001^{c}	1.16 ± 0.22^{b}
ARE	25 µg/mL	0.370 ± 0.001^{a}	0.61 ± 0.05^{a}
ARE	50 µg/mL	0.360 ± 0.001^{b}	0.42 ± 0.03^{a}

Data are presented as Mean ± SEM. Different superscript letters (a, b, c) within the same column indicate significant differences (P < 0.05). Abbreviations: TAC: Total Antioxidant Capacity; MDA: Malondialdehyde; ARE: Ashwagandha root extract.

Biochemical Parameters

ARE supplementation significantly altered post-thaw seminal oxidative stress markers (Table 3). Total Antioxidant Capacity (TAC) increased in a dose-dependent manner up to 25 µg/mL. The control group had the lowest TAC (0.348 ± 0.001 mM/L), which did not differ significantly from the 10 µg/mL treatment (0.352 ± 0.001 mM/L). The highest TAC was observed in the 25 µg/mL group (0.370 ± 0.001 mM/L), followed by the 50 µg/mL group (0.360 ± 0.001 mM/L; $P < 0.05$).

For malondialdehyde (MDA), a marker of lipid peroxidation, all ARE-treated groups showed significantly lower concentrations than the control group ($P < 0.05$). The control group recorded the highest oxidative damage (1.54 ± 0.08 nmol/mL). Supplementation with 10 µg/mL reduced MDA levels to 1.16 ± 0.22 nmol/mL, whereas 25 and 50 µg/mL achieved the lowest lipid peroxidation levels (0.61 ± 0.05 and 0.42 ± 0.03 nmol/mL, respectively), with no significant difference between them.

Table 4: Effect of different concentrations of Ashwagandha root extract (ARE) supplementation in the cryo-extender on post-thaw sperm DNA integrity in rams

Group	Concentration	Tailed Sperm (%) *Mean ± SEM*	Tail Length (µm) *Mean ± SEM*	Tail DNA (%) *Mean ± SEM*	Tail Moment *Mean ± SEM*
Control	0 µg/mL	21.43 ± 0.72^{c}	11.63 ± 0.51^{ab}	11.47 ± 0.70^{a}	1.49 ± 0.01^{b}
ARE	10 µg/mL	10.37 ± 0.32^{a}	9.55 ± 0.26^{a}	10.04 ± 0.36^{a}	0.96 ± 0.05^{a}
ARE	25 µg/mL	13.13 ± 0.19^{b}	12.90 ± 0.73^{b}	11.48 ± 0.44^{a}	1.18 ± 0.09^{a}
ARE	50 µg/mL	12.47 ± 0.09^{b}	16.88 ± 0.94^{c}	13.55 ± 0.15^{b}	2.38 ± 0.06^{c}

Data are presented as Mean ± SEM. Different superscript letters (a, b, c) within the same column indicate significant differences (P < 0.05). ARE: Ashwagandha root Extract.

Figure 4. Representative fluorescence micrograph of ram spermatozoa subjected to single-cell gel electrophoresis (Comet assay) to assess DNA integrity. Sperm cells displaying comet-like structures with distinct heads and extended tails (white arrows) indicate varying degrees of DNA fragmentation. In contrast, compact fluorescent heads without tails (green arrows) indicate intact nuclear DNA.

DNA Integrity

Comet assay-based DNA fragmentation parameters differed significantly among experimental groups ($P < 0.05$; Table 4). The control group exhibited marked nuclear DNA damage (21.43 ± 0.72% tailed sperm, tail length of 11.63 ± 0.51 µm, and tail moment of 1.49 ± 0.01). The 10 µg/mL ARE treatment provided maximum genomic protection, with the lowest percentage of tailed sperm (10.37 ± 0.32%), the shortest tail length (9.55 ± 0.26 µm), and the lowest tail moment (0.96 ± 0.05). The 25 µg/mL group also effectively protected DNA integrity relative to the control. However, the 50 µg/mL concentration induced a pro-oxidant or cytotoxic response, yielding the highest tail length (16.88 ± 0.94 µm), tail DNA percentage (13.55 ± 0.15%), and tail moment (2.38 ± 0.06), all of which exceeded the control values ($P < 0.05$; Figure 4).

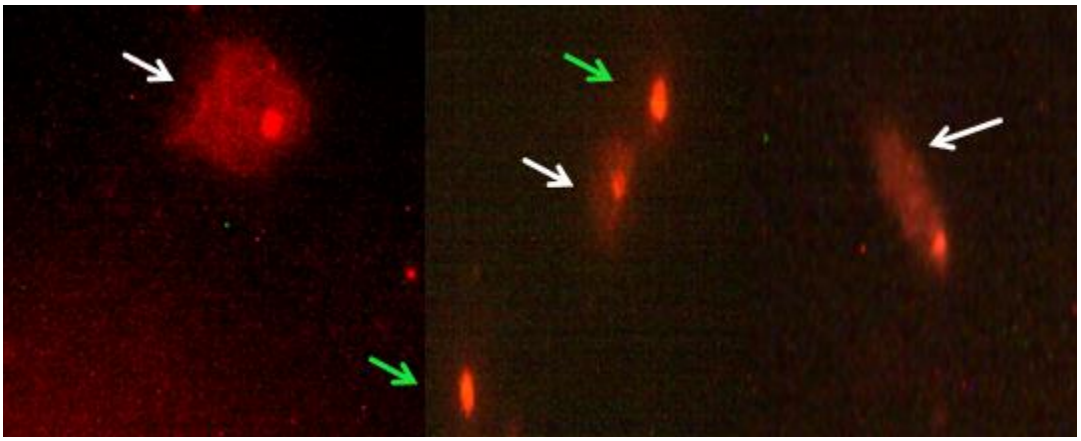


Figure 4. Representative fluorescence micrograph of ram spermatozoa subjected to the comet assay to assess DNA integrity. Sperm cells exhibiting comet-like structures with discernible heads (white arrows) and extended tails indicate varying degrees of DNA fragmentation (white arrows), whereas compact fluorescent heads without tails reflect intact nuclear DNA (green arrows). The heterogeneity in comet tail length and fluorescence intensity suggests differential levels of DNA damage among spermatozoa within the sample.

Conception Rate

As shown in **Table 5,** ewes inseminated with frozen-thawed semen supplemented with 10 µg/mL ARE had a higher total conception rate (51.72%) than the control group (30.43%). Conception rates in the first and second cycles were numerically higher in the ARE group (27.59% and 24.13%) than in the control group (17.39% and 13.04%). Although these differences did not reach statistical significance ($P > 0.05$), the odds ratio (2.45; 95% CI) indicated that ewes inseminated with ARE-treated semen were 2.45 times more likely to conceive than those in the control group.

Table 5: Effect of Ashwagandha root extract (ARE, 10 µg/mL) in the cryo-extender on *in vivo* conception rate in ewes

Group	Total Inseminated (n)	1st Cycle Conception, n (%)	2nd Cycle Conception, n (%)	Total Conception Rate, n (%)	Non-Conceived Ewes, n (%)
Control	23	4 (17.39%)	3 (13.04%)	7 (30.43%)	16 (69.57%)
ARE (10 µg/mL)	29	8 (27.59%)	7 (24.13%)	15 (51.72%)	14 (48.28%)

No statistically significant differences were observed between groups (P > 0.05); ARE: Ashwagandha root

Discussion

Effect of *Withania somnifera* on Post-Thaw Sperm Motility and Kinematics

The present study demonstrated that supplementing the ram semen cryo-extender with *Withania somnifera* root extract (ARE) significantly improved post-thaw sperm CASA total and progressive motility compared with the non-supplemented control. Doses of 10 and 20 µg/mL yielded optimal kinematic outcomes, with the highest motility parameters and the lowest proportion of immotile spermatozoa. Conversely, increasing the concentration to 50 µg/mL reduced protective efficacy, highlighting a clear dose-dependent biological response. These findings align with those of Kanwar *et al*. (2026), who reported modest improvements in motility at lower concentrations (10 and 20 µg/mL), with values of 71.81 ± 1.58 % and 71.89 ± 1.61%, respectively, relative to the control group (70.36 ± 1.44%). In contrast, higher doses (30 and 40 µg/mL) triggered a decline in motility (63.73 ± 2.718). Similarly, Ahmad *et al*. (2010) demonstrated that administration of *W. somnifera* mitigates reactive oxygen species (ROS) accumulation and bolsters antioxidant defense mechanisms, thereby preserving sperm motility.

The superior performance observed at 10 and 20 µg/mL can be attributed to the high levels of bioactive phytochemicals in Ashwagandha—specifically withanolides, flavonoids, and phenolic compounds. These constituents effectively scavenge free radicals and safeguard mitochondrial ATP production, which is essential for flagellar propulsion (Bashir *et al*., 2023; Zhu *et al*., 2023). In contrast, the low motility in the control group stems from cryoinjury, characterized by excessive ROS generation and structural damage to the membrane (Shehab-El-Deen *et al*., 2022). The reduced efficacy at 50 µg/mL suggests that excessive antioxidant levels disrupt physiological ROS-antioxidant homeostasis. Low levels of endogenous ROS are critical for

normal sperm function, and an overabundance of exogenous antioxidants can induce reductive stress or mild cytotoxicity (Avdatek *et al*., 2024; Fleming & Thomson, 2025).

Plasma Membrane and Acrosomal Integrity

Cryopreservation severely compromises sperm structural integrity due to cold shock, osmotic stress, and lipid peroxidation. Ram spermatozoa are particularly susceptible to oxidative damage during freeze-thawing due to high levels of polyunsaturated fatty acids (PUFAs) in their plasma and acrosomal membranes (Zhu *et al*., 2023). In the current study, ARE supplementation significantly enhanced post-thaw plasma membrane and acrosomal integrity, with the 10 and 20 µg/mL doses providing optimal acrosomal protection (87.2 ± 1.43% intact acrosomes vs. 12.8 ± 1.43% defects).

The tudypresent s results agree with Munir *et al*. (2022), who observed dose-dependent improvements in membrane stabilization, total motility, and viability in human spermatozoa supplemented with 25-50 µg/mL *W. somnifera* extract. Furthermore, Kanwar *et al*. (2026) demonstrated that Ashwagandha-derived nanoparticles restored membrane integrity and suppressed lipid peroxidation in cryopreserved bovine semen (71.50 ±1.47% intact acrosomes vs. 68.83 ±1.35% in control). Similar trends were reported by Gamage et al. (2024) in preserved boar semen, where Ashwagandha supplementation (1-20 mg/mL) significantly preserved acrosomal integrity during extended storage compared with untreated controls ($P < 0.05$).

The protective capacity of ARE on cellular membranes is largely mediated by its membrane-stabilizing and lipophilic radical-scavenging properties, which prevent oxidative cleavage of membrane phospholipids (Castro *et al*., 2025). The slight decline in membrane preservation at 50

μg/mL may be linked to changes in extender osmolarity or to mild pro-oxidant activity at higher extract concentrations (Riesco *et al*., 2021; Khalil *et al*., 2023).

Antioxidant Capacity and Lipid Peroxidation (TAC and MDA)

ARE supplementation significantly increased Total Antioxidant Capacity (TAC) and reduced Malondialdehyde (MDA) levels across all treatment groups, demonstrating potent inhibition of lipid peroxidation. MDA is a key biomarker of oxidative degradation of sperm membrane lipids; thus, its suppression directly reflects improved structural preservation (Riesco *et al*., 2021).

The highest TAC and the lowest MDA levels were recorded at 25 and 50 μg/mL ARE, corroborating the findings of Kanwar *et al*. (2026), who reported a significant decrease in MDA formation in bull semen supplemented with Ashwagandha-derived formulations (305.32 ±575.60 nmol/mL vs. 460.88 ±961.70nmol/mL in control). Phytochemical constituents of *W. somnifera* neutralize ROS generated during cryopreservation, thereby reinforcing endogenous antioxidant enzymatic systems (Aitken & Drevet, 2020; Munir *et al*., 2022).

Notably, although the 50 μg/mL concentration produced the highest biochemical antioxidant status (highest TAC, lowest MDA), it did not translate into optimal sperm motility or DNA preservation. This discrepancy underscores that maximal biochemical radical scavenging does not always equate to optimal physiological performance, as maintaining physiological levels of intracellular ROS is essential for functional competence (Zhu *et al*., 2023).

Sperm DNA Integrity (Comet Assay Parameters)

Spermatozoa lack active nuclear DNA repair mechanisms, rendering their genomic material highly vulnerable to cryo-induced oxidative cleavage (Sikka, 2020; Wang *et al*., 2023). In this study, control spermatozoa exhibited marked chromatin damage, as evidenced by elevated comet

assay parameters (% tailed DNA, tail length, and tail moment). ARE supplementation at 10 and 20 µg/mL provided substantial genoprotection, yielding the lowest %tailed DNA (10.37 ± 0.32%), shortest tail length (9.55 ± 0.26µm), and minimal tail moment (0.96 ±9.55)

compared with the control (21.43 ±0.72%, 11.63 ±0.51 µm, and 1.49 ±0.01, respectively). This protective effect is primarily attributed to withanolides, which scavenge hydroxyl radicals before they inflict oxidative single- and double-strand breaks on nuclear DNA (Ahmad *et al*., 2010). Moderate preservation was also maintained at 25 µg/mL ARE. However, the 50 µg/mL dose induced a paradoxical increase in DNA migration parameters (tail length: 16.88 ± 0.94µm; tail moment: 2.38 ±0.06). This biphasic hormetic response is characteristic of high-dose botanical antioxidants, in which excessive polyphenolic concentrations can undergo auto-oxidation, thereby promoting DNA strand breakage and cellular stress (Calabrese, 2021; Singh *et al*., 2025).

Across all parameters, the alignment among reduced lipid peroxidation (MDA), maintained membrane integrity, and minimized comet assay metrics confirms that ARE protects sperm genomic integrity primarily by attenuating upstream oxidative stress (Yue, 2016).

In Vivo **Fertility**

Information on the direct use of *W. somnifera*-supplemented cryopreserved semen in artificial insemination (AI) trials remains limited. In the present study, insemination with ram semen supplemented with ARE achieved a conception rate of 51.72%. This outcome aligns with previous findings by Singh *et al*. (2025) on the positive impact of Ashwagandha on overall reproductive efficiency in livestock. The improvement in field fertility confirms that *in vitro* enhancements in motility, membrane integrity, and genomic stability translate into superior fertilizing potential *in vivo*

Conclusion

Supplementing ram semen cryo-extender with *Withania somnifera* root extract (ARE) markedly improves post-thaw sperm quality by enhancing motility kinematics, preserving plasma membrane and acrosomal integrity, reinforcing antioxidant defenses, and preventing oxidative DNA fragmentation. The 10 and 20 μg/mL concentrations represent the optimal protective doses, whereas higher concentrations (50 μg/mL) show a biphasic effect that compromises motility and genomic stability. Furthermore, the encouraging pregnancy outcome (51.72%) demonstrates the practical utility of ARE as a natural cryoprotectant supplement to optimize ram semen preservation and increase efficiency in sheep artificial insemination programs.

Declarations

Author Contributions (CRediT Statement)

- **Ayat Abd-El Maksoud Elshamy:** Methodology, Investigation, Semen Processing & Cryopreservation, Formal Analysis, Data Curation, Writing – Original Draft.
- **Amira Khaled Khattab:** Methodology, Investigation, Post-Thaw Sperm Evaluation, Data Curation, Writing – Original Draft.
- **Nehal Ali Abu Elnaga:** Conceptualization, Methodology, Supervision, Project Administration, Writing – Review & Editing final.
- **Magdy Ramadan Badr:** Conceptualization, Methodology, *In Vivo* Artificial Insemination, Supervision, Writing – Review & Editing final.
- **Zaher Ibrahim Rawash:** Investigation, Validation, Data Curation, Writing – Review & Editing.

- **Asmaa Medhat:** Writing draft, primary Revisions.

All authors have read and agreed to the published version of the manuscript.

Ethics Statement

All experimental procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Agricultural Research Center (ARC-IACUC), Egypt (Protocol No. ARC-AZU-136-24). All animal handling complied with institutional guidelines for the care and use of agricultural research animals.

Disclosure Statement

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