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# Effect of Ashwagandha (*Withania somnifera*) supplementation on oxidative stress and freezability of breeding bull semen

N SINGH[1], A PATEL[1], S KUMAR[2], V SINGH[1], M PANDE[3]✉, N CHAND[2],
AS SIROHI[4], S MAHAJAN[2], SARIKA[2] and C RANI[2]

*ICAR-Central Institute for Research on Cattle, Meerut, 250 001, Uttar Pradesh, India*

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## ABSTRACT

*Withania somnifera* (ashwagandha), a well-known Ayurvedic adaptogen, is recognized for its potent antioxidant, cytoprotective, and anticancer properties. Although its efficacy in improving semen quality under oxidative stress is well documented in humans, its potential application in bull semen cryopreservation, where oxidative damage is a key determinant of reduced sperm survival and function, remains largely unexplored. The present study provided the first experimental evidence on the effect of ashwagandha supplementation in bull semen extenders. A total of 18 bull ejaculates (n = 18; six per bull) were cryopreserved in tris-based extender and divided into four groups: control (no supplementation) and three treatment groups supplemented with 1%, 2.5%, and 3.5% (v/v) aqueous ashwagandha extract. Post-thaw assessments included progressive motility, viability, acrosome integrity, plasma membrane integrity, and oxidative stress markers [reactive oxygen species (ROS) and malondialdehyde (MDA)]. Supplementation at 2.5–3.5% significantly ($p < 0.05$) improved motility, viability, acrosome- and plasma membrane integrity, while reducing ROS and MDA levels. Although DNA fragmentation did not differ statistically, numerical improvements were observed. The results demonstrated that ashwagandha supplementation enhanced post-thaw semen quality and mitigates cryo-induced oxidative stress in breeding bulls. Overall, the findings established *Withania somnifera* as a promising natural additive for bull semen extenders, with practical potential to improve the freezability and functional resilience of bull spermatozoa.

**Keywords**: Ashwagandha, Cryopreservation, Frieswal, Oxidative stress, Semen quality

*Withania somnifera* (ashwagandha), a widely revered herb in Ayurveda and a cornerstone of India's Indigenous Technical Knowledge (ITK) system, has traditionally been used to promote vitality, fertility and resilience against stress. Its bioactive constituents, particularly withanolides and withaferin-A, exhibit potent antioxidant, adaptogenic and cytoprotective effects (Sengupta *et al.* 2018, Mirjalili *et al.* 2009). In human clinical trials, ashwagandha supplementation has been reported to alleviate oxidative stress and improve semen quality parameters such as sperm concentration, motility and morphology (Ahmad *et al.* 2010, Shukla *et al.* 2011, Munir *et al.* 2022, Mikulska 2023). In boars, supplementation of semen extenders with Ashwagandha extract improved sperm motility, viability, acrosome integrity and reduced intracellular ROS during storage (Gamage *et al.* 2024). In rams, dietary supplementation of *W. somnifera* through feed-based interventions has been associated with improved libido, sperm quality and reproductive parameters (Rodriguez-Sánchez *et al.* 2024).

Artificial insemination (AI) is one of the most widely adopted reproductive technologies in livestock production, enabling genetic improvement and conservation of germplasm. However, the efficiency of AI is highly dependent on quality of cryopreserved semen, which often declines due to cryo-induced oxidative stress. The sequential steps of cryopreservation (dilution, cooling, freezing and thawing) compromise sperm membrane stability, motility and enzymatic activity, while also promoting the overproduction of reactive oxygen species (ROS). Excessive ROS induces lipid peroxidation (LPO), reduces sperm viability, disrupts acrosomal integrity and impairs fertilizing potential (Agarwal *et al.* 2014, Wang *et al.* 2025). Although spermatozoa possess intrinsic antioxidant defenses, their limited cytoplasmic content restricts this capacity. Moreover, during semen processing, seminal plasma is further diluted, which further reduces the natural antioxidant protection. Therefore, external supplementation become necessary to counteract oxidative stress.

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Present address: [1]Department of Veterinary Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, Sardar Vallabhbhai Patel University of Agriculture and Technology, Modipuram, Meerut, Uttar Pradesh, India. [2]Division of Cattle Physiology and Reproduction, ICAR-CIRC, Meerut. [3]Division of Animal Physiology and Biochemistry, ICAR-CSWRI, Avikanagar. [4]Division of Cattle Nutrition and Management, ICAR-CIRC, Meerut. ✉Corresponding author email: Megha.Pande@icar.org.in; megha985@gmail.com

Several antioxidant additives have been evaluated to improve sperm cryosurvivability, including ascorbic acid (Kocabaş *et al.* 2022), vitamin E (Sabetian *et al.* 2021), glutathione (Abdullah *et al.* 2021), synthetic antioxidants like butylated hydroxytoluene (Kumar *et al.* 2018) and novel peptides such as humanin (Pande *et al.* 2024). Nevertheless, natural supplements with minimal adverse effects are considered more desirable for routine application in semen cryopreservation.

Although Ashwagandha has been extensively studied in humans and evaluated in some livestock species, its role in bull semen cryopreservation remains largely unexplored. Therefore, the present study was conducted with two specific objectives: (i) to evaluate the effect of Withania somnifera extract supplementation in semen extenders on post-thaw sperm quality traits, and (ii) to assess its influence on oxidative stress parameters in breeding bulls. It was hypothesized that the antioxidant properties of Ashwagandha would enhance sperm functional attributes and mitigate cryo-induced damage.

## MATERIALS AND METHODS

The present investigation was undertaken at Cattle Physiology and Reproduction Division, ICAR-Central Institute for Research on Cattle (CIRC), Meerut Cantt, Uttar Pradesh, India.

*Experimental animals and semen collection:* For the present study, three clinically healthy Frieswal breeding bulls, each weighing between 500–600 kg, were selected as semen donors. Experimental bulls were maintained under standardized management and biosafety conditions, and all procedures were carried out in compliance with the guidelines of the Institute's Animal Ethics Committee. Semen was collected during the winter season (November–February) in the morning (08:00–09:00 h) using an artificial vagina, following standard procedures.

*Fresh semen evaluation:* Among the ejaculates collected, 18 samples fulfilling the selection criteria of initial progressive motility (IPM) ≥70% and sperm concentration above $500 \times 10^6$ sperm/mL were selected for the study. Semen volume was measured in milliliters using a graduated centrifuge collection tube, while colour and consistency were assessed visually; samples with abnormalities were discarded. Semen pH was recorded using a digital pH meter (Orion Star A211, Thermo Fisher Scientific, Chelmsford, USA). Sperm concentration was assayed using bull-semen-specific photometer (Accucell, IMV-France), calibrated monthly using a Neubauer haemocytometer. Progressive sperm motility was assessed by placing a 20 µL drop of diluted semen on a pre-warmed glass slide, gently covering it with a coverslip to avoid air bubble formation, and examining it under a phase-contrast light microscope (Olympus BX40, Japan) equipped with a thermostatically regulated stage (Tokai Hit, Japan) maintained at 37 °C. The movement of spermatozoa was evaluated at 400× magnification across several randomly selected fields, and the percentage of progressively motile sperm cells was recorded as the proportion of motile spermatozoa to the total observed. Live-dead ratio, acrosome integrity, and plasma membrane integrity were assessed following established protocols and described earlier (Pande *et al.* 2022).

*Preparation and addition of W. somnifera extract in semen extender:* Dried roots of *W. somnifera* (ashwagandha) were procured from a local herbal market, thoroughly rinsed, and dehydrated in a hot air oven at 80 °C for 24 hours. The dehydrated roots were ground into a fine powder, sieved, and processed into an aqueous extract. A 0.225% w/v solution was made by mixing 9 mg of powder in 4 mL of Tris-citrate buffer. It was thereafter kept at 37 °C for 2 h, and then subsequently passed through a 0.22 µm syringe filter to ensure sterility and removal of fine particulate matter prior to use.

For semen dilution, a Glycerol-Egg Yolk-Tris (GEYT) extender was prepared. The semen was diluted to obtain a final sperm concentration of $80\times10^6$ spermatozoa/mL, corresponding to $20\times10^6$ spermatozoa per straw, across all the groups. The experiment included a control group (CG) and three treatment groups (TG-I, TG-II, and TG-III). In control group, spermatozoa were frozen in GEYT without supplementation, whereas TG-I, TG-II, and TG-III were supplemented with Ashwagandha extract at final concentrations of 1.0% v/v (20 µL/2 mL), 2.5% v/v (50 µL/2 mL), and 3.5% v/v (70 µL/2 mL), respectively.

*Equilibration, cryopreservation, thawing, and post-thaw semen evaluation:* Diluted semen samples from control and treatment groups were filled into 0.25 mL French mini straws at a final concentration of 20 million spermatozoa per straw, sealed, and equilibrated at 4°C for 4 h in a horizontal cold-handling cabinet (Minitube). After equilibration, the straws were frozen in a programmable biofreezer (IMV, France) following a controlled freezing curve: from 4 °C to −10 °C at a rate of −3 °C/min, held for 1 min for seeding, then cooled to −100 °C at −20 °C/min, and finally plunged into liquid nitrogen (−196 °C) for storage. For post-thaw assessments, semen straws were thawed using a digital thawing unit at 37°C for 30 seconds to restore them to physiological conditions before analysis. The thawed sample was assessed for progressive motility, livability, percent acrosomal intactness, sperm membrane integrity, and DNA integrity. For consistency, the contents of at least two straws were pooled into a borosilicate test tube and maintained at 34°C in a water bath prior to analysis.

*Acridine orange staining for DNA integrity assessment:* DNA fragmentation in spermatozoa was assessed using the Acridine Orange (AO) staining method (Srivastava and Pande 2017). Briefly, frozen–thawed semen (0.5 mL) was diluted with 2 mL of non-capacitation medium in a 3 mL cryovial and centrifuged at 1000 × g for 300 seconds. The supernatant was removed, and the procedure was repeated. A smear was prepared from a 0.5 mL aliquot, fixed in Carnoy's fixative for 3 h, and air-dried. The slide was subsequently treated with AO working solution for 300 seconds before mounting, and examined under an epifluorescent microscope (Nikon, 400×, excitation 450–490 nm). A total of 200 spermatozoa were randomly evaluated for DNA integrity.

*Lipid Peroxidation (LPO) estimation:* Lipid peroxidation was measured as malondialdehyde (MDA), a key by-product of lipid degradation, using the thiobarbituric acid (TBA) assay with modifications (Sanocka and Kurpisz 2004). In brief, 20 million spermatozoa were mixed in 1 mL Tris-citrate buffer and mixed with 2 mL TBA–TCA reagent (15% trichloroacetic acid, 375 mg thiobarbituric acid, 2.21 mL HCl, made up to 100 mL with Milli Q water). The reaction mixture was kept in a boiling water bath for 45 minutes, allowed to cool to room temperature, and centrifuged at 2800 rpm for 10 minutes. The absorbance of the resulting supernatant was measured at 535 nm using a spectrophotometer (UV-1900, Shimadzu, Japan), and the malondialdehyde concentration was determined based on an extinction coefficient of $1.56 \times 10^5$ mol$^{-1}$ cm$^{-1}$.

*Reactive oxygen species estimation:* Reactive oxygen species (ROS) levels in semen was estimated using the method of Hayashi *et al.* (2007) with minor modifications. In brief, equal amount (5 µL) of seminal plasma and hydrogen peroxide standards (100–1000 mg/L) were dispensed in duplicate into a transparent microtitre plate. Each well then received 140 µL of 0.1 M sodium acetate buffer (pH 4.8) along with 100 µL of the reaction mixture (R1:R2 in a ratio of 1:25). The absorbance was measured at 492 nm at both 0 and 2 minutes with the aid of a spectrophotometer, and a standard curve was prepared by plotting optical density (OD) against $H_2O_2$ concentrations. ROS levels in the test samples were extrapolated from this curve, where an increase in absorbance indicated elevated ROS production. The reagents used for the assay included 0.1 M sodium acetate buffer (prepared by dissolving 4.2 g sodium acetate in 300 mL distilled water, adjusted to pH 4.8, and made up to 500 mL), R1 (DEPPD, 100 µg/mL in buffer), and R2 (ferrous sulfate, 4.37 µM in buffer, pH 4.8).

*Statistical analysis:* Data were examined using standard statistical approaches. Percentage values were subjected to arcsine transformation before analysis. One-way ANOVA was carried out with SPSS version 20.0 (SPSS Inc., Chicago, USA), and significance was determined at $p < 0.05$. Post-hoc comparisons among treatment means were conducted using Duncan's test. Results are expressed as mean ± standard error of mean, and graphical representations were prepared using GraphPad Prism 5.0 (GraphPad, USA).

## RESULTS AND DISCUSSION

*Fresh semen characteristics*: The ejaculates that exhibited superior quality at the fresh stage were selected for the experiment. The mean sperm concentration was 923.44 ± 63.61 million/mL, ranging from 610 to 1431 million/mL. The average initial progressive motility was 71.11 ± 0.76%, while sperm viability varied between 81% and 90% with a mean of 85.17 ± 0.65%. The mean values for acrosome integrity and plasma membrane integrity were 77.50 ± 1.20% and 81.44 ± 0.56%, respectively. The average pH recorded was 6.71 ± 0.78. These baseline attributes were in close agreement with previous reports on semen quality in breeding bulls utilized for artificial insemination (Pande *et al.* 2018, Sirohi *et al.* 2022).

*Effect of W. somnifera supplementation on post-thaw semen quality:* Supplementation with Ashwagandha significantly improved post-thaw semen quality in Frieswal breeding bulls (Table 1).

Values with different superscripts (a, b) within the same column indicate significant differences ($p < 0.05$). CG: Control group (semen cryopreserved without Ashwagandha extract). TG-I, TG-II, and TG-III: Treatment groups supplemented with 1.0% v/v (20 µL/2 mL), 2.5% v/v (50 µL/2 mL), and 3.5% v/v (70 µL/2 mL) Ashwagandha extract, respectively.

Table 1. Effect of ashwagandha extract supplementation on semen quality parameters in cryopreserved Frieswal Breeding Bull Spermatozoa (n = 18 ejaculates; 6 per bull) (Mean ± SEM)

| Groups | Post-thaw Motility (%) | Viability (%) | Acrosome Integrity (%) | Plasma Membrane Integrity (%) |
|---|---|---|---|---|
| CG (Control) | 33.06 ± 1.81$^{a}$ | 36.94 ± 2.09$^{a}$ | 62.17 ± 1.98$^{a}$ | 35.50 ± 1.76$^{a}$ |
| TG-I (1.0 %) | 33.33 ± 1.47$^{a}$ | 38.67 ± 1.93$^{a}$ | 64.17 ± 1.67$^{a}$ | 37.06 ± 1.79$^{ab}$ |
| TG-II (2.5%) | 41.94 ± 1.52$^{b}$ | 44.00 ± 1.84$^{b}$ | 70.83 ± 1.19$^{b}$ | 41.61 ± 1.35$^{b}$ |
| TG-III (3.5%) | 42.11 ± 1.87$^{b}$ | 47.44 ± 1.75$^{b}$ | 72.56 ± 1.36$^{b}$ | 44.83 ± 1.18$^{b}$ |

Motility was higher in TG-II and TG-III compared with the control group, while TG-I remained comparable to the control. A similar trend was observed for sperm viability, where the higher supplementation levels showed better survival rate following thawing. These observations are in consistent with earlier reports indicating that ashwagandha enhances sperm function through its antioxidant and adaptogenic properties (Ahmad *et al.* 2010, Munir *et al.* 2022). Acrosome integrity also improved in the groups receiving higher levels of supplementation, indicating better preservation of sperm structural integrity during cryopreservation. Comparable findings have been reported in boar semen, where ashwagandha extract improved acrosomal stability during storage (Gamage *et al.* 2024). Plasma membrane integrity followed a similar pattern, with better membrane preservation in TG-II and TG-III than in the control group, whereas TG-I showed only a marginal improvement. Since the sperm plasma membrane is rich in polyunsaturated fatty acids and highly susceptible to cryo-induced oxidative damage, the antioxidant components of ashwagandha may help stabilize the membrane during freezing and thawing. Representative photomicrographs of HOS-positive and HOS-negative spermatozoa are presented in Fig. 2.

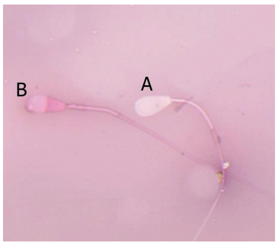

Fig. 1. Photomicrographs of live (white, A) and dead (pink, B) spermatozoa (Eosin-Nigrosin stain, 1000× magnification).

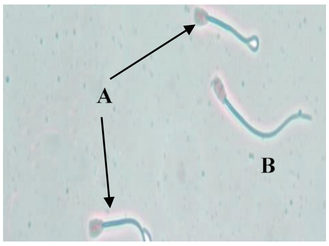

Fig. 2. Photomicrographs depicting Hypo-Osmotic Swelling (HOS) Test reactive (A) and non-reactive (B) spermatozoa (150 mOsm/L hypo-osmotic solution, 400× magnification).

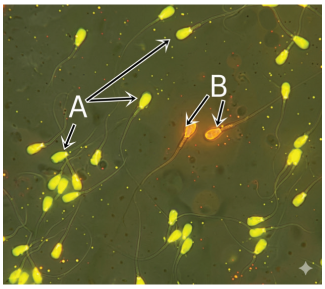

Fig. 3. Photomicrographs showing DNA integrity under an epifluorescent microscope (400×). Spermatozoa with intact DNA (green fluorescence on the head) and non-intact DNA (orange/red fluorescence on the head).

Table 2. Effect of Ashwagandha supplementation on sperm DNA integrity in cryopreserved Frieswal bull semen (n = 18 ejaculates, 6 per bull) (Mean ± SEM)

| Groups | Spermatozoa with Intact DNA (%) |
|---|---|
| CG (Control) | 80.33 ± 1.40 |
| TG-I (1.0 %) | 84.94 ± 0.51 |
| TG-II (2.5%) | 82.39 ± 0.39 |
| TG-III (3.5%) | 83.95 ± 0.49 |

These results highlighted ashwagandha's cytoprotective potential in maintaining membrane functionality under cryogenic stress. Previous studies have also shown that ashwagandha extract possesses strong antioxidant properties, stabilizing cell membranes, reducing oxidative stress, and minimizing cellular injury, while also exhibiting notable anticancer potential (Barnes *et al.* 2016). Recent studies have also confirmed that crossbred bulls, such as Frieswal, are more vulnerable to oxidative stress than purebred or exotic breeds (Kumaresan *et al.* 2021), underscoring the practical significance of ashwagandha supplementation in enhancing their semen cryosurvivability.

Cryopreservation-induced oxidative stress is a major cause of sperm DNA fragmentation, which can impair fertilization capacity and embryo development due to excessive generation of reactive oxygen species (ROS) that damage sperm chromatin and membrane integrity (Ribas-Maynou *et al.* 2024). *W.* somnifera contains bioactive compounds such as withanolides and flavonoids with strong antioxidant and free-radical scavenging properties, which may help mitigate oxidative stress and stabilize sperm chromatin during cryopreservation. Supporting evidence shows that ashwagandha supplementation improves semen quality and reproductive function, including enhanced sperm count, motility, and semen volume in oligospermic men (Ambiye *et al.* 2013), improved antioxidant status and reduced lipid peroxidation in bovine semen using ashwagandha-derived nanoparticles (Kanwar *et al.* 2024), and better CASA-based sperm motion traits and post-thaw motility in subfertile buffalo bulls following herbal supplementation containing W. somnifera (Shivkumar *et al.* 2018).

In the present study, the effect of ashwagandha supplementation on sperm DNA integrity at the post-thaw stage is summarized in Table 2. Although a numerical improvement was observed in the treated groups, the differences were not statistically significant. Representative AO stained spermatozoa with intact DNA (green fluorescence on the head, A) and fragmented DNA (orange/red fluorescence, B) are shown in Fig. 3. Comparable findings have been reported in liquid-stored boar semen, where ashwagandha supplementation significantly preserved chromatin stability and reduced DNA fragmentation (Gamage *et al.* 2024). Although statistical significance was not achieved in the present study, the consistent trend toward improved DNA integrity suggested a potential protective role of ashwagandha, likely to be mediated through its antioxidant and chromatin-stabilizing properties.

*Effect of ashwagandha supplementation on oxidative stress markers (ROS and lipid peroxidation):* Following freeze–thawing, ashwagandha supplementation reduced oxidative stress in cryopreserved Frieswal bull semen, as reflected by lower ROS and malondialdehyde (MDA) levels (Figs. 4 and 5). ROS concentrations were significantly lower ($p < 0.05$) in TG-II and TG-III compared with the control and TG-I, whereas TG-I did not differ from the control. Similarly, MDA levels, an indicator of lipid peroxidation, were reduced in TG-II and TG-III relative to the control and TG-I groups, while no difference was observed between TG-II and TG-III. These findings suggest that the antioxidative effect of ashwagandha becomes evident at moderate supplementation levels and tends to plateau beyond 2.5%.

Excessive ROS generation is widely recognized as a major cause of sperm dysfunction, promoting lipid peroxidation, protein oxidation and DNA damage (Wang *et al.* 2025, Raz *et al.* 2025). In the present study, the reduction in ROS and MDA in ashwagandha-treated groups indicated effective suppression of oxidative stress during cryopreservation. Such antioxidant action may be attributed to the bioactive phytochemicals present in *W. somnifera*, which possess strong free radical scavenging properties. Earlier studies in humans have also reported that ashwagandha supplementation improves semen quality by lowering oxidative stress markers (Ahmad *et al.* 2010, Munir *et al*. 2022), and similar benefits have been observed in stress-related male infertility (Mahdi *et al.* 2011). Recent reports further indicate that ashwagandha can inhibit lipid peroxidation and reduce oxidative damage in semen (Mikulska *et al.* 2023).

The comparable ROS and MDA levels between TG-II and TG-III indicate a possible threshold effect, where higher concentrations do not confer additional antioxidative advantage. Similar saturation patterns have been reported for other antioxidant additives in bovine semen cryopreservation (Kocabaş *et al*. 2022, Pande *et al.* 2024). Overall, the reduction in oxidative stress markers observed in the present study provides mechanistic support for the improved post-thaw motility, viability and membrane integrity recorded in the ashwagandha-supplemented groups, highlighting the importance of antioxidant supplementation in protecting spermatozoa from cryo-induced oxidative damage.

Taken together, these findings establish *W. somnifera* (Ashwagandha) as a promising natural additive in semen extenders, improving sperm motility, viability, acrosome integrity, and plasma membrane stability while reducing oxidative stress in cryopreserved bull semen. The similar responses observed between 2.5% and 3.5% (v/v) indicate that 2.5% may be an optimal supplementation level. To our knowledge, this study provides the first experimental evidence of Ashwagandha improving semen freezability in breeding bulls, highlighting its potential as an ITK-based strategy to mitigate cryopreservation-induced oxidative stress in bovine reproduction.

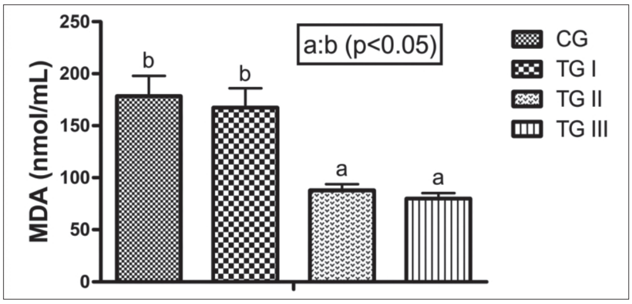

Fig. 4. Malondialdehyde (MDA) levels in control and ashwagandha-supplemented groups at the post-thaw stage. CG: Control (no ashwagandha); TG-I, TG-II, and TG-III: 1.0% (20 µL/2 mL), 2.5% (50 µL/2 mL), and 3.5% (70 µL/2 mL) ashwagandha, respectively.

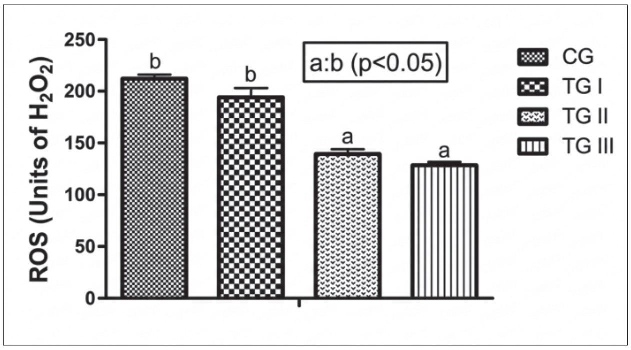

Fig. 5. Reactive oxygen species (ROS) levels (units of $H_2O_2$) in control and ashwagandha-supplemented groups at the post-thaw stage. CG: Control (no ashwagandha); TG-I, TG-II, and TG-III: 1.0% (20 µL/2 mL), 2.5% (50 µL/2 mL), and 3.5% (70 µL/2 mL) ashwagandha, respectively.

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