di Fonzo et al.
CABI Agriculture and Bioscience (2025) 6:1, 0048
https://doi.org/10.1079/ab.2025.0048

CABI Agriculture and Bioscience

RESEARCH

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Llama semen cooling: Use of zinc oxide nanoparticles as potential antioxidants

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Abstract

Background: There are no reports evaluating the effect of antioxidant supplementation during semen cooling in llamas. This study evaluates the impact of zinc oxide nanoparticles (ZnO-NPs) on sperm ultrastructure and characteristics in cooled llama semen. *Methods:* Eighteen ejaculates obtained from six male llamas were divided into three aliquots and diluted with: AndroMed®-20% egg-yolk (AM-EY); AM-EY with 50 µg/ml of ZnO-NPs; and AM-EY with 100 µg/ml of ZnO-NPs. Samples were cooled to 5°C in an Equitainer®. Evaluations were carried out in raw semen, after dilution (pre-cooling; 0 h), and after 24 and 48 h of storage. Additionally, cooled samples were evaluated after 15, 90, and 180 min of incubation at 37°C. Data were analyzed using Friedman tests, conventional ANOVA, or split-plot design models, depending on the structure and distribution of the data. *Results:* Oscillatory sperm motility remained unchanged after 24 or 48 h of cooling ($P > 0.05$). Progressive sperm motility (PM) was similar between all samples cooled for 24 h and pre-cooling samples. A significant decrease in PM was observed in all cooled samples for 48 h compared to pre-cooling samples with ZnO-NPs. Total sperm motility also decreased in cooled samples, particularly after 48 h of storage. No significant differences were found in sperm membrane functionality, morphology, or DNA integrity between raw semen and samples cooled for 24 and 48 h. Regarding viability and acrosomal integrity and intact DNA, no changes were observed among the cooled samples at any of the evaluated storage times (24 and 48 h), nor compared to pre-cooling samples. Live lipoperoxidation-negative sperm decreased in all cooled semen samples compared to raw semen, showing no differences among cooled samples. Plasma membrane damage was evident in all cooled samples, more clearly seen with transmission electron microscopy. Finally, ZnO-NPs did not improve the preservation of cooled samples at 37°C for 180 min. *Conclusions:* The addition of ZnO-NPs at 50 or 100 µg/ml to AM-EY extenders does not fully protect llama sperm from cooling damage. Further evaluation using other concentrations may be necessary to determine the optimal dose of ZnO-NPs for cooling llama semen. Exploring other nanoparticles or antioxidants could be beneficial. Electron microscopy shows notable structural damage in all samples (with and without ZnO-NPs), highlighting the sensitivity of llama spermatozoa to cooling.

Keywords: antioxidants, camelids, cooling, DNA, lipoperoxidation, nanoparticles, semen, zinc oxide

Introduction

South American camelids (SAC) are a tribe within the Camelidae family, encompassing llamas, alpacas, guanacos, and vicuñas. These species are essential to the economy and culture of various South American regions and have historically been valued for their fiber, meat, load-carrying capabilities, and their significance in the Andean worldview. Furthermore, as native species of South America, they exhibit remarkable adaptations to their original biomes, thriving under harsh to extreme climatic conditions, with limited and low-quality forage and minimal oxygen pressure.

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Submitted: 15 January 2025. Accepted: 18 May 2025. Published: 19 June 2025

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Despite these evolutionary advantages, SACs present reproductive characteristics – such as induced ovulation, low sperm concentration, and viscous seminal plasma – that limit their reproductive efficiency. These features pose challenges for the implementation of assisted reproductive technologies (ARTs), ultimately restricting the potential for genetic improvement and productivity in these species. In particular, artificial insemination (AI) is limited to the use of raw semen due to the very low post-thaw sperm quality observed in SAC. Studies report a loss of 60 to 75% of motile and viable sperm with intact acrosomes in frozen/thawed semen samples (Aller *et al*., 2003; Santiani Acosta *et al*., 2013; Carretero *et al*., 2015a; Fumuso *et al*., 2019, 2020, 2021; Flores Huarco *et al*., 2021). Conversely, SAC spermatozoa exhibit better survival rates after cooling compared to freezing. However, both preservation methods yield low pregnancy rates following AI (0–33.3%: Bravo *et al*., 2000; Aller *et al*., 2003; Vaughan *et al*., 2003; Huanca *et al*., 2007; Giuliano *et al*., 2012; Fumuso *et al*., 2018; Stuart *et al*., 2019; Aisen *et al*., 2021; Contreras Huamani *et al*., 2024). In this regard, Zampini *et al*. (2020) identified several ultrastructural injuries in cooled and frozen/thawed llama sperm using both scanning electron microscopy (SEM) and transmission electron microscopy (TEM). These injuries might not be readily detectable during routine seminal assessments.

The mammalian sperm cell contains very low cytoplasm volume, which is associated with inefficient free radical scavenging systems. Additionally, its plasma membrane has a high proportion of polyunsaturated fatty acids, making it highly susceptible to peroxidation and oxidative stress (Guthrie and Welch, 2012). Cryopreservation, on the other hand, greatly stimulates reactive oxygen species (ROS) production, which extracts electrons from membrane lipids, oxidizing them in the process. This breaks the balance between oxidative and antioxidative agents in sperm cells subjected to cryopreservation (Aitken, 2017). Oxidative stress induces changes in the organization and functionality of the sperm plasma membrane and compromises chromatin stability (Khalifa *et al*., 2008). These effects result in reduced semen quality, lower sperm motility, functional integrity, viability, and finally, diminished sperm fertility (Gautier and Aurich, 2022).

In various species, the addition of antioxidant agents to the cryopreservation medium has been shown to mitigate the effects of ROS and improve sperm quality after thawing (Hezavehei *et al*., 2018, Review). Zinc is naturally present in both sperm cells and seminal plasma (Allouche-Fitoussi and Breitbart, 2020), and zinc complexes are effective free radical scavengers (Zago and Oteiza, 2001). This metal acts as a cofactor for enzymes involved in the antioxidant defense system, such as superoxide dismutase, thereby enhancing the sperm's antioxidant capacity and effectively inhibiting membrane lipid peroxidation (Kowalczyk, 2022). Nanoparticle-formulated zinc oxide (ZnO-NPs) carries a neutral charge, rendering it unable to penetrate the negatively charged membrane of live sperm (Asadi *et al*., 2023). Studies using TEM and fluorescence microscopy have shown that ZnO-NPs surround sperm cells and are partially adsorbed by them, exerting their protective features during cryopreservation (Isaac *et al*., 2017). In addition, in various species, the inclusion of ZnO-NPs in extenders has been reported to optimize sperm cryopreservation protocols, leveraging their antioxidant properties (human: Isaac *et al*., 2017; ram: Heidari *et al*., 2019; bull: Farhadi *et al*., 2022; dromedary: Shahin *et al*., 2020, 2021; goat: Abedin *et al*., 2023).

There are limited reports on the use of antioxidant agents in the cryopreservation of SAC sperm. In alpacas, higher sperm motility values have been reported with the use of superoxide dismutase analogs in the cryopreservation of semen and sperm obtained via deferent duct deviation (Santiani Acosta *et al*., 2013; Gómez-Quispe *et al*., 2016). In llamas, however, no studies have yet evaluated the effects of antioxidant supplementation during semen cooling. Hence, given the advantages of nanoparticles (NPs) and the promising results observed in other species regarding their use as antioxidants during cryopreservation, this study aims to evaluate the effect of zinc oxide nanoparticles (ZnO-NPs) in extenders on sperm ultrastructure and characteristics in cooled llama semen. Additionally, the experimental design includes evaluations after maintaining post-cooled sperm cells at body temperature (37°C) over time to simulate the biological conditions experienced by sperm during transport in the female genital tract before fertilization.

Methods

ANIMALS AND LOCATION

Six male *Lama glama*, aged 6–10 years with an average weight of 129.6 ± 10.5 kg, participated in this study at the Faculty of Veterinary Sciences, University of Buenos Aires, Argentina. The llamas were sheared in November, housed in grass enclosures, supplemented with alfalfa, and had unrestricted access to fresh water. All experimental procedures received approval from the Committee for the Use and Care of Laboratory Animals (CICUAL) at the Faculty of Veterinary Sciences, University of Buenos Aires (protocol 2024/15).

SEMEN COLLECTION

Semen collection was performed between April and November using electroejaculation under general anesthesia, following the protocol described by Director *et al*. (2007).

EVALUATION OF SEMINAL PARAMETERS

After collection, the following semen characteristics were examined: volume, thread formation, sperm motility, sperm concentration, viability and acrosome integrity, sperm membrane function, sperm morphology, DNA integrity, lipid peroxidation, and sperm ultrastructure.

Volume was measured in milliliters (ml) using a micropipette.

Thread formation was classified as either present or absent based on the samples' ability to form a thread when 20 µl was pipetted onto a slide (Bertuzzi *et al*., 2022).

Sperm concentration was determined using a Neubauer chamber (Bertuzzi *et al*., 2022).

Despite computer-assisted semen analysis (CASA) being the most objective method for evaluating sperm motility, it poses significant challenges when applied to semen from SAC due to the variability in sperm morphology and the differing shapes and sizes of sperm heads that are considered normal (Buendía *et al*., 2002; Casaretto *et al*., 2012). This variability makes it unreliable to adjust the system settings specifically for this species. Moreover, attempts to use CASA on raw llama semen (Fumuso *et al*., 2014) revealed that the system fails to recognize sperm exhibiting oscillatory motility – the predominant motility pattern observed in raw semen – and incorrectly classifies such sperm as immotile. To ensure our findings are comparable with most previous studies in sperm analysis of SAC, we evaluated sperm motility subjectively using a phase-contrast microscope with a 100× magnification and a warm stage maintained at 37°C. The motility patterns were classified as follows: Oscillatory Motility (OM): sperm moving in place without forward progression; Progressive Motility (PM): sperm moving in a straight line or making wide circles; and Circular Motility (CM): sperm moving in very tight circles. Total sperm motility (TM) was calculated as the sum of OM, PM, and CM. Additionally, sperm vigor, defined as the intensity of motility, was assessed using a scale from 1 to 5, where 1 indicates very low vigor and 5 represents maximum vigor (Bertuzzi *et al*., 2022).

Combined evaluation of sperm viability and acrosome integrity was performed using the dual stain FITC-PNA/PI (Fluorescein isothiocyanate conjugated with *Arachis hypogaea* agglutinin and propidium iodide), as described by Carretero *et al*. (2015b). In brief, samples were incubated in a staining medium consisting of 2 µl of

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PNA (2 mg/ml in PBS) and 500 µl of saline solution (as outlined by Harrison and Vickers, 1990) for 10 min at 37°C. Following this, 2 µl of PI (0.5 mg/ml in physiological solution) was added, and the mixture was incubated for an additional 10 min. A minimum of 200 spermatozoa were examined using a fluorescence microscope (1000x magnification) (Leica®, DMLS, Heerbrugg, Switzerland). The sperm were classified into the following categories: (LI) live sperm with intact acrosomes (sperm cells with intact head plasma and acrosome membranes, showing no fluorescence in either the head or acrosome cap); (LD) live sperm with damaged outer acrosomal membranes (no fluorescence in the head with green fluorescence in the acrosome cap or green fluorescent equatorial segment); (DI) dead sperm with intact acrosomes (red fluorescent head and no fluorescence in the acrosome cap) and (DD) dead sperm with damaged outer acrosomal membranes (red fluorescent head with green fluorescence in the acrosome cap or green fluorescent equatorial segment).

The sperm membrane function was assessed using the hypoosmotic swelling (HOS) test described by Giuliano *et al*. (2008). In brief, 50 µl of sperm samples were incubated for 20 min at 37°C in 200 µl of a hypoosmotic solution composed of fructose (2.45 mg/ml) and sodium citrate (4.5 mg/ml) in Milli-Q deionized water, adjusted to 50 mOsm/L and a pH of 7.4. After incubation, a minimum of 200 spermatozoa were evaluated using a phase-contrast microscope at 400× magnification. Sperm exhibiting the characteristic tail swelling were classified as HOS+, indicating functional plasma membranes.

Sperm morphology was evaluated using phase-contrast microscopy at a magnification of 1000×. A micro-droplet of the sample was placed on a glass slide and covered with a coverslip. For each sample, 200 spermatozoa were assessed and classified into the following categories: normal, abnormal head, detached head, abnormal midpiece, abnormal tail, and presence of cytoplasmic droplet. The percentages of spermatozoa with normal and abnormal morphology were calculated (Bertuzzi *et al*., 2022).

The Sperm Chromatin Dispersion (SCD) assay was used to evaluate DNA integrity according to Carretero *et al*. (2012). In this procedure, sperm samples were embedded in an agarose matrix and treated with denaturing and lysis solutions. After treatment, the samples were stained with Giemsa dye and examined under brightfield microscopy at 1000× magnification. The sperm were classified into two groups based on the degree of DNA fragmentation: those exhibiting a large or medium halo, which indicates intact DNA, and those with a small or no halo, indicative of fragmented DNA.

Sperm membrane lipoperoxidation (LPO): the BODIPY C11-581/591 probe was used to evaluate lipid peroxidation (Caldevilla *et al*., 2024). Sperm samples were incubated with 2 µM BODIPY (from a stock solution of 2 mM in DMSO) for 30 min at 37°C. After incubation, the samples were washed with HAM medium to remove excess fluorochrome by centrifugation at 750 g for 5 min. Then, 1 µl of propidium iodide (PI) was added to achieve a final concentration of 2.5 µg/ml before evaluation with a cytometer (BD FACSCanto II, Germany). A minimum of 100,000 sperm were analyzed, with BODIPY fluorescence detected using the 520 nm filter (FL-1) and PI using the 610 nm filter (FL-3), both on a logarithmic scale. The results were analyzed using the FACSDiva program, and the following populations were defined: live, LPO-negative sperm (live non-peroxidized); live, LPO-positive sperm (live peroxidized); dead, LPO-negative sperm (dead non-peroxidized); and dead, LPO-positive sperm (dead peroxidized). Samples exposed to UV light for 60 minutes served as controls for lipid peroxidation (LPO).

Sperm ultrastructure was analyzed using scanning (SEM) and transmission (TEM) electron microscopy at the Centro Integral de Microscopía Electrónica (CIME), CONICET, Tucumán-Argentina. Briefly, the samples were diluted 1:3 in PBS and then centrifuged at 600 *g* for 8 min. After discarding the supernatant, 1 ml of PBS was added, followed by another round of centrifugation for 5 min. Once the supernatant was discarded, 100 µl of Karnovsky fixative (1.7% glutaraldehyde and 2.7% paraformaldehyde in phosphate buffer at pH 7.2) was added. The samples were then refrigerated and sent to CIME for processing according to the method described by Zampini *et al*. (2020).

For SEM, 100 µl of fixed sperm suspension was placed on a glass slide that had been coated with 2% agar and allowed to cure for 1 h. The samples were then dehydrated in increasing concentrations of ethanol (30, 50, 70, 90, and 100%) with each concentration maintained for 10 min. Following this, the samples underwent two acetone baths, also lasting 10 min each. The samples were critical-point dried with liquid carbon dioxide (Denton Vacuum DCP-1 Critical Point Dryer, NJ, USA), mounted on aluminum stubs, and metalized with gold (JEOL ion sputter JFC-1100, Tokyo, Japan) for later observation using a scanning electron microscope (Zeiss SUPRA 55VP, Oberkochen, Germany). Different fields were randomly chosen, and 100 sperm per sample were examined.

For TEM, sperm fixed in Karnovsky's reagent were centrifuged, and the resulting pellets were embedded in a 1.2% agar solution. The samples were washed three times with sodium phosphate buffer, post-fixed in 2% osmium tetroxide in the same buffer at 4°C overnight and then treated with an aqueous solution of 2% uranyl acetate for 30 min. The samples underwent serial dehydration in ethanol, were passed through acetone, and were finally embedded in Spurr resin overnight at 60°C. Ultrafine sections (60–70 nm) were examined on copper grids using a Zeiss Libra 120 electron microscope (Carl Zeiss, Oberkochen, Germany). Micrographs were analyzed with a focus on different sperm structures, including the plasma membrane, acrosome, nucleus, mitochondria in the midpiece, and axoneme along with periaxonemal structures in the principal piece. Observations were made to determine whether these structures were preserved or exhibited any alterations. A total of 100 sperm heads and tails per sample were examined, including both longitudinal and cross-sections, to describe the sperm quality characteristics in the different groups (raw semen and cooled samples).

EXPERIMENTAL DESIGN

The effect of different Zinc oxide nanoparticles (ZnO-NPs) concentrations on semen cooled at 5°C was evaluated. A total of 18 ejaculates were collected from six adult male llamas. Semen characteristics were evaluated immediately after collection, and each ejaculate was divided into three aliquots of equal volume. To standardize sperm concentration, each aliquot was diluted to 80×10^6 sperm/ml with one of the following extenders, pre-warmed to 37°C: (1) AndroMed® with 20% EY (AM-EY, control); (2) AM-EY with 50 µg/ml of ZnO-NPs (AM-EY50), and (3) AM-EY with 100 µg/ml of ZnO-NPs (AM-EY100). Using a micropipette, samples were blended approximately ten times, depending on the ejaculates, to ensure a homogeneous mixture of semen and extender.

After dilution, thread formation was re-evaluated, along with sperm motility, viability, and acrosome integrity. These assessments were designated as time 0 h (pre-cooling samples: AM-EY 0 h, AM-EY50 0 h and AM-EY100 0 h). After this, samples were cooled to 5°C in an Equitainer® under anaerobic conditions in 0.5 ml Eppendorf® and were maintained at this temperature for 48 h, following the method described by Bertuzzi *et al*. (2022). An aliquot was taken from each sample at 24 and 48 h of storage and all semen characteristics were reassessed: thread formation, sperm motility, viability and acrosome integrity, sperm membrane function, sperm morphology, and DNA integrity after 15, 90, and 180 min of incubation at 37°C. Lipid peroxidation and sperm ultrastructure were specifically evaluated in samples cooled for 24 h. The experimental design and evaluation times are summarized in Fig. 1.

The selected nanoparticle concentrations were based on the findings of Isaac *et al*. (2017) in human sperm and Shahin *et al*. (2020) in dromedary sperm.

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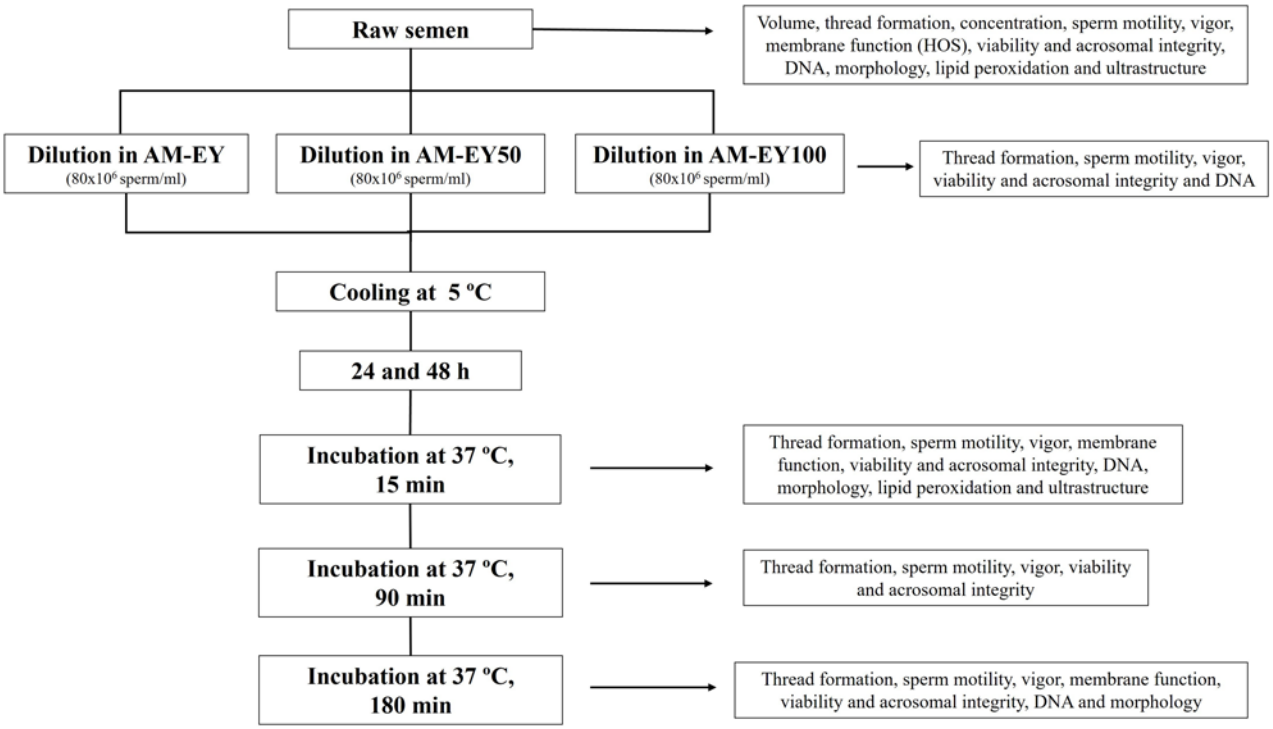


Fig. 1. Experimental design used for cooling llama semen. AM-EY: AndroMed® extender with 20% egg yolk, AM-EY50: AM-EY extender with 50 µg/ml of Zinc oxide nanoparticles and AM-EY100: AM-EY extender with 100 µg/ml of Zinc oxide nanoparticles.

REAGENTS

The components for the HOS test, FITC-PNA/PI dual stain, and ZnO-NPs were purchased from Global Lab S.A. (Argentina) and Sigma Chemicals (St. Louis, MO, USA). The commercial extenders were acquired from Minitube, Argentina. The saline medium used for the FITC-PNA/PI technique was prepared according to the protocol described by Harrison and Vickers (1990): NaCl (140 mM), Glucose (10 mM), KCl (2.5 mM), Polyvinyl-pyrrolidone (0.5 mM), Hepes (20 mM). The solution was adjusted to a pH of 7.55 at 20°C, and an osmolarity of 300 mOsm/L.

STATISTICAL ANALYSIS

Statistical analyses were performed using InfoStat software (Di Rienzo *et al*., 2008), and the significance level was set at 0.05. Data normality and homogeneity of variances were checked using the Shapiro–Wilk and Bartlett´s tests, respectively.

- *Comparison of raw semen with diluted samples at 37°C (pre-cooling):* for variables with normal distribution and homogeneity of variance, a factorial ANOVA was conducted, blocking by individual males. For variables that did not meet these assumptions, the Friedman test was applied.
- *Comparison of sperm membrane functionality and morphology:* ANOVA with a factorial design was used to compare raw semen with samples cooled for 24 and 48 h.
- *Comparison of pre-cooling samples with cooled samples (24 and 48 h):* depending on data distribution, ANOVA or the Friedman test was used.
- *Evaluation of different incubation times at 37°C (15, 90, and 180 min):* for samples cooled for 24 and 48 h, a split-plot design and Friedman tests were applied, depending on data normality.

Finally, a Pearson test was used to correlate the percentages of live, LPO-negative sperm (BODIPY) with live sperm with intact acrosomes (FITC-PNA/PI) in all samples evaluated.

Results

The volume of the ejaculates obtained was 583.0 ± 336.1 µl, and the concentration was 395.3 ± 503.3 × 10^6 sperm/ml (mean ± SD). Of the 18 ejaculates evaluated, 13 exhibited thread formation.

COMPARISON BETWEEN RAW SEMEN AND DILUTED SAMPLES (PRE-COOLING)

A significant decrease in oscillatory sperm motility and a significant increase in progressive sperm motility were observed in all pre-cooling diluted samples compared to raw semen ($P < 0.05$), with no differences in total sperm motility (Table 1). Sperm vigor was higher in samples diluted with extenders containing nanoparticles (NPs) compared to raw semen ($P < 0.05$) (Raw semen: 3.4 ± 0.7, AM-EY 0 h: 4.0 ± 0.6, AM-EY50 0 h: 4.3 ± 0.5, and AM-EY100 0 h: 4.3 ± 0.4; mean ± SD).

Regarding viability and acrosomal integrity, a significant decrease in live sperm with intact acrosomes and a significant increase in dead sperm with damaged acrosomes were observed in pre-cooling samples diluted with AM-EY and AM-EY50 compared to raw semen ($P < 0.05$). No significant differences were found among the pre-cooling samples in these parameters. The percentage of sperm with intact DNA was also similar (Table 1). Thread formation decreased in all diluted samples.

COMPARISON BETWEEN RAW SEMEN AND SAMPLES COOLED FOR 24 AND 48 H (INCUBATED AT 37°C FOR 15 MIN)

No significant differences were observed in sperm membrane functionality (HOS+) and sperm morphology between raw semen and samples cooled for 24 and 48 h with different ZnO-NPs concentrations (0, 50, and 100 µg/ml). The values for HOS+ sperm were (mean ± SD): 57.3 ± 13.2% (raw semen), 47.8 ± 16.3%

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(AM-EY 24 h), 50.4 ± 14.5% (AM-EY50 24 h), 52.0 ± 18.2% (AM-EY100 24 h), 41.4 ± 19.3% (AM-EY 48 h), 42.0 ± 19.8% (AM-EY50 48 h) and 41.7 ± 21.7% (AM-EY100 48 h). The values for normal sperm morphology were (mean ± SD): 46.0 ± 16.0% (raw semen), 49.8 ± 13.0% (AM-EY 24 h), 50.6 ± 12.2% (AM-EY50 24 h), 50.3 ± 11.6 (AM-EY100 24 h), 48.1 ± 13.2% (AM-EY 48 h), 50.0 ± 14.1% (AM-EY50 48 h) and 46.7 ± 14.0% (AM-EY100 48 h).

Live LPO-negative sperm decreased significantly in all cooled semen samples (AM-EY 24 h, AM-EY50 24 h, and AM-EY100 24 h) compared to raw semen ($P < 0.05$); however, no differences were observed among the cooled samples ($P > 0.05$) (Figs. 2 and 3). A positive correlation was observed between live LPO-negative sperm evaluated by BODIPY and live sperm with intact acrosomes assessed by FITC-PNA/PI ($r = 0.74$, $P = 0.003$).

Table 1. Percentages of the different sperm characteristics in raw llama semen and in pre-cooling samples diluted in AndroMed®-egg yolk (AM-EY) with different concentrations of ZnO nanoparticles (0, 50, and 100 µg/ml).

	OM (%)	PM (%)	CM (%)	TM (%)	LI (%)	DD (%)	DNA integrity (%)
Raw semen	34.5 ± 17.3 (32.5)[a]	5.3 ± 9.5 (0)[a]	0 (0)[a]	39.8 ± 15.4 (42.5)[a]	65.1 ± 15.5 (70.0)[a]	23.7 ± 14.8 (21.0)[a]	93.6 ± 3.4 (94.1)[a]
AM-EY 0 h	17.4 ± 13.1 (15.0)[b]	18.1 ± 14.7 (17.5)[b]	3.1 ± 5.1 (0)[a]	38.6 ± 13.6 (40.0)[a]	52.9 ± 15.7 (51.5)[b]	34.3 ± 13.6 (32.0)[b]	94.7 ± 2.6 (94.0)[a]
AM-EY50 0 h	17.8 ± 13.1 (11.0)[b]	21.0 ± 16.8 (22.5)[b]	3.0 ± 4.3 (0)[a]	41.8 ± 17.8 (39.5)[a]	51.4 ± 14.5 (52.0)[b]	35.4 ± 10.0 (36.0)[b]	94.0 ± 1.2 (93.7)[a]
AM-EY100 0 h	18.4 ± 13.3 (12.5)[b]	21.1 ± 15.1 (20.0)[b]	1.5 ± 3.9 (0)[a]	41.0 ± 14.2 (40.0)[a]	54.3 ± 16.2 (57.0)[ab]	31.6 ± 13.4 (30.0)[ab]	93.5 ± 3.7 (94.0)[a]

[a,b]Different letters indicate significant differences between the different samples in each variable analyzed ($P < 0.05$). OM = oscillatory sperm motility, PM = progressive sperm motility, CM = circular sperm motility, TM = total sperm motility, LI = live sperm with intact acrosomes, DD = dead sperm with damaged outer acrosomal membranes.

The values are mean ± SD. The median values are indicated in parentheses ($n = 6$, $r = 3$).

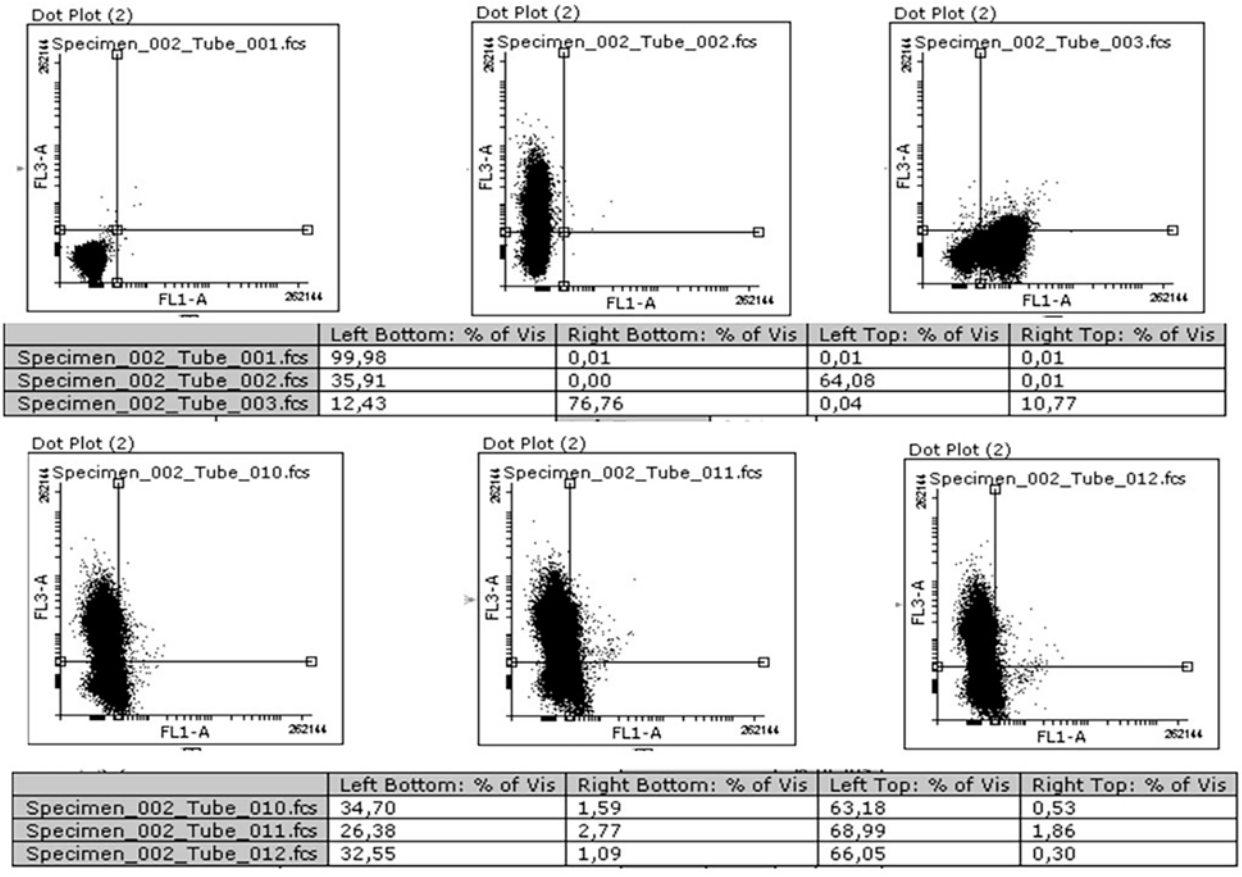


Fig. 2. Sperm membrane lipoperoxidation (LPO) evaluated using BODIPY C11-581/591 probe. BODIPY fluorescence was detected using the 520 nm filter (FL-1) and PI with the 610 nm filter (FL-3), both in the logarithmic scale. The figure show the samples and controls used: tube_001.fcs: autofluorescence sperm samples (without fluorochromes), tube_002.fcs: sperm samples stained with propidium iodide (PI), tube_003.fcs: sperm samples exposed to UV light and stained with BODIPY, tube_010.fcs: sperm samples cooled with AM-EY (stained with BODIPY and PI), tube_011.fcs: sperm samples cooled with AM-EY50 (stained with BODIPY and PI) and tube_012.fcs: sperm samples cooled with AM-EY100 (stained with BODIPY and PI).

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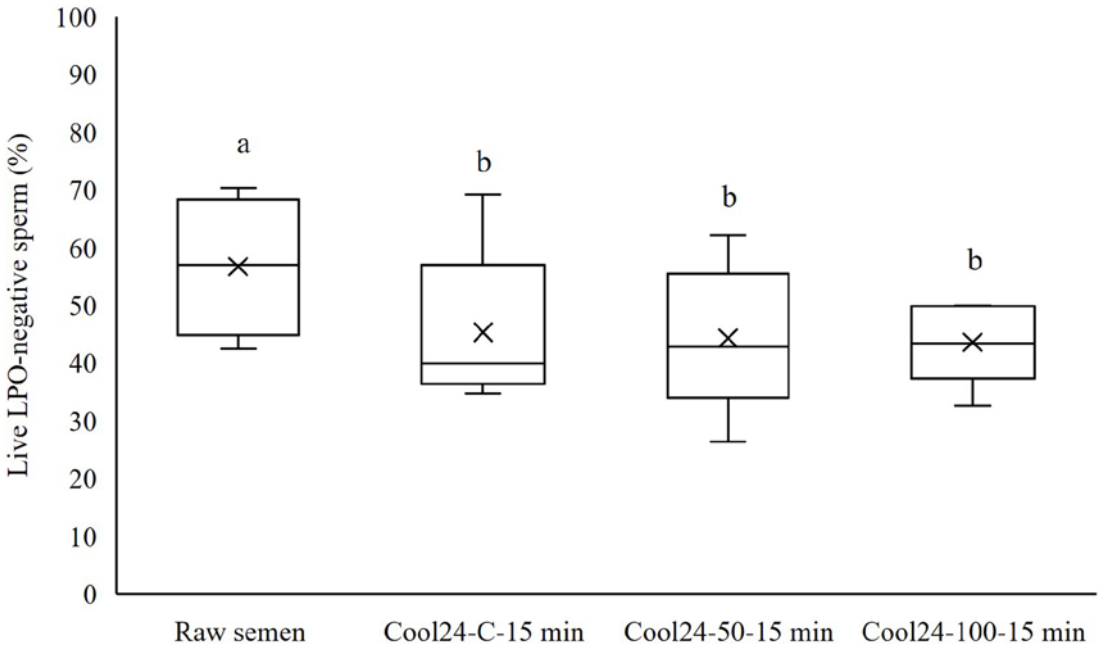


Fig. 3. Live LPO-negative sperm evaluated by BODIPY in raw llama semen and in cooled samples. The cross inside the box indicates the mean and the line inside the box indicates the median (n = 6, r = 3). (a, b) Different letters indicate significant differences between the different samples evaluated (P < 0.05). Cool24-C-15min: control sample cooled at 5°C for 24 h and incubated at 37°C for 15 min, Cool24-50-15min: sample cooled at 5°C for 24 h with 50 µg/ml of ZnO nanoparticles and incubated at 37°C for 15 min, Cool24-100-15min: sample cooled at 5°C for 24 h with 100 µg/ml of ZnO nanoparticles and incubated at 37°C for 15 min.

COMPARISON BETWEEN DILUTED SAMPLES (PRE-COOLING) AND SAMPLES COOLED FOR 24 AND 48 H (INCUBATED AT 37°C FOR 15 MIN)

The percentage of spermatozoa with oscillatory motility did not change after cooling at either 24 or 48 h of storage (P > 0.05). Progressive sperm motility (PM) was similar between samples cooled for 24 h and pre-cooling samples. However, a significant decrease in PM was observed in all cooled samples for 48 h compared to pre-cooling AM-EY50 and AM-EY100 (P < 0.05). Total sperm motility showed a decrease in cooled samples compared to all pre-cooling samples; this was particularly evident after 48 h of cooling (Table 2).

No significant differences were observed in sperm vigor among the cooled samples at different evaluation times (AM-EY 24 h: 3.4 ± 0.5, AM-EY50 24 h: 3.7 ± 0.7, AM-EY100 24 h: 3.7 ± 0.6, AM-EY 48 h: 3.1 ± 0.7, AM-EY50 48 h: 3.3 ± 0.6, and AM-EY100 48 h: 3.3 ± 0.9; mean ± SD).

Regarding viability and acrosomal integrity and intact DNA, no changes were observed among the cooled samples at any of the evaluated storage times (24 and 48 h at 5°C), nor compared to pre-cooling samples (Table 2).

COMPARISON OF DIFFERENT INCUBATION TIMES AT 37°C (15, 90, AND 180 MIN) IN SAMPLES COOLED FOR 24 H AT 5°C

Oscillatory sperm motility did not change in samples cooled for 24 h (with and without nanoparticles) throughout the 180 min of incubation. Progressive sperm motility of cooled samples incubated for 180 min decreased with regard to 15 min incubation, being significant in AM-EY50. Total sperm motility decreased after 180 min of incubation in the AM-EY and AM-EY50 samples compared to 15 min of incubation, whereas remained steady in AM-EY100 samples (Table 3).

No significant differences were observed in the FITC-PNA/PI patterns at different incubation times (15, 90, and 180 min) in the samples cooled for 24 h. However, there was a trend toward a decrease in LI and an increase in DD (P = 0.05) as the incubation time increased to 180 min in all cooled samples (Table 3).

The percentage of spermatozoa with functional membranes (HOS+) decreased significantly after 180 min compared to 15 min of incubation at 37°C in all samples cooled for 24 h. The percentage of HOS+ sperm in cooled samples with AM-EY (control) decreased after 180 min of incubation compared to all cooled samples that were incubated for just 15 min. In contrast, the cooled samples containing nanoparticles (NPs) after 180 min of incubation did not show a significant difference from those in the cooled AM-EY samples at 15 min (Fig. 4).

COMPARISON OF DIFFERENT INCUBATION TIMES AT 37°C (15, 90, AND 180 MIN) IN SAMPLES COOLED FOR 48 H AT 5°C

No differences were observed in all motility patterns, sperm vigor, and FITC-PNA/PI patterns as incubation time increased in samples cooled for 48 h, nor among the different samples with and without nanoparticles (Supplementary Material Table 4).

Regarding membrane functionality, no significant differences were found between the samples at the evaluated times (Fig. 5).

EVALUATION OF SPERM ULTRASTRUCTURE

Damage to the plasma membrane was observed in all cooled samples, both with and without ZnO-NPs, being more evident in the TEM evaluation than in the SEM analysis (Figs. 6–8). Sperm head membrane disruptions were observed by SEM (Fig. 7B and C), and irregular membrane swelling was noted by TEM at both the head (Fig. 8B and C) and tail region (Fig. 8F and G).

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Table 2. Percentages of the different sperm characteristics in pre-cooling samples diluted in AndroMed®-egg yolk (AM-EY) with different concentrations of ZnO nanoparticles (0, 50, and 100 µg/ml) and in the respective samples cooled at 5°C for 24 and 48 h.

	OM (%)	PM (%)	CM (%)	TM (%)	LI (%)	DD (%)	DNA intact (%)
AM-EY 0 h	17.4 ±13.1 (15.0)^{a}	18.1 ± 14.7 (17.5)^{ab}	3.1 ± 5.1 (0)^{ab}	38.6 ± 13.6 (40.0)^{ab}	52.9 ± 15.7 (51.5)^{a}	34.3 ± 13.6 (32.0)^{a}	94.7 ± 2.6 (94.0)^{a}
AM-EY50 0 h	17.8 ±13.1 (11.0)^{a}	21.0 ± 16.8 (22.5)^{a}	3.0 ± 4.3 (0)^{ab}	41.8 ± 17.8 (39.5)^{a}	51.4 ± 14.5 (52.0)^{a}	35.4 ± 10.0 (36.0)^{a}	94.0 ± 1.2 (93.7)^{a}
AM-EY100 0 h	18.4 ±13.3 (12.5)^{a}	21.1 ± 15.1 (20.0)^{a}	1.5 ± 3.9 (0)^{a}	41.0 ± 14.2 (40.0)^{a}	54.3 ± 16.2 (57.0)^{a}	31.6 ± 13.4 (30.0)^{a}	93.5 ± 3.7 (94.0)^{a}
AM-EY 24 h	12.0 ± 8.7 (10.0)^{a}	11.8 ± 11.2 (8.5)^{ab}	7.9 ± 8.0 (6.0)^{ab}	31.7 ± 13.1 (25.0)^{abc}	44.5 ± 16.2 (46.5)^{a}	42.9 ± 14.4 (42.0)^{a}	90.4 ± 6.6 (92.1)^{a}
AM-EY50 24 h	11.7 ± 8.2 (9.5)^{a}	12.6 ± 12.6 (10.0)^{ab}	9.9 ± 10.0 (10.0)^{b}	34.2 ± 15.5 (34.5)^{abc}	44.1 ± 16.5 (47.5)^{a}	45.4 ± 15.3 (44.5)^{a}	90.2 ± 4.2 (90.9)^{a}
AM-EY100 24 h	12.4 ± 9.2 (10.0)^{a}	10.6 ± 8.6 (10.0)^{ab}	8.1 ± 10.2 (5.0)^{ab}	31.1 ± 13.2 (29.0)^{abc}	43.0 ± 14.3 (46.5)^{a}	44.4 ± 10.5 (45.0)^{a}	93.1 ± 4.3 (93.0)^{a}
AM-EY 48 h	11.3 ± 8.8 (12.5)^{a}	5.0 ± 5.5 (3.0)^{b}	4.2 ± 6.5 (1.5)^{ab}	20.5 ± 7.6 (20.0)^{c}	40.5 ± 15.2 (41.5)^{a}	48.7 ± 15.8 (48.0)^{a}	91.0 ± 0.9 (91.0)^{a}
AM-EY50 48 h	9.8 ± 8.3 (9.5)^{a}	6.8 ± 7.3 (4.0)^{b}	6.5 ± 7.8 (4.5)^{ab}	23.1 ± 14.5 (20.0)^{bc}	40.8 ± 17.6 (45.5)^{a}	49.8 ± 17.2 (44.0)^{a}	91.3 ± 0.3 (91.3)^{a}
AM-YH100 48 h	8.3 ± 9.2 (6.0)^{a}	4.8 ± 5.6 (4.5)^{b}	6.7 ± 8.4 (5.0)^{ab}	19.8 ± 13.1 (20.0)^{c}	40.4 ± 15.7 (40.0)^{a}	50.3 ± 15.9 (52.5)^{a}	91.7 ± 1.9 (91.7)^{a}

^{a,b,c}Different letters indicate significant differences between the different samples in each variable analyzed ($P < 0.05$). OM = oscillatory sperm motility, PM = progressive sperm motility, CM = circular sperm motility, TM = total sperm motility, LI = live sperm with intact acrosomes, DD = dead sperm with damaged outer acrosomal membranes.

The values are mean ± SD. The median values are indicated in parentheses ($n = 6$, $r = 3$).

Table 3. Percentages of the different sperm characteristics in samples cooled for 24 h in AndroMed®-egg yolk (AM-EY) with different concentrations of ZnO nanoparticles (0, 50, and 100 µg/ml), evaluated at different incubation times at 37°C (15, 90, and 180 min).

	OM (%)	PM (%)	CM (%)	TM (%)	LI (%)	DD (%)
AM-EY 15 min	12.0 ± 8.7 (10.0)^{a}	11.8 ± 11.2 (8.5)^{ab}	7.9 ± 8.0 (6.0)^{ab}	31.7 ± 13.1 (25.0)^{ab}	44.5 ± 16.2 (46.5)^{a}	42.9 ± 14.4 (42.0)^{a}
AM-EY50 15 min	11.7 ± 8.2 (9.5)^{a}	12.6 ± 12.6 (10.0)^{a}	9.9 ± 10.0 (10.0)^{a}	34.2 ± 15.5 (34.5)^{a}	44.1 ± 16.5 (47.5)^{a}	45.4 ± 15.3 (44.5)^{a}
AM-EY100 15 min	12.4 ± 9.2 (10.0)^{a}	10.6 ± 8.6 (10.0)^{abc}	8.1 ± 10.2 (5.0)^{ab}	31.1 ± 13.2 (29.0)^{ab}	43.0 ± 14.3 (46.5)^{a}	44.4 ± 10.5 (45.0)^{a}
AM-EY 90 min	12.3 ± 5.8 (13.0)^{a}	10.3 ± 10.5 (9.0)^{abc}	5.6 ± 6.0 (5.0)^{ab}	28.2 ± 14.1 (29.5)^{abc}	43.0 ± 13.6 (44.0)^{a}	46.0 ± 9.6 (44.5)^{a}
AM-EY50 90 min	10.5 ± 6.9 (8.0)^{a}	9.9 ± 9.1 (9.0)^{abc}	6.6 ± 6.7 (5.0)^{ab}	27.0 ± 12.0 (26.0)^{abc}	42.7 ± 14.0 (44.0)^{a}	47.0 ± 11.9 (47.5)^{a}
AM-EY100 90 min	9.8 ± 7.3 (9.0)^{a}	8.4 ± 11.5 (2.5)^{abc}	6.4 ± 8.0 (5.0)^{ab}	24.6 ± 13.0 (23.5)^{abc}	43.1 ± 16.9 (43.5)^{a}	46.8 ± 16.4 (45.5)^{a}
AM-EY 180 min	7.6 ± 5.7 (5.0)^{a}	3.1 ± 4.6 (0)^{bc}	2.1 ± 2.9 (0)^{b}	12.8 ± 9.2 (12.5)^{c}	33.1 ± 17.1 (34.0)^{a}	58.2 ± 19.0 (54.0)^{a}
AM-EY50 180 min	7.5 ± 6.4 (6.0)^{a}	2.4 ± 4.3 (0)^{c}	2.5 ± 4.0 (0)^{ab}	12.4 ± 11.0 (10.0)^{c}	30.9 ± 14.7 (35.0)^{a}	59.7 ± 19.6 (58.0)^{a}
AM-EY100 180 min	8.6 ± 7.8 (7.5)^{a}	3.6 ± 7.6 (0)^{abc}	2.6 ± 4.1 (0)^{ab}	14.8 ± 12.8 (15.0)^{bc}	33.9 ± 18.9 (38.5)^{a}	58.6 ± 21.0 (53.0)^{a}

^{a,b,c}Different letters indicate significant differences between the different samples in each variable analyzed ($P < 0.05$). OM = oscillatory sperm motility, PM = progressive sperm motility, CM = circular sperm motility, TM = total sperm motility, LI = live sperm with intact acrosomes, DD = dead sperm with damaged outer acrosomal membranes.

The values are mean ± standard deviation. The median values are indicated in parentheses ($n = 6$, $r = 3$).

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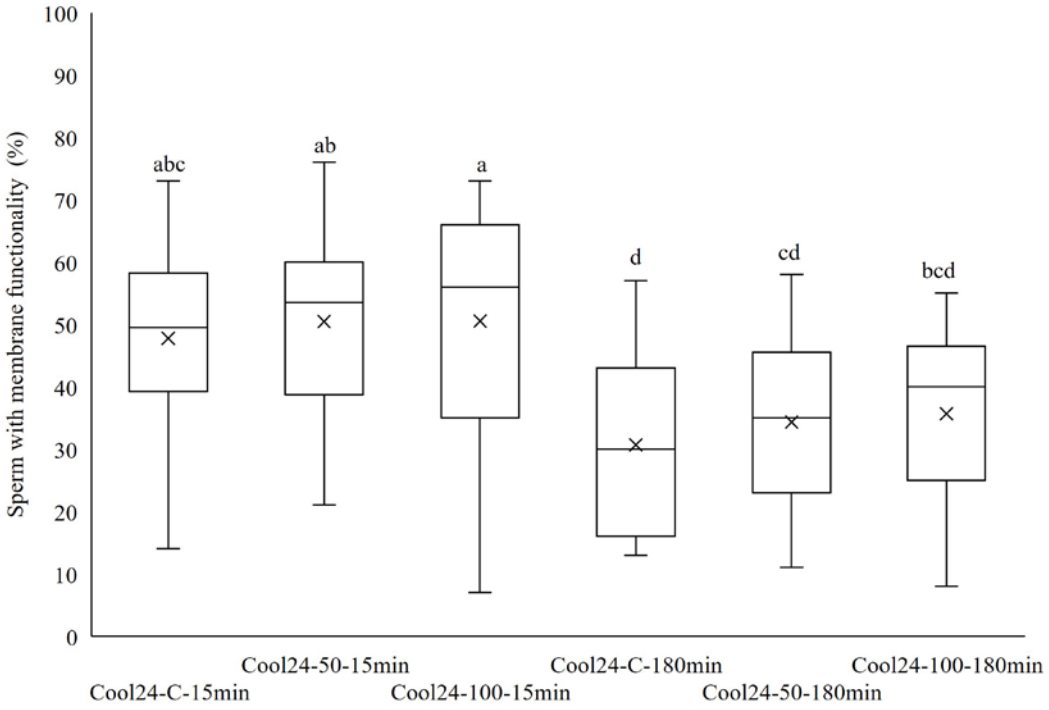


Fig. 4. Llama spermatozoa with membrane functionality (HOS+) in samples cooled for 24 h and evaluated at 15 and 180 min of incubation at 37°C. The cross inside the box indicates the mean and the line inside the box indicates the median (n = 6, r = 3). (a,b,c,d) Different letters indicate significant differences between the different samples and times evaluated ($P < 0.05$). Cool24-C-15 min: control sample cooled at 5°C for 24 h and incubated at 37°C for 15 min, Cool24-50-15 min: sample cooled at 5°C for 24 h with 50 µg/ml of ZnO nanoparticles and incubated at 37°C for 15 min, Cool24-100-15 min: sample cooled at 5°C for 24 h with 100 µg/ml of ZnO nanoparticles and incubated at 37°C for 15 min, Cool24-C-180 min: control sample cooled at 5°C for 24 h and incubated at 37°C for 180 min, Cool24-50-180 min: sample cooled at 5°C for 24 h with 50 µg/ml of ZnO nanoparticles and incubated at 37°C for 180 min and Cool24-100-180 min: sample cooled at 5°C for 24 h with 100 µg/ml of ZnO nanoparticles and incubated at 37°C for 180 min.

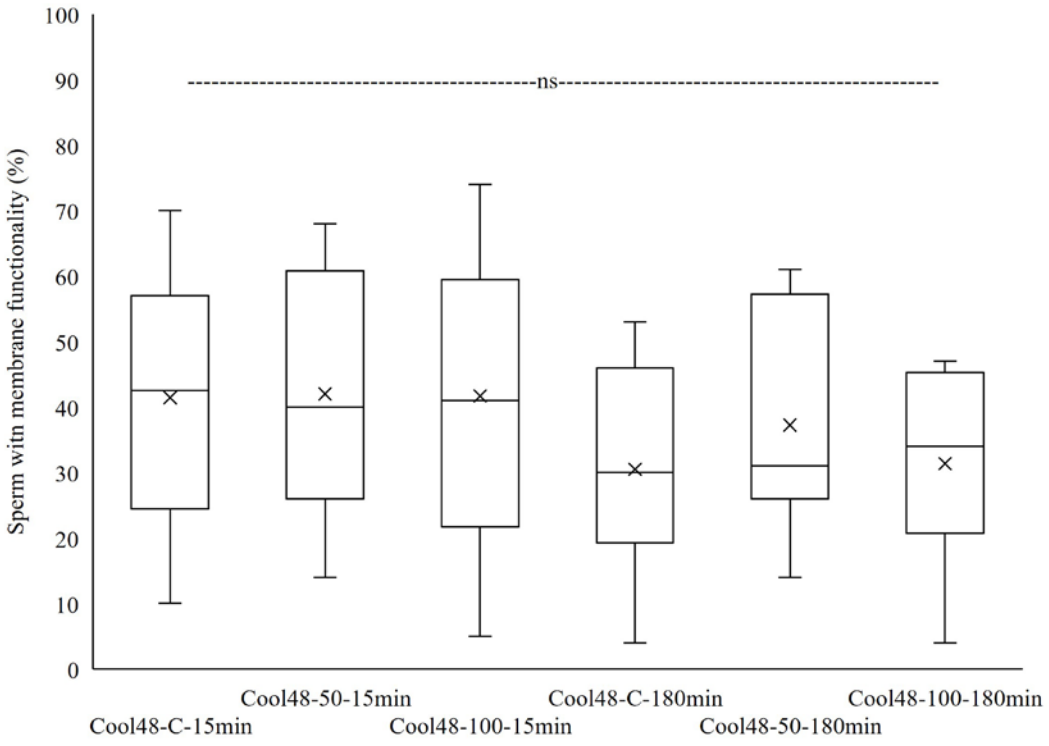


Fig. 5. Llama spermatozoa with membrane functionality (HOS+) in samples cooled for 48 h and evaluated at 15 and 180 min of incubation at 37°C. The cross inside the box indicates the mean and the line inside the box indicates the median (n = 6, r = 3). ns: no significant differences between treatments and evaluation times. Cool48-C-15 min: control sample cooled at 5°C for 48 h and incubated at 37°C for 15 min, Cool48-50-15 min: sample cooled at 5°C for 48 h with 50 µg/ml of ZnO nanoparticles and incubated at 37°C for 15 min, Cool48-100-15min: sample cooled at 5°C for 48 h with 100 µg/ml of ZnO nanoparticles and incubated at 37°C for 15 min, Cool48-C-180 min: control sample cooled at 5°C for 48 h and incubated at 37°C for 180 min, Cool48-50-180 min: sample cooled at 5°C for 48 h with 50 µg/ml of ZnO nanoparticles and incubated at 37°C for 180 min and Cool48-100-180 min: sample cooled at 5°C for 48 h with 100 µg/ml of OZn nanoparticles and incubated at 37°C for 180 min.

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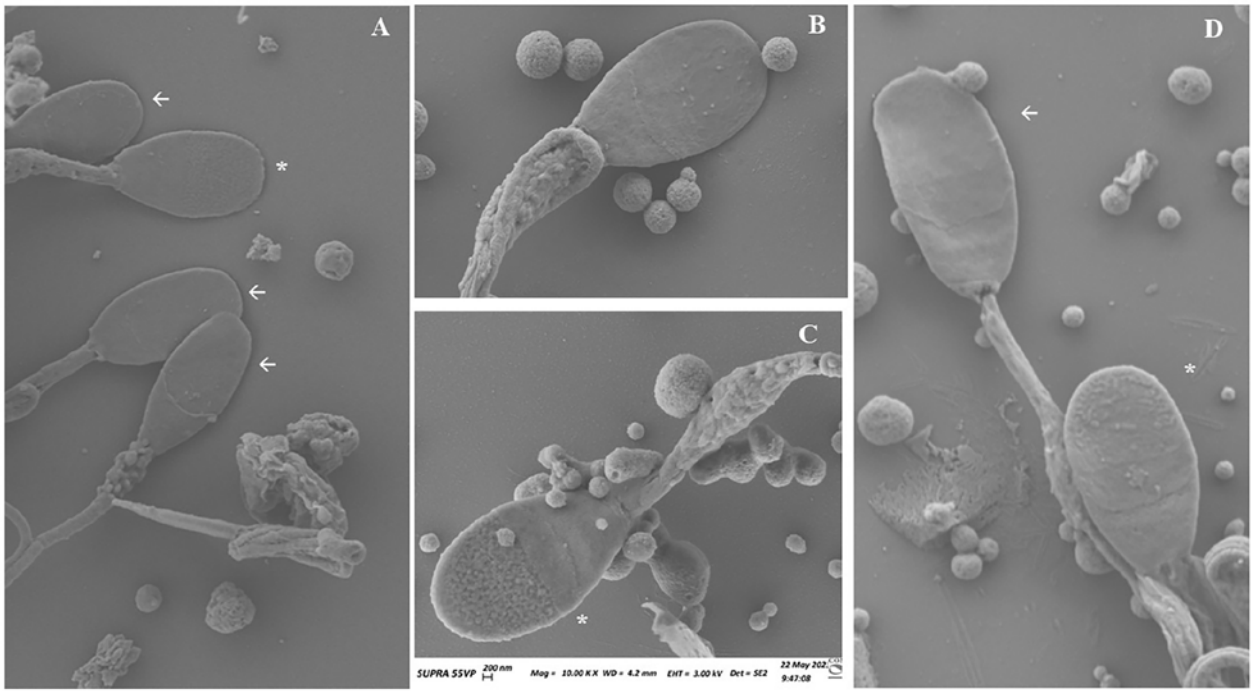


Fig. 6. Llama sperm ultrastructure evaluated by SEM (Scanning Electron Microscopy). A: acrosome reaction (asterisk) and intact acrosomes (arrows) in sperm from raw semen. B: incipient acrosome vesiculation in sperm cooled with AM-EY. C: advanced acrosomal reaction in sperm cooled with AM-EY100. D: acrosome reaction (asterisk) and intact acrosome (arrow) in sperm cooled with AM-EY50.

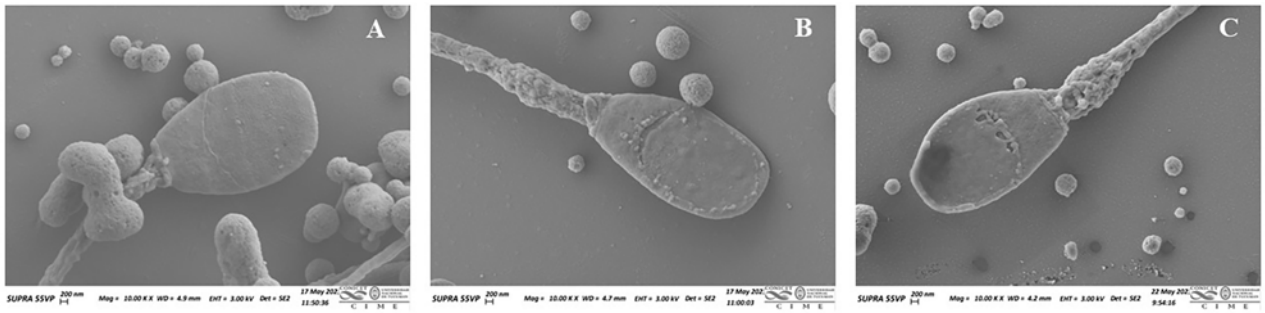


Fig. 7. Llama sperm ultrastructure evaluated by SEM (Scanning Electron Microscopy). A: incipient acrosomal reaction in sperm cooled with AM-EY50. B and C: membrane damage in sperm cooled with AM-EY and AM-EY100, respectively.

Unlike raw semen spermatozoa, where the plasma membrane surface was observed mostly smooth, with a clear distinction between the acrosomal and post-acrosomal regions (Fig. 6A), a high number of spermatozoa in different stages of the acrosomal reaction were observed in the cooled samples. These included incipient vesiculation of the acrosome (Fig. 6B), incipient acrosomal reaction (Fig. 7A), and advanced stages of acrosome reaction (Figs. 6A, C, D and 8B, D, E).

Discussion

This report is the first to use ZnO-NPs for cooling llama semen. The results of our study indicate that the addition of 50 and 100 µg/ml ZnO-NPs to the commercial extender AM-EY does not enhance the preservation of sperm characteristics from cooling damage, though, some favorable trends were observed in sperm membrane functionality during the incubation at 37°C.

There are still very scarce reports on the use of antioxidants for the cooling or freezing of SAC semen. Santiani Acosta *et al*. (2013) reported that adding superoxide dismutase (SOD) analogs such as Tempo (1 mM) or Tempol (1 mM) to the semen extender partially preserved sperm motility, membrane functionality, and DNA integrity in cryopreserved alpaca semen. However, analogous to our study, these authors found no beneficial effects on sperm viability or acrosomal status when including these antioxidants. Likewise, Gómez-Quispe *et al*. (2016) observed that the addition of 0.5 and 1 mM of Tempol preserved the progressive motility of sperm from the alpaca's deferent duct in samples cooled to 5°C for 2 h and in frozen-thawed samples, compared to cryopreserved samples without Tempol or those treated with higher concentrations (1.5 mM).

Regarding the use of NPs, studies in dromedaries have reported benefits from adding zinc oxide (50 µg/ml) and selenium (1 µg/ml) nanoparticles, such as improved sperm motility, viability, membrane function, and ultrastructural morphology in epididymal sperm stored at 5°C for 2 h or cryopreserved and thawed (Shahin *et al*., 2020). Furthermore, these same authors reported that adding zinc or selenium nanoparticles preserved motility and viability in

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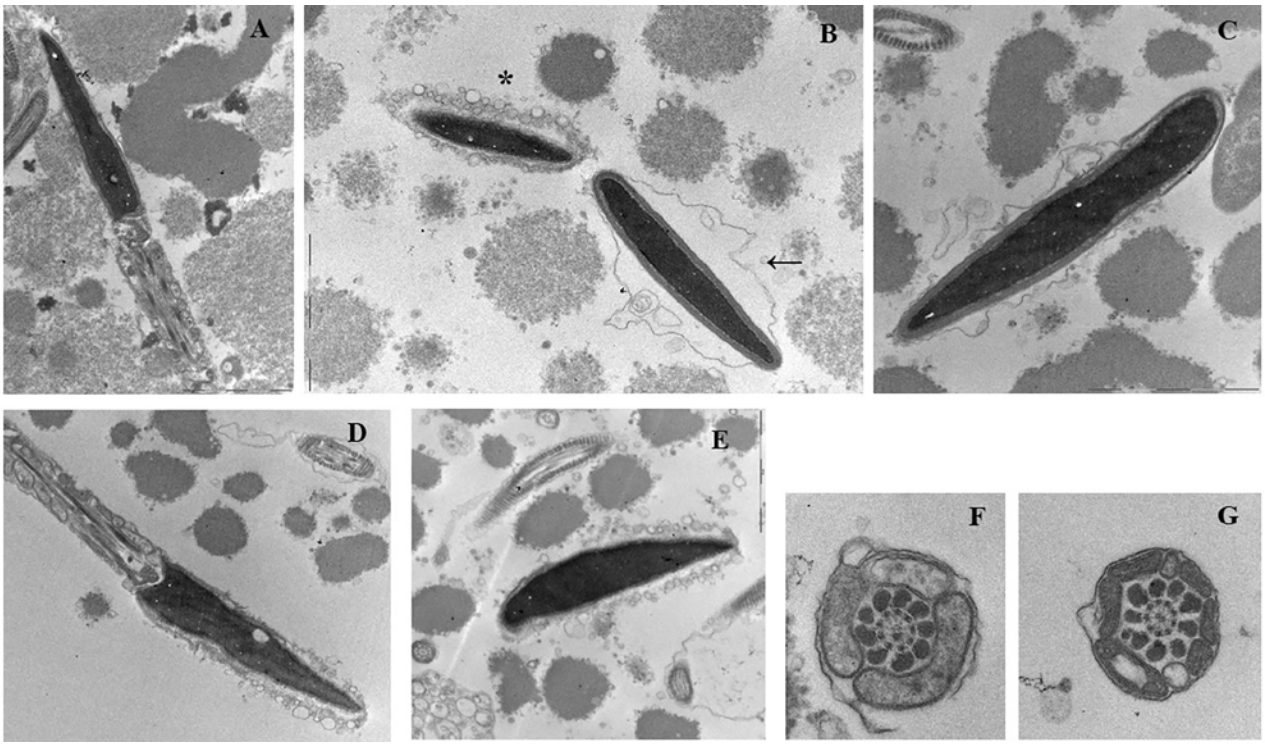


Fig. 8. Llama sperm ultrastructure evaluated by TEM (Transmission Electron Microscopy). A: normal head llama sperm in raw semen. B: acrosomal reaction (asterisk) and membrane damage (arrows) in sperm cooled with AM-EY. C: membrane damage in sperm cooled with AM-EY100. D: acrosome reaction in sperm cooled with AM-EY100. E: acrosome reaction in sperm cooled with AM-EY50. F and G: tail membrane damage in sperm cooled with AM-EY.

epididymal camel sperm stored at 4°C for up to 144 h (Shahin *et al*., 2021). Similarly, Heidari *et al*. (2019) cryopreserved ram semen with different ZnO-NPs concentrations and observed that 100 µg/ml yielded higher post-thaw total and progressive motility, along with a greater percentage of live sperm, compared to the control and higher concentrations of NPs (500 and 1000 µg/ml). They also noted a decline in sperm motility and viability at 1000 µg/ml. Likewise, in frozen-thawed goat semen, Abedin *et al*. (2023) reported higher percentages of sperm with intact plasma membranes and intact acrosomes in the ZnO-NPs (100 µg/ml) group compared to the frozen control group. While similar to our results, Isaac *et al*. (2017) cryopreserved ejaculates from normozoospermic men with 100 µg/ml of ZnO-NPs and found no significant differences in post-thaw total and progressive sperm motility compared to the control. In addition, in bull semen, Farhadi *et al*. (2022) observed that membrane integrity, functionality, and acrosome integrity were significantly higher in samples treated with 1 µg/ml ZnO-NPs compared to the control, although no differences in post-thaw total and progressive motility were noted. In the present study, we examined the effects of ZnO-NPs at concentrations of 50 and 100 µg/ml, which have proven effective in other species. We observed higher percentages of sperm with membrane function in cooled samples treated with nanoparticles. However, we found no significant differences or beneficial effects regarding sperm viability, acrosomal status, motility, DNA integrity, or morphological characteristics. Overall, evidence from various species indicates that antioxidant nanoparticles can enhance sperm motility during cooling or freezing processes, which contrasts with our findings in llama semen. One possible explanation is that some components of seminal plasma can bind to the sperm membrane, forming a barrier that may limit the effectiveness of certain antioxidants. Notably, camelid seminal plasma exhibits unique properties, such as high thread formation and structural viscosity, which could further interfere with nanoparticle activity. This perspective may help to explain the discrepancies between our results and those reported in previous research on dromedary epididymal sperm (Shahin *et al*., 2020, 2021) and alpaca sperm from the *vas deferens* (Gómez-Quispe *et al*., 2016). In both instances, the sperm cells had not interacted with seminal plasma, which underscores the importance of this factor in our findings.

Concerning the LPO sperm membrane evaluated by BODIPY, again no positive effect was observed on the percentage of live non-peroxidized sperm in the cooled samples in the presence of 50 and 100 µg/ml of ZnO-NPs. Heidari *et al*. (2019) reported significantly lower levels of malondialdehyde (MDA), a membrane lipid peroxidation biomarker, in frozen-thawed ram semen cryopreserved with 100 µg/ml ZnO-NPs. However, higher concentrations of ZnO-NPs (500 and 1000 µg/ml) resulted in MDA levels comparable to the control group without nanoparticles. In bulls, Farhadi *et al*. (2022) used lower concentrations of ZnO-NPs (0, 0.1, 1, and 10 µg/ml) for cryopreservation and found reduced MDA levels only with the 1 µg/ml concentration. These findings suggest that the optimal NPs concentration varies significantly among species.

When comparing refrigeration times (24 and 48 h), although the evaluated parameters did not differ between the two preservation periods, higher values for PM, TM, and LI were observed at 24 h. Notably, the addition of ZnO-NPs to the cooling extender did not provide a protective effect on these parameters, either at 24 or 48 h of preservation. Using the same extender as in our study (AM-EY), Bertuzzi *et al*. (2020) found no differences in TM, viable sperm, or the percentages of sperm with membrane functionality and intact acrosomes between 24 and 48 h of cooling in alpaca sperm collected from the deferent ducts. These findings suggest that the Andromed® commercial extender, supplemented with 20% egg yolk, could serve as a feasible alternative for cooling llama semen and alpaca sperm obtained from deviated deferent ducts. This extender effectively preserves all sperm parameters for up to 48 h and, being a commercial product, is convenient for farm producers as it requires minimal equipment and supplies for preparation.

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When evaluating the effect of ZnO-NPs on refrigerated semen incubated at 37°C for 180 min, no protective effect of the NPs was observed on the evaluated parameters over time, except for membrane functionality. The highest values for this parameter were recorded at 3 h of incubation in the samples refrigerated with NPs. Similarly, Isaac *et al*. (2017) found no significant differences in total and progressive human sperm motility after 24 h of incubation at 37°C between the control group and the ZnO-NPs groups. However, the highest sperm motility values were observed in samples treated with nanoparticles at a concentration of 100 µg/ml (Isaac *et al*., 2017). In addition, the significant decrease in membrane functionality (HOS+) observed in our study during incubation, especially after 180 min at 37°C, highlights the impact of refrigeration on the thermal resistance of llama spermatozoa. Interestingly, this reduction in membrane integrity and functionality was not mirrored by viability assessments (FITC-PNA/PI), which did not show a marked decline over the same period. Similar results were observed in frozen/thawed llama sperm incubated at 37°C for 180 min (Fumuso *et al*. 2020). This suggests that the HOS test is more sensitive in detecting subtle alterations in membrane functionality that may not be evident through viability measurements, providing complementary insights into sperm quality. These findings may partially explain the limited success rates in artificial insemination programs using cooled semen, as refrigeration compromises sperm thermal resistance. Even though ZnO-NPs did not significantly improve thermal resistance during prolonged incubation, this strengthens the need to explore alternative antioxidants or optimize nanoparticle concentrations to enhance their protective effects. Another important aspect to consider is incubating cooled samples at 37°C without the presence of antioxidants (by washing them) to evaluate the potential resistance of sperm to the female genital tract. In this environment, many components of the protective solutions are eliminated through dissolution in female fluids. However, in SACs, spermatozoa are highly sensitive to centrifugation procedures, and washing steps often lead to a significant loss of sperm quality. This limitation reflects one of the aforementioned challenges in applying assisted reproductive technologies in these species.

While ZnO-NPs may have potential benefits for preserving llama semen, the concentrations tested in this study might not be sufficient to produce significant improvements in the sperm characteristics assessed. This points toward the need to explore other concentrations (lower and/or higher) than those used here, as well as alternative nanoparticles or other types of antioxidants. In this context, increasing the concentration of ZnO-NPs could surpass the threshold required to achieve significant effects on semen quality. Further research should aim to identify the concentration that optimally balances efficacy and safety. In addition, the unique characteristics of llama semen may influence its response to the antioxidants used in this study. The composition of the sperm membrane, particularly its proportion of polyunsaturated fatty acids, could be a key factor contributing to its vulnerability to oxidative stress during cooling and freezing. A more detailed analysis of the lipid composition of this membrane would provide valuable insights for optimizing the use of antioxidants in preservation protocols. Furthermore, unidentified biochemical factors in llama spermatozoa or seminal plasma may influence their interaction with antioxidants. Molecules such as proteins or peptides present in seminal plasma, could play a role in modulating the antioxidant response, thereby affecting the efficacy of nanoparticles and other applied antioxidants. Since the mechanisms of damage and protection in llama semen may differ from those in other species, it is essential to conduct more in-depth studies on the composition of the sperm membrane and the biochemical environment of seminal plasma. Gaining insight into these factors could help clarify the role of antioxidants in semen cryopreservation. Moreover, the possibility of a chemical incompatibility between components of the extender – particularly the high concentration of egg yolk – and ZnO-NPs cannot be ruled out, as such interactions could diminish the protective effects of the nanoparticles on sperm quality. Future studies should explore the use of alternative extenders specifically tested and optimized for camelid species, as well as varying percentages of egg yolk, since they may offer better compatibility and enhance the efficacy of antioxidant additives, such as nanoparticles, during semen preservation.

Regarding the ultrastructural evaluation of refrigerated sperm, the results indicate that using ZnO-NPs at 50 and 100 µg/ml concentrations does not effectively preserve sperm membranes after refrigeration at 5°C for 24 hours. Similarly, Zampini *et al*. (2020) reported that SEM and TEM analyses revealed cryopreservation-induced ultrastructural damage in llama sperm, which began during the cooling phase and aggravated during freezing. In contrast, Shahin *et al*. (2020) used different antioxidants (vitamin C, vitamin E, zinc sulfate, ZnO-NPs, and Se-NPs) to freeze dromedary epididymal sperm and observed a significant increase in the percentage of intact plasma membranes in the nano-selenium group compared to the control and zinc sulfate-supplemented groups. Additionally, they reported variability in the occurrence of abnormal ultra-structures (e.g., swollen and slightly swollen plasma membranes) across experimental groups, with a significantly higher prevalence of swollen membranes in the control group (without antioxidants) paralleled to the other groups with antioxidants. It is worth noting, however, that Shahin *et al*. (2020) relied exclusively on SEM for sample evaluation. In our study, both SEM and TEM were used, with TEM providing higher resolution and allowing for a more detailed observation of membrane damage.

Finally, this study reveals that refrigeration induces significant damage to the membranes of llama spermatozoa, which cannot be detected using routine laboratory techniques. This finding also highlights the high susceptibility of llama spermatozoa not only to the freezing process but also to refrigeration, which could explain the low reproductive success rates reported in artificial insemination, limiting the potential benefits of semen cooling.

In conclusion, the addition of zinc oxide nanoparticles to the AndroMed®-egg yolk extender at concentrations of 50 or 100 µg/ml does not provide comprehensive protection for sperm characteristics against damage induced by the cooling process in llama semen. Further evaluation using other concentrations may be necessary to determine the optimal dose of ZnO-NPs for cooling llama semen. Assessing other types of nanoparticles or antioxidant agents would also be beneficial. The structural damage observed through electron microscopy further highlights the high sensitivity of llama spermatozoa not only to freezing but also to cooling. Developing effective cooling protocols for llama semen is essential to optimize preservation methods, enhance the feasibility of artificial insemination programs, and improve reproductive efficiency and genetic management in this species.

CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

ETHICS STATEMENT

All necessary ethical approval has been received for the research (Protocol 2024/15, University of Buenos Aires).

AUTHOR CONTRIBUTIONS

dFA carried out the study and wrote the manuscript. BML assisted with sample collection and critically revised and corrected the manuscript. ASA and ZR assisted with the preparation and interpretation of the electron microscopy samples, critically revised the manuscript and discussed the results. CMI designed the study, collaborated in data analysis, interpreted electron microscopy images, and critically revised and corrected the manuscript.

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FUNDING STATEMENT

This research was supported by grants from the University of Buenos Aires (UBACyT 20020220300139BA) and from the Agencia Nacional de Promoción Científica y Tecnológica (PICT 2020-01478 and PICT-2021-I-A-00265). The authors would like to thank OneLab, Argentina for donating laboratory materials such as pipet tips, Eppendorf, slides and coverslip.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPLEMENTARY MATERIAL

The supplementary material is available in the online version of this article.

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