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Novel approaches to predict and improve sperm function during semen storage

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Novel approaches to predict and improve sperm function during semen storage

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Editorial: Novel approaches to predict and improve sperm function during semen storage

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Editorial on the Research Topic
Novel approaches to predict and improve sperm function during semen storage

Artificial insemination (AI) is the most popular assisted reproductive technology (ART) applied to domestic animals thanks to the advantages that it provides compared to natural mating (1). In addition, ARTs represent a powerful tool in conservation breeding programs to save wild endangered species from extinction (2). Because sperm quality is critical for ARTs success, different approaches like semen refrigeration (liquid or encapsulated) and cryopreservation are being employed for preserving sperm fertilizing capacity (3–5). Therefore, methods for predicting and assessing sperm function are of great relevance to ensure optimal fertilization outcomes, which may have important economic and ecological implications. The articles published in this Research Topic address different perspectives related to sperm analysis and preservation across different animal species.

A preliminary assessment of male reproductive ability in domestic animals is usually carried out by the evaluation of some anatomical characteristics (e.g., testes size) and health status (6). For AI purposes, basic semen analyses should be conducted to filter out low quality seminal samples such as those with reduced sperm motility and concentration. The prediction of sperm quality before semen collection can lead to early detection of superior sires and help to establish their optimal semen collection regime. In rams, the use of testicular ultrasonography (Montes-Garrido et al.) is a non-invasive and predictive tool for estimating variations in the sperm quality of sires subjected to different frequencies of semen collection. Semen, besides spermatozoa, also consists of seminal plasma (SP) that not only acts as a transport medium for sperm cells within the female reproductive tract but also influences sperm function and offspring development (7, 8). Recently, the extracellular vesicles (EVs) present in the SP have gained special attention (9) as they have been linked to sperm function (10). Thus, the characterization and identification of the miRNA expression profiles in the SP-EVs isolated from fertile and sub-fertile males could serve as fertility biomarkers (11). Moreover, the importance of EVs does not lie solely in their characterization, but also in their application in ARTs. For this reason, it is important to know if the cryopreservation process as well as the addition of extenders, with animal or

vegetal lipid content, could interfere in the correct profiling of these EVs for being used as biomarkers of sperm quality and fertility (Capra et al.).

Despite the advances reached during the last decades, sperm damage induced by semen storage still represents a common and almost unavoidable side effect of semen handling and preservation procedures. Genetics and ejaculate traits have been shown to be implied in the sperm ability to withstand the preservation protocols (12, 13), but there are still gaps to fill to identify the causes of a poor sperm tolerance to semen storage. For instance, it has been reported that the differences observed in sperm cryotolerance in goats may be related to certain amino acids and metabolic intermediates present in the SP, which can therefore be used as potential biomarkers of sperm freezability in this species (Xu et al.).

Yet, several procedures (e.g., centrifugation, dilution, addition of cryoprotectants) and factors (e.g., osmotic and thermal changes) that occur before, during, and after sperm storage can induce a state of oxidative stress that finally impairs the sperm function (14). For this reason, the characterization and localization of reactive oxygen species (ROS) by using different fluorescent probes are crucial to study their effects on sperm function (Palacin-Martinez et al.). A plethora of antioxidants have been developed to scavenge specific ROS such as those produced in the mitochondria. The mito-TEMPO, a mitochondria-targeted antioxidant, has been successfully tested in tomcat (Ali Hassan et al.) and bull (Elkhawagah et al.) spermatozoa during the cryopreservation process at a concentration ranging from 10 to 55 μM. While in the tomcat spermatozoa this antioxidant preserves the acrosome structure, in bull it also improves sperm kinetics, organelles and DNA integrity, cleavage and blastocyst formation rates. Thanks to their antioxidant and antimicrobial properties, natural compounds also represent a promising supplement for semen extenders that can be economically and environmentally sustainable (15, 16). Thus, several natural-based products [i.e., alpha-lipoic acid (Sun et al.), myo-inositol (Jawad et al.), phosphorus/vitamin B12 (Suwimonteerabutr et al.), and selenium (Paul et al.)] have been used as alternative additives in the preservation of rooster (Suwimonteerabutr et al.), ram (Sun et al.), and boar (Jawad et al.; Paul et al.) semen with positive effects on sperm function (e.g., decreased ROS and lipid peroxidation levels during semen preservation) and higher pregnancy rates. Once the sperm is deposited into the female genital tract, either by natural mating or AI, neutrophil extracellular traps (NETs), triggered by spermatozoa, are recruited into the female reproductive tract often causing reduced sperm function and fertilizing ability (17, 18). The synthetic glucocorticoid methylprednisolone (MPS) has shown promising results inhibiting the adverse effects of lipopolysaccharide-induced polymorphonuclear neutrophils on boar spermatozoa (Li et al.). Additionally, MPS supplementation also exerts positive effect on sperm function during liquid semen preservation at 17°C and acts as a ROS scavenger.

Even though the cryopreservation is considered the gold standard for long-term sperm storage, the use of liquid nitrogen that is required for this purpose shows a considerable carbon footprint (manufacture and transportation) and is also associated with large amount of waste. Alternatively, the freeze-drying is an interesting method for long-term sperm storage at room temperature and at 4°C. Despite freeze-dried spermatozoa are motionless and dead (conventional sense), their nuclei can support normal embryonic development after being injected into oocytes (19). In the present issue, a technique known as vacuum-drying encapsulation (VDE), which was originally developed for nucleic acid conservation in anhydrous state, has been successfully adapted to ram spermatozoa. Compared to canonical lyophilization, VDE better preserves the structural and fertilization potential of ram spermatozoa stored for 2 years both at room temperature and at 4°C (Palazzese et al.).

Alongside the advancements in ARTs highlighted in this Research Topic, improvements in sperm function analyses are essential for predicting fertility outcomes. Under this perspective, multi-parametric flow cytometry analyses have become an interesting tool for determining sperm quality parameters during the cryopreservation process, but they miss other important parameters such sperm morphology. The use of hybrid imaging flow cytometry, which combines flow cytometry and light microscopy features, may help to standardize sperm quality assessment protocols (Umirbaeva et al.).

Taking together, this Research Topic provides a series of articles dealing with novel techniques and analyses for predicting sperm quality and improving semen preservation protocols with the ultimate goal of maximizing ARTs' efficiency.

Author contributions

JR-S: Writing – original draft. FG-V: Writing – review & editing. FT: Writing – review & editing. EP: Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Application of ultrasound technique to evaluate the testicular function and its correlation to the sperm quality after different collection frequency in rams

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The frequency of semen collection is a crucial factor to consider in the rams performance inside breeding centers workout. To evaluate this factor, ram Breeding Soundness Evaluation could include sperm quality evaluation and new predictive and non-invasive tools such as ultrasound technique. In this work, an advanced ultrasonography technology, analyzing the testicular volume, echotexture, and vascular function, was used in three different frequencies of semen collection (abstinence frequency, AF; standard frequency, SF; and intensive frequency, IF). Semen samples were cooled (15°C, 6 h) and evaluated in terms of production, motility, viability, apoptosis, and content of reactive oxygen species. Correlation coefficients were calculated between ultrasonography measurements of echotexture and blood flow and sperm quality parameters. Our results showed an increase in the testicular echotexture when the frequency of semen collection was intensified. Doppler parameters (PSV, RI, PI, TABF) increased ($P \leq 0.05$) when the frequency of semen collection was intensified. The sperm motility and functionality decreased in the samples of IF ($P \leq 0.05$), evidencing the frequency of semen collection's influence. Moreover, moderate positive correlations were established among echotexture and different Doppler parameters with motility parameters in SF. Furthermore, the influence of abstinence days on AI success was analyzed in a field assay. The highest fertility rates were obtained when males had two to five abstinence days. To conclude, frequency of semen collection could be influenced in terms of semen quantity and sperm quality,

showing changes in parenchyma echotexture and testicular vascularization. The standard semen collection frequency was the most adequate option. In addition, ultrasonography may be a predictive tool for estimating variations in the sperm quality of donor rams subjected to different frequencies of semen collection in reproduction centers.

KEYWORDS

Doppler, fertility, ovine, semen collection frequency, sperm quality, ultrasonography

Introduction

Reproductive performance is the most important parameter affecting flock profitability (1), and the reproductive capacity of the rams plays a key role (2). MacLaren (3) suggests that 50% of the reproductive potential of a flock is provided by the ram (3). Testis evaluations are performed to assess the overall potential capacity of rams to serve and impregnate ewes, also known as the Breeding Soundness Evaluation (BSE) (2). A BSE may include anatomical and structural examinations and assessment for health status, body condition score (BCS), testicular measurements, sperm quality, and libido (4). In this context, sexual behavior and semen characteristics are the main parameters limiting male reproductive efficiency, and both of them are greatly influenced by the frequency of semen collection. Variations in ejaculation frequency induce changes in the sperm maturation process, sperm functional characteristics, ionic composition, and enzymatic activity of the seminal plasma (5, 6). In a previous study, Ollero et al. (5) documented that different periods of abstinence could affect sperm quality in terms of viability, motility, and acrosome integrity in the ovine species. To our knowledge, there are no studies in a large number of rams assessing the effect of semen collection frequency in male reproductive capacity, semen production, and quality employing innovative and predictive techniques.

Traditional methods, such as measurement of scrotal circumference and libido or palpation and manipulation of the genital organs, have been used to evaluate the potential reproductive capacity of rams (3, 7, 8). In recent years, several new tools, including ultrasonography, have been used to predict variations in semen characteristics and the ram's reproductive capacity in reproduction centers and flocks (9, 10). Specifically, ultrasonography is a non-invasive, non-ionizing, and non-damaging technique and an indispensable tool in reproductive clinics that provides real-time and sequential information on male reproductive performance (11, 12). The B-mode ultrasound has been used in different domestic animals species as a valid tool to estimate the testicular volume (13–15), estimate the parenchyma echotexture (16–18), and identify uncertain clinical findings, such as early stages of macroscopic pathological processes or monitoring changes in lesions (12). Color and Pulse Doppler ultrasonography has been employed to characterize and quantify testes' blood flow in different species, such as stallions (19) and dogs (20). This technology has also been used to evaluate scrotal disorders in dogs (14) and camelids (21) and correlate them with sperm quality. Several studies have assessed using B-mode ultrasound in physiological and pathological conditions (12, 22–24), and the relationship between the puberty and the changes in the echogenicity of the parenchyma (25–28) in ovine andrology. In addition, several studies on the use of Doppler ultrasonography for the evaluation of testicular blood flow in rams are available (9, 10, 26, 29) that consider the influence of this parameter in testicular function. Testicular blood flow is the main route through which all the required nutrients, oxygen, regulatory hormones, and secretory products are regulated and exchanged to and from the testes (30). In this respect, different studies have evidenced an association between testicular blood flow and sperm quality in several species such as humans (31), stallions (13, 32), or rams (9, 10), and used Doppler parameters to diagnose fertility rates in camelids (21) or dogs (14). Because of this, we hypothesize that semen collection frequency could be a crucial factor causing testicular changes in those parameters detected by ultrasound and in sperm quality.

Considering the importance of rams management in terms of performance within a breeding center, the first objective of this study is to investigate the effects of the frequency of semen collection on male reproductive performance including testicular function, and semen quantity and quality employing a multiparametric approach based on testicular morphometry, echotexture and vascularization, blood testosterone level, libido, and sperm motility and functionality parameters. Secondly, the study aims to approach the possible association between sperm quality parameters and ultrasonography measurements in the different semen collection frequencies. As a third aim, we study the direct consequences of different semen extraction frequencies in terms of sperm quality and fertility in a field assay.

Materials and methods

Animals

Twent-five sexually mature (age range 2–7 years) Assaf rams were used during the breeding season in the current study. All the rams were previously examined, and they did not have any disease. Animals were housed grouped (five animals per each group) in closed pens with access to an open area at the Animal Selection and Reproduction Center of the Junta de Castilla y León (CENSYRA) (Villaquilambre, León, Spain), where they were fed on a standard balanced diet. The current study was performed in accordance with the Guidelines of the European Union Council (2010/63/EU), following Spanish regulations (RD/1201/2005, abrogated by RD/53/2013) for the use of laboratory animals. All the experiments were approved by the Institutional Animal Care and Use Committee at the University of León (ÉTICA-ULE-013-2018).

Experimental design

Experiment 1: Evaluation of different semen collection frequencies

Sexually mature Assaf rams were used during the breeding season. All rams were enrolled in the abstinence semen collection frequency (AF) where the males were sexually rested and semen was not collected for 1 month. At the end of this period, testicular volume, testicular echotexture, and Doppler parameters were assessed. After the animal's evaluation, two consecutive ejaculates per ram were collected and mixed, measuring the following parameters: ejaculate volume, sperm concentration, and total sperm production. Ejaculates were analyzed including motility and sperm physiology parameters (detailed in section "Sperm evaluation"). Then, all rams were enrolled in the standard semen collection frequency (SF) for 1 month: two consecutive ejaculates per day/two collection days per week. At the end of this period, all ultrasonographic measurements were repeated, and, after the animal's evaluation, two consecutive ejaculates per ram were collected, mixed, and analyzed. To conclude this experiment, the 25 rams were enrolled in the intensive semen collection frequency (IF): two consecutive ejaculates per day/five collection days per week (Monday–Friday). Again, 1 month later, all ultrasonographic measurements were repeated. In this scenario, after 2 days of abstinence by the weekend, two consecutive ejaculates per ram were collected and analyzed on Monday (IFM) and, after five consecutive days of semen collection, two consecutive ejaculates per ram were collected and analyzed on Friday (IFF). The experimental design is shown in Figure 1.

Experiment 2: Ram's performance and sperm yield: A field assay

In this part of the experiment, we tried to extrapolate our findings to field assay. In a reproduction center simulation, ejaculates obtained from our experimental rams (25 males) in experiment 1 were classified following the reproduction center criteria in valid (ejaculate volume $>$ 0.5 ml, mass motility $>$ 3, sperm concentration $>$ 3,000 $\times$ 10^6 sperm/ml) and non-valid ejaculates (some of the criteria below the minimum value) as previously described Neila-Montero et al. (33). Then, sperm quality, including motility and cytometry, was assessed in both experimental groups.

For the fertility trial, the experimental groups were classified by male abstinence period before semen collection for insemination: the fertility standard interval (SI), when seminal doses were obtained from males with two–five abstinence days before semen collection for AI; the fertility high interval (HI), when seminal doses were obtained from males with at least 10 abstinence days before semen collection for AI; the fertility low interval (LI), when seminal doses were obtained from males with semen collection the previous day for AI; the annual fertility (AN), annual average fertility from the National Association of Breeders of the Assaf Sheep Breed (ASSAF.E). A descriptive assay was performed to assess the effect of the abstinence period of semen collection on fertility AI.

For this purpose, 357 seminal doses (400 $\times$ 10^6 sperm/dose) from 10 mature Assaf rams housed in the Sheep and Goat Selection and Genetic Improvement Center of Castilla y León (Ovigén; Villalazán, Junta de Castilla y León, Spain) were used during the breeding season. Ejaculates were diluted to 1,600 $\times$ 10^6 sperm/ml with INRA 96 and cooled using a rate of -0.5°C/min from 30°C down to 15°C using a programmable water bath (CC-K8, Huber, Germany). A total of 357 Assaf adult ewes from six commercial farms were inseminated 6–8 h after semen collection. Females were subjected to treatment for estrus induction and synchronization using intravaginal sponges with 20 mg fluorogestone acetate (Chronogest®, MSD, Kenilworth, NJ, USA) over 14 days. The sponges were removed, and ewes were treated with 500 IU of eCG intramuscular (Folligon®, MSD, Bogotá, Colombia). Cervical artificial inseminations (54 $\pm$ 1 h post sponges removal) were performed by two experienced veterinarians of ASSAF.E. Reproductive success was evaluated in terms of fertility (lambing ewes/inseminated ewes (%) according to the births registered at 137–154 days post-insemination).

Previous clinical examination

Before being included in the study, every single male underwent a general clinical examination, visual inspection of the scrotum, and testicular palpation for the evaluation of consistency, symmetry, mobility, and sensitivity of testes.

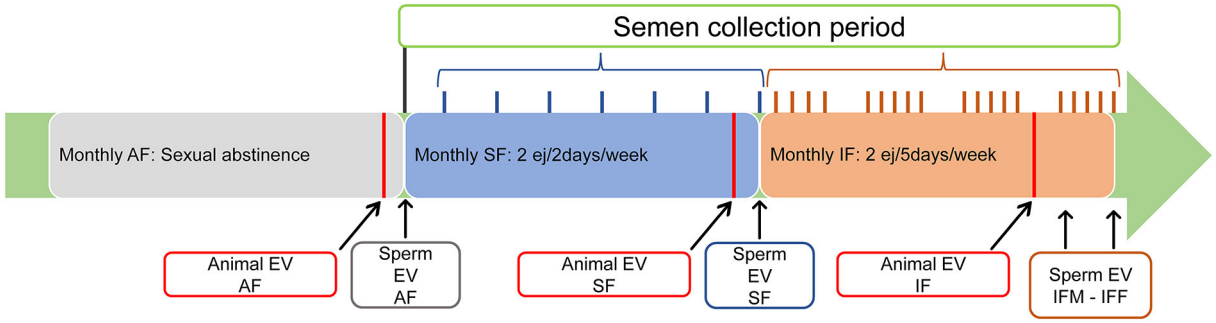

FIGURE 1
Experimental design. All three semen collection frequencies were extended for 1 month. Animal evaluation (Animal EV): clinical examination (inspection and palpation of external genitalia, libido, and blood collection) and ultrasonography evaluation (testicular volume, echotexture, and blood flow) were carried out at least 24 h before experimental semen collection). Sperm evaluation (Sperm EV): sperm production, motility, and physiology were performed at the end of the evaluated semen collection frequency. AF, abstinence semen collection frequency; SF, standard semen collection frequency; IFM, intensive semen collection frequency on Monday; IFF, intensive semen collection frequency on Friday.

Epididymis and pampiniform plexus were also palped in order to ensure that no observable gross pathology was resent on the external genitalia. Then, the rams were mildly sedated with xylazine (0.05 mg/kg), administered intramuscularly, and restrained in the standing position using a containment rack. The wool on both sides of the scrotum was shaved.

Testosterone levels

After all clinical measures, blood samples were collected into a vacutainer tube without anticoagulant from the jugular vein. The samples were refrigerated at 5°C, and the blood serum was collected and stored at −20°C until assayed. A commercial ELISA kit using the Immulite 2000 XPi Immunoassay System (Siemens, Eschborn, Germany) was used to determine the serum testosterone. According to the manufacturer's instructions, the sensitivity was 0.15 ng/ml, and the intra- and inter-assay coefficients of variation were 5.1 and 7.2% when the average samples were 9.91 ng/ml.

Libido evaluation

Libido was subjectively categorized (zero to 10 score). We calculated time spent from contact with a female decoy to ejaculation in both daily semen collections. Time intervals were used to qualify the rams. If ejaculation occurred in 1 min or less, the male obtained the maximum score (10). If ejaculation occurred in 1–2 min, the male obtained a score of nine. If ejaculation occurred in 2–3 min, the male obtained a score of eight, etc. When ejaculation occurred after more than 10 min, the male obtained a score of zero. The average score of the two ejaculations was calculated.

Testicular volume

All the ultrasonographic measurements were carried out by the same technician. All examinations were performed using a real-time ultrasound scanner, EXAPAD (IMV, France), equipped with a 7.5 MHz linear array. The transducer was covered with a copious amount of gel to facilitate ultrasonographic imaging. Scanning was performed without pressure to avoid a distortion of the testicular shape. Images of the caudocranial, lateral-lateral, and ventrodorsal axis of the testes were obtained. The testicular width, height, and length were measured using electronic calipers integrated into the ultrasound machine. Cursors were set at the borders of the tunica albuginea. The measurements were performed in triplicate of three different images (technical replicates). The echogenicity, homogeneity, and surface of the scrotal contents were also assessed. The testicular volume was calculated using the formula described by Hedia et al. (9): $L \times H \times W \times 0.71$.

Initial semen handling

Semen collection was performed during the breeding season. At all the sampling times, the ejaculate volume, sperm concentration, and total sperm output (ejaculate volume × sperm concentration) were calculated immediately after collection. Ejaculates were collected by artificial vagina at 40°C (IMV Technologies, L'Aigle, France) in the presence of a female decoy, and the tubes were maintained at 30°C before cooling. All the semen collections were carried out by the same investigator. The ejaculate volume was estimated by collecting them in Falcon® type graduated semen collection tubes. Sperm concentration was assessed by a cell counter (NucleoCounter SP-100, ChemoMetec, Allerod, Denmark). Ejaculates were diluted 1:1 (v/v) with INRA 96. The samples were then refrigerated

using a rate of −0.5°C/min from 30°C down to 15°C. After that, the final concentration was adjusted to 1,600 × 10^6 sperm/ml and the samples were packed into 0.25 ml plastic straws. The seminal doses were stored at 15°C. Sperm evaluation was performed 6–8 h after semen collection.

Ultrasonography evaluation of testicular function

For testicular evaluation function, all examinations were carried out by the same technician. Measurements were performed using the same real-time ultrasound scanner equipped with a 7.5 MHz linear array and 10 MHz high-frequency linear array transducers to evaluate the testicular echotexture and testicular blood flow, respectively.

Testicular echotexture

The probe was positioned by pressing on the center of the testicle transversely, and at least three clips per testicle were measured (technical replicates). The software Ecotext® (HUMECO; Huesca, Spain) was used for analyzing the parenchyma echotexture. The following parameters were measured: Ecotext 1 (EC1: black pixels number), Ecotext 2 (EC2: white pixels number), Ecotext 3 (EC3: mean gray level of pixels), the tubular density (density of tubules/cm^2), the tubular diameter [mean diameter (μm) of the lumen of the seminiferous tubules] and the tubular area [proportion (%) of the total area that was occupied by the lumen of the tubules in the parenchyma].

Testicular blood flow

Doppler parameters were measured in the supratesticular artery located in the spermatic cord region, and the transducer was positioned at a midway point between the inguinal ring and the testicle (34). For distinguishing between a testicular artery and vein by Doppler analysis, an artery, for example, will have a waveform on the spectral graph that reflects the arterial pulse in each cardiac cycle (systole and diastole). However, the flow in a vein is almost constant, that is, without a pulse. At least three consecutive waveforms were measured per testicle (technical replicates), and the Doppler parameters were automatically calculated by the software package provided with the ultrasound machine. Peak systolic velocity (PSV) was measured, and the Doppler indices studied were resistive index [RI = (maximum velocity-minimum velocity)/maximum velocity] and pulsatility index [PI = (maximum velocity-minimum velocity)/mean velocity]. Furthermore, the total artery blood flow (TABF) was calculated [mean velocity × A; A (cross-section of the artery): πr^2]. Based on previous studies (11, 19, 35), the angle between the long axis of the vessel and the Doppler beam was from 20 to 60 degrees in the direction of the blood flow. Additionally, the Doppler gate was kept constant at 1 mm. To minimize variations in measurements, the ultrasound settings (focus, gains, brightness, and contrast) were standardized, fixed, and used equally for all examinations like others authors (9).

Sperm evaluation

Motility and kinetic parameters by CASA

The motility and kinetic parameters were assessed using the Assisted Sperm Analysis (CASA) (Sperm Class Analyzer - SCA- software V 6.3.0.59; Microptic S.L., Barcelona, Spain). The parameters setting was set to capture at 100 frames/s a total of 50 frames, and particles with an area of 20–70 μm^2 were considered compatible with the head area. Aliquots of each ejaculate were diluted to 25 × 10^6 sperm/ml in an extender (TES-Tris-fructose and 1% egg yolk) and tempered on a 37°C plate for 5 min. After that, a 5 μl drop was placed in a Makler counting cell chamber (10 μm depth; Sefi Medical Instruments, Haifa, Israel). Samples were examined with an ×10 negative phase contrast objective in a microscope equipped with a warmed stage at 38°C (Eclipse E400, Nikon, Tokyo, Japan). At least 400 sperm from four different randomly selected fields were captured and analyzed. The reported kinetic parameters were the velocity according to the straight path (VSL, μm/s); the amplitude of the lateral displacement of the sperm head (ALH, μm); the head beat-cross frequency (BCF, Hz); the total motility (TM), defined as the percentage of sperm with VCL (curvilinear velocity) >15 μm/s; the progressive motility (PM), defined as the percentage of sperm with VCL >45 μm/s; and the rapid progressive motility (RAP PM), defined as the percentage of sperm with VCL >75 μm/s. All parameters were previously described by Palacin-Martinez et al. (36).

Sperm functionality by flow cytometry

Staining for determination of viability, caspases 3 and 7 activity, and mitochondrial functionality

Fluorescence probe Zombie Violet™ Fixable Viability Kit was acquired from BioLegend (San Diego, CA, USA), and CellEvent™ Caspase-3/7 Green Detection Reagent and CellROX™ Deep Red Reagent were supplied from ThermoFisher (Invitrogen, Eugene, Oregon, USA).

For staining, a protocol previously described by Riesco et al. (37) was used. Sperm samples were diluted in PBS to a concentration of 2 × 10^6 sperm/ml to wash the cells by short centrifugation (15"; MiniSpin plus, Eppendorf, Hamburg, Germany) with the removal of the supernatant. Then, cells were incubated at room temperature and in the dark for 30 min with 96 μl Zombie Violet™ (1:1,000 final dilution), 2 μl CellEvent™ Caspase-3/7 (4 μM final concentration), and 2 μl CellROX™ (5 μM final concentration). After that, a new wash

was performed to stop cell staining and avoid an over-staining effect, and the pellet was resuspended in 1 ml PBS, immediately conducting the analysis by flow cytometry.

The combination Zombie Violet™ Fixable Viability Kit, CellEvent™ Caspase-3/7 Green Detection Reagent, and CellROX™ Deep Red was used to simultaneously determine the viability through plasma membrane integrity, caspases 3 and 7 activity as a marker of apoptosis, and mitochondrial function through reactive oxygen species (ROS) content, respectively.

Flow cytometry analyses

Flow cytometry analyses were conducted in the flow cytometer MACSQuant Analyzer 10 (Miltenyi Biotech, Bergisch Gladbach, Germany) equipped with three lasers emitting at 405, 488, and 635 nm (violet, blue, and red, respectively) and 10 photomultiplier tubes. Violet fluorescence was detected in V1 (excitation 405 nm, emission 450/50 nm), green fluorescence was detected in B1 (excitation 488 nm, emission 525/50 nm), and red fluorescence was detected in R1 [excitation 635 nm, emission 655–730 nm (655 LP + split 730)]. Samples were acquired using MACS Quantify software (Miltenyi Biotech, Bergisch Gladbach, Germany), recording a total of 40,000 cells per sample at a flow rate of 200–300 cells/s. Data were analyzed using FlowJo V 10.2 (Ashland, Wilmington, DE, USA). The interest sperm subpopulations assessed were plotted as follows: viable sperm (Zombie Violet™ low intensity -alive-), apoptotic sperm (CellEvent™ Caspase 3/7 positive), and sperm with high mitochondrial activity (CellROX™ positive).

Statistical analyses

Data were analyzed with SAS/STAT® version 9.1 statistical package (SAS Institute, Cary, NC, USA). Data were analyzed by a mixed linear model (MIXED procedure), considering the male effect as a random factor. Libido was analyzed by Kruskal–Wallis test (NPAR1WAY procedure). Relations between sperm quality parameters and ultrasonography measurements were studied by Pearson's correlation. The results are displayed as the mean ± standard error of the mean (SEM). Differences were statistically significant at $P \leq 0.05$.

Results

Testicular yield

Significant differences ($P \leq 0.05$) were found among all the frequencies of semen collection in the serum testosterone levels (Figure 2A), with the highest levels in the IF. Libido was significantly ($P \leq 0.05$) higher in intensive semen collections (IFM and IFF; Figure 2B) compared to AF and SF. Concerning testicular measurements, a significant increase in volume was observed in IF compared to AF ($P \leq 0.05$; Figure 2C). According to sperm production, the ejaculate volume was significantly decreased ($P \leq 0.05$) in SF and IFF in comparison with AF, but non-significant differences ($P > 0.05$) between both were found (Figure 2D). Sperm concentration decreased in both sperm evaluations of IF (Figure 2E), and sperm production was gradually lower ($P \leq 0.05$) with increasing intensity of semen collection frequency. However, non-significant differences concerning sperm production were revealed between SF and IFM ($P > 0.05$; Figure 2F).

Ultrasonography evaluation of testicular function

Right and left testis did not show significant differences with respect to echotexture and Doppler parameters (data not shown). Thus, the means of the right and lefts testis were used for further analysis.

The testicular echotexture results are shown in Figure 3. EC1, tubular area and tubular diameter decreased significantly ($P \leq 0.05$) when the frequency of semen collection increased. However, EC2 and EC3 increased significantly ($P \leq 0.05$) under the same conditions. The tubular density was similar among the semen collection frequencies ($P > 0.05$).

The testicular vascularization results are shown in Figures 4, 5. The PSV parameter was significantly higher ($P \leq 0.05$) in SF and IF with respect to AF. In relation to RI and TABF parameters, both increased significantly ($P \leq 0.05$) in IF in comparison to AF and SF. In addition, there were significant differences among all semen collection frequencies in PI ($P \leq 0.05$).

Sperm quality evaluation

Motility and kinetic parameters

The sperm motility parameters are shown in Figure 6. There were significant differences between AF and IFF in TM ($P \leq 0.05$), decreasing when the semen collection intensity increased. In the same way, the TM was significantly higher ($P \leq 0.05$) in SF in comparison with IFF (Figure 6A). Regarding RAP PM, the highest percentage was observed in AF ($P \leq 0.05$; Figure 6C). PM, VSL, and ALH were significantly higher ($P \leq 0.05$) in AF and SF in comparison with IFM and IFF (Figures 6B,D,E). BCF was significantly higher ($P \leq 0.05$) in AF in comparison with IFM and IFF. In the same way, SF was significantly higher ($P \leq 0.05$) than IFF (Figure 6F).

Sperm functionality

Attending to the flow cytometry analysis, total viability was significantly lower ($P \leq 0.05$) in IFM with respect to AF and SF (Figure 7A). The percentage of apoptotic sperm,

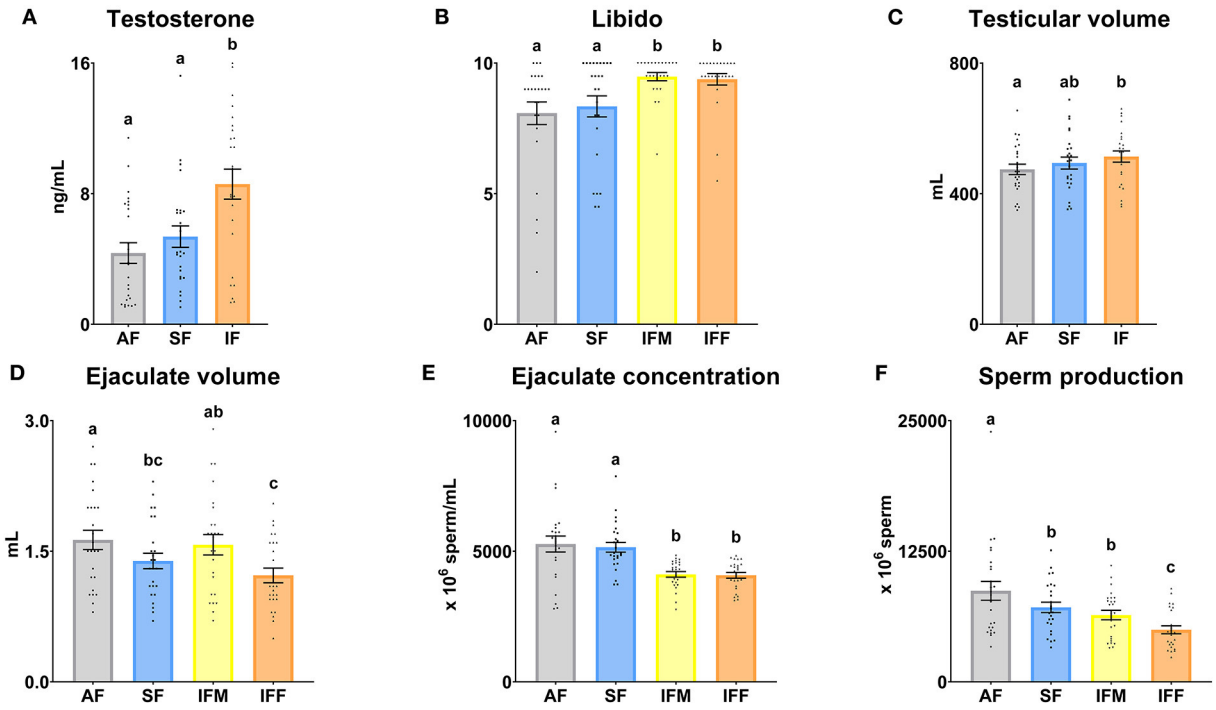

FIGURE 2
Ram reproductive values according to semen collection frequencies. **(A)** Testosterone, serum testosterone levels (ng/ml); **(B)** Libido, subjective evaluation (zero to 10); **(C)** Testicular volume (ml); **(D)** Ejaculate volume (ml); **(E)** Ejaculate concentration ($\times 10^6$ sperm/ml); **(F)** Sperm production ($\times$ 10^6 sperm). The same 25 males were analyzed in each experimental group (AF, abstinence semen collection frequency; SF, standard semen collection frequency; IFM, intensive semen collection frequency on Monday; and IFF, intensive semen collection frequency on Friday). Graph dots represent individual male values [graphs **(A–C)**] and ejaculates [graphs **(D–F)**]. Means (±SEM) are shown. Different lowercase superscripts letters (a–c) indicate differences ($P \leq 0.05$) among the semen collection frequencies.

with active caspases 3 and 7, was significantly higher ($P \leq 0.05$) in IFF compared with the other experimental groups. Moreover, this parameter also was significantly higher ($P \leq 0.05$) in SF with respect to AF (Figure 7B). Finally, the percentage of sperm with high mitochondrial activity showed the same statistically significant differences as the previous parameter analyzed (Figure 7C) but with an opposite trend.

Correlations between ultrasonography parameters and sperm quality

Correlations between ultrasonography parameters and sperm quality are shown in Figures 8–10, related to AF, SF, and IF, respectively. The highest correlation between ultrasonography and sperm quality in AF was found between Tubular density and RAP PM ($R^2 = -0.408$; Figure 8). Concerning SF, the pulsatility index presented the highest correlation with RAP PM ($R^2 = 0.637$). Also, RI, TABF, Area, and Diameter showed significant moderate positive correlations with motility parameters (Figure 9). Attending to IF, Doppler indexes (RI and PI) correlated strongly and positively with C3&7A. However, the same Doppler indexes showed strong negative correlations with ROS ($P \leq 0.05$). All the correlations studied are included in a correlation matrix (Figure 10).

Field results: Semen yield, sperm quality and fertility trials

Concerning ejaculate yield, a higher percentage of valid ejaculates was recorded in SF (76%) compared with IF (60%) (Figure 11A). In terms of ejaculation yield, the percentage of valid sperm numbers and doses were higher in SF in comparison with IF (Figure 11C). Motility parameters registered significant differences ($P \leq 0.05$) between SF and IF in valid ejaculates in terms of TM, PM, and RAP PM, being lower in IF. On the other hand, significant differences were found between valid and non-valid ejaculates in IF with respect to TM and PM ($P \leq 0.05$; Figure 11B). Cytometry analyses showed that apoptosis was significantly lower in valid ejaculates in SF compared to valid ejaculates in IF ($P \leq 0.05$). Contrary to this, mitochondrial activity was higher in valid ejaculates in SF with respect to valid ejaculates in IF ($P \leq 0.05$). Regarding the valid

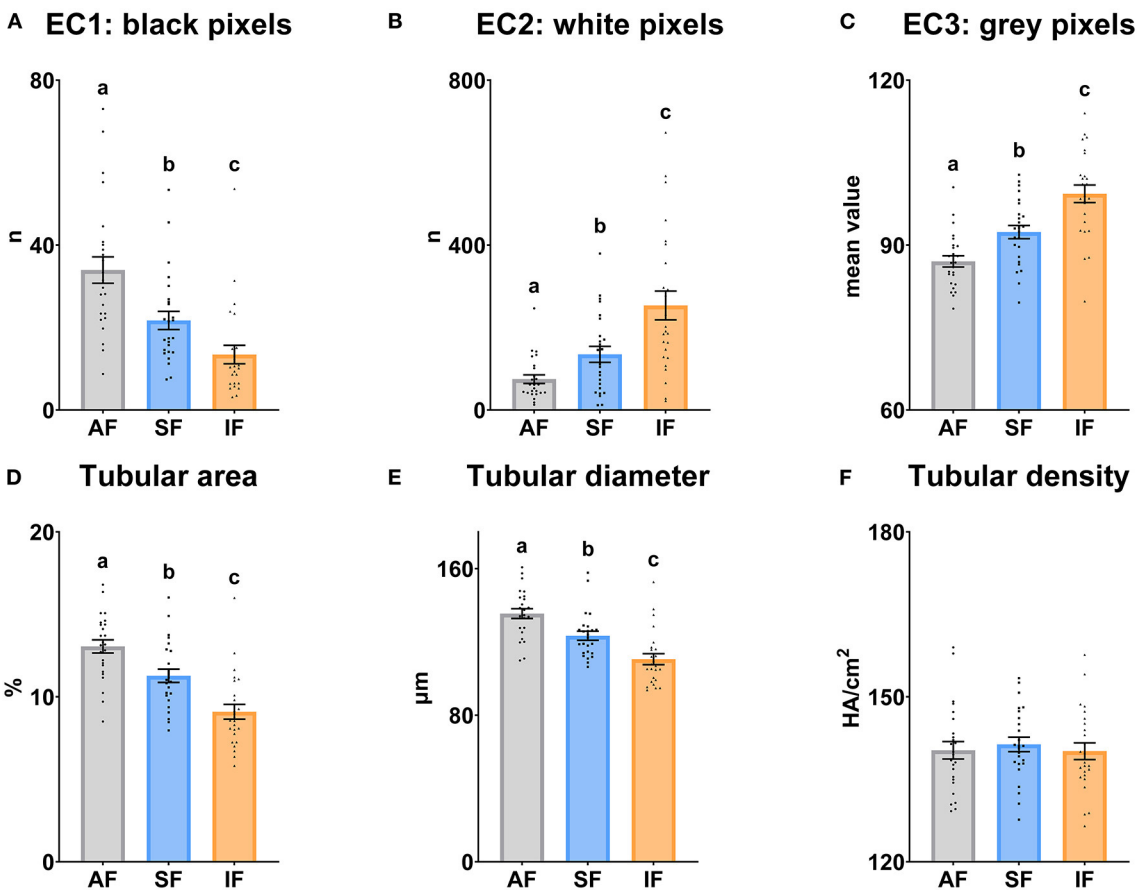

FIGURE 3
Echotexture characteristics of the ram testes according to semen collection frequencies. **(A)** EC1, Ecotext 1 (black pixels); **(B)** EC2, Ecotext 2 (white pixels); **(C)** EC3, Ecotext 3 (mean value of gray pixels); **(D)** Tubular area, proportion (%) of the total area corresponding with the lumen of the seminiferous tubules; **(E)** Tubular diameter, mean diameter (µm) of the lumen of the seminiferous tubules; **(F)** Tubular density, the density of hypoechogenic areas per cm^2 corresponding with the seminiferous tubules. The same 25 males were analyzed in each experimental group (AF, abstinence semen collection frequency; SF, standard semen collection frequency; IF, intensive semen collection frequency). Graph dots represent individual male values. Means (±SEM) are shown. Different lowercase superscripts letters (a–c) indicate differences ($P \leq 0.05$) among the semen collection frequencies.

ejaculates comparison, there were significant differences ($P \leq 0.05$) in viability, apoptosis, and mitochondrial activity in SF (Figure 11D).

A fertility trial was carried out considering an abstinence period before semen collection for AI (Figure 12A). According to this, the highest fertility rates were obtained with SI of semen collection ($P \leq 0.05$). However, when males were submitted both high or low intervals (HI and LI, respectively), fertility rates decreased significantly ($P \leq 0.05$), following the same trend with respect to SI and AN. Additionally, there was no identification of differences between them. In addition, the annual fertility was significantly lower than SI ($P \leq 0.05$). With respect to the descriptive study per male, we observed the same trend in most of them.

According to this, most males presented higher fertility rates with SI of semen collection. However, when males were submitted both high or low intervals (HI or LI, respectively), fertility rates decreased following the same trend (Figure 12B).

Discussion

The improvement of semen donor rams in terms of sperm quality and yield in reproduction centers is a feasible alternative to increase the results of artificial insemination (AI). In this context, semen collection frequency is a common factor studied in several species such as ram (38), boar (39), stallion (40), pigeon (41) or human (42). In our study, sperm production and quality parameters decreased when the semen collection frequency increased. These evidences are in accordance with findings from other studies carried out in rams (6) or humans (43), where it was also reported that increased semen collection

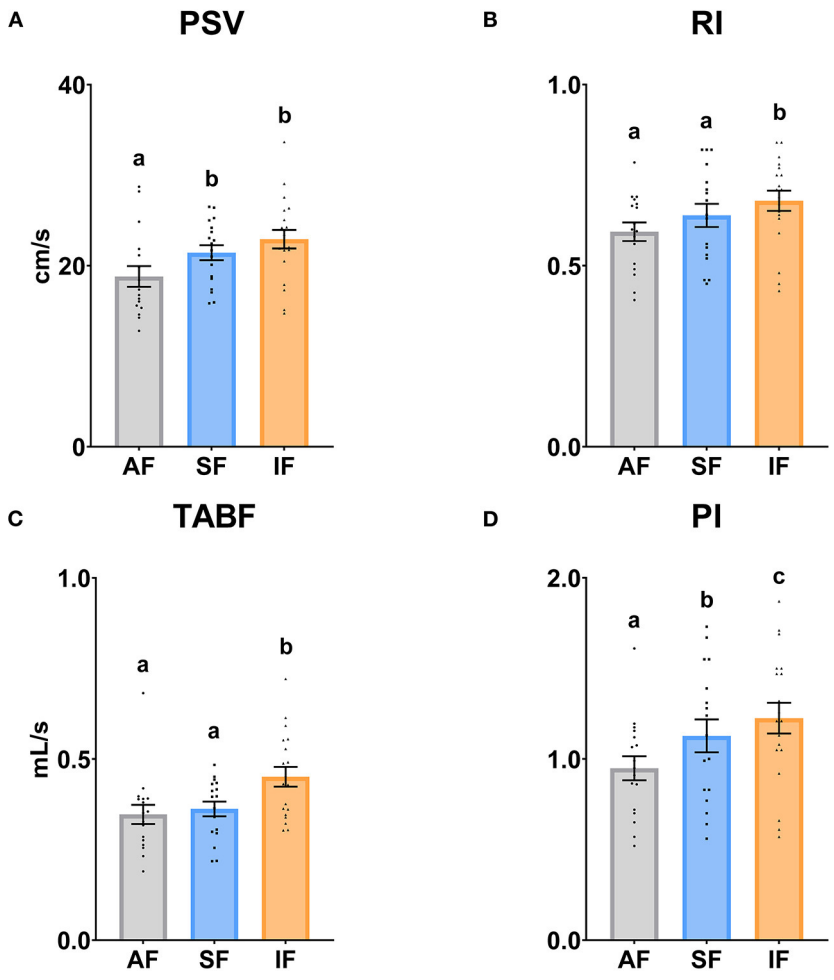

FIGURE 4
Ram supratesticular artery Doppler parameters according to semen collection frequencies. **(A)** PSV, peak systolic velocity (cm/s); **(B)** RI, resistive index; **(C)** TABF, total artery blood flow (ml/min); **(D)** PI, pulsatility index. The same 17 males were analyzed in each experimental group (AF, abstinence semen collection frequency; SF, standard semen collection frequency; IF, intensive semen collection frequency). Graph dots represent individual male values. Means (±SEM) are shown. Different lowercase superscripts letters (a–c) indicate differences ($P \leq 0.05$) among the semen collection frequencies.

frequency had a negative impact on sperm count. Our current findings were consistent with those in a report from Ollero et al. (5), where about 27% less ejaculate volume was obtained after 1 or 2 days of abstinence than after abstinence for 3 days, and sperm concentration decreased significantly as the abstinence period decreased. In our study, 72 h of sexual abstinence (weekend) were enough for a partial recuperation of ejaculate volume and sperm production, allowing us to discard a loss of testicular functionality phenomenon. Linked to our findings on sperm quantity, sperm motility, which is used in the routine evaluation of sperm (5), showed a significant ($P \leq 0.05$) decrease in IF, demonstrating the influence of semen collection frequency in sperm motility parameters. Our findings in ovine species are consistent with those reported for rams (6) and boars (39). Concerning the sperm functionality, there were no significant differences ($P > 0.05$) in viability. In contrast, more advanced cytometry parameters in sperm preservation showed interesting changes. Caspases 3 and 7 activity presented the highest value in IF. These caspases are specific cysteinyl aspartate proteases that execute the breakdown of structural proteins and DNA (44). For this reason, this finding was related to apoptotic changes, which could compromise the ability to fertilize the oocyte (45, 46). Moreover, the lowest percentage of ROS content was found in IF. The ROS content measured by the CellROX probe in sperm mainly reflects intense mitochondrial activity rather than oxidative stress (47–50). Thus, a high frequency of semen collection could reduce the mitochondrial activity of ram sperm. Contrary to our findings, cytometry parameters in humans were not significantly affected after a 2-week period of daily ejaculation, although a decreasing trend in intracellular

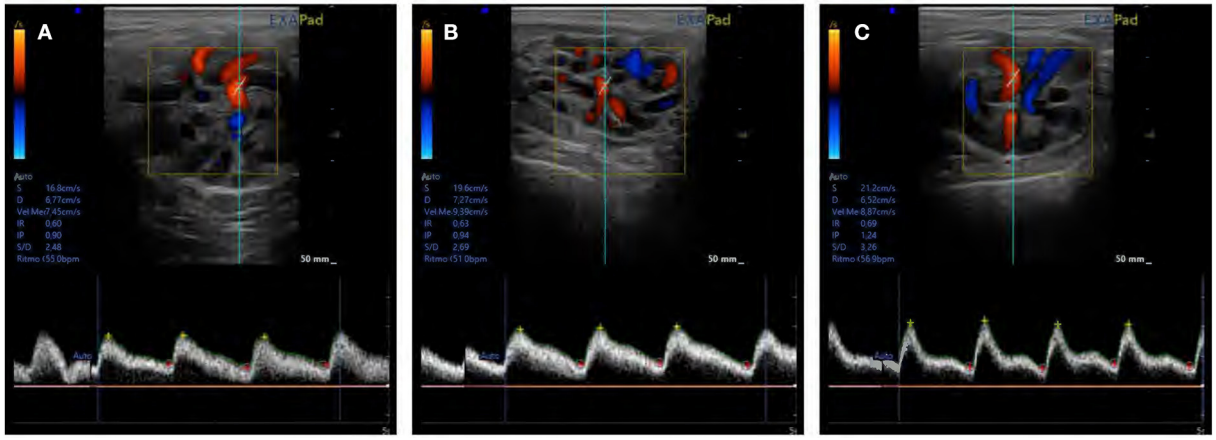

FIGURE 5
An assessment of testicular blood flow in supratesticular artery using pulse Doppler ultrasonography in **(A)** abstinence, **(B)** standard, and **(C)** intensive semen collection frequencies.

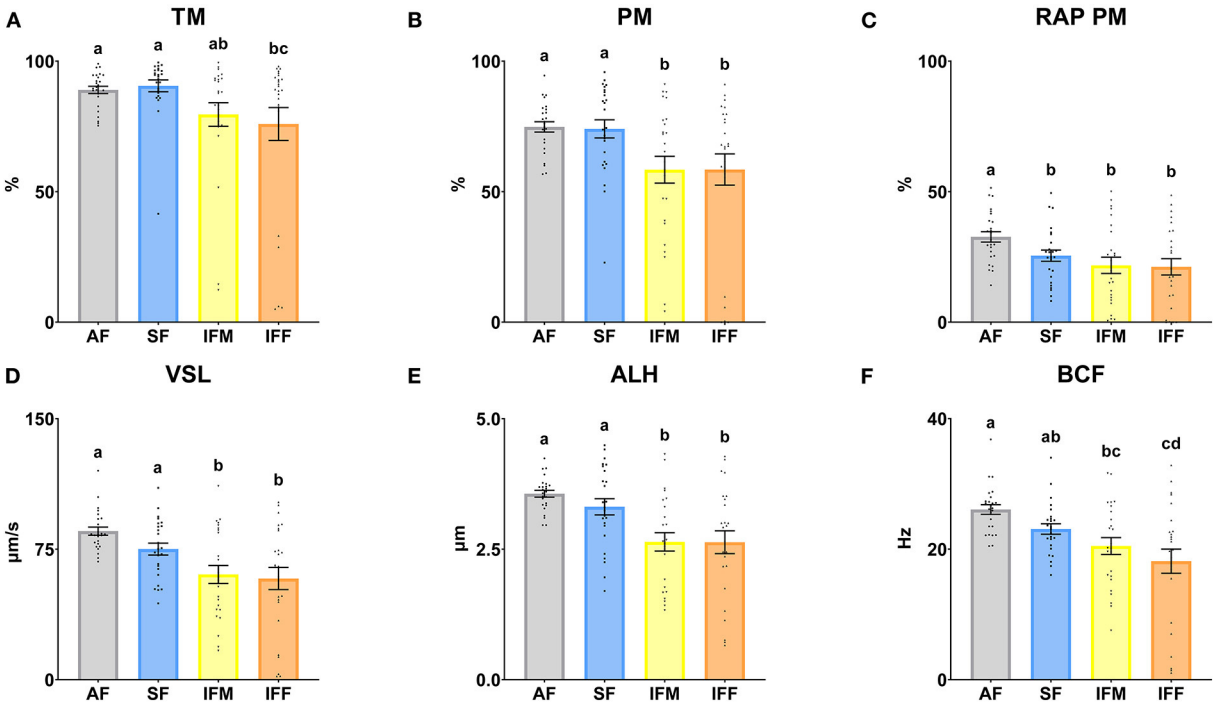

FIGURE 6
Ram sperm motility according to ejaculate collection frequencies. **(A)** TM, total motility (%); **(B)** PM, progressive motility (%); **(C)** RAP PM, rapid progressive motility (%); **(D)** VSL, straight-line velocity (µm/s); **(E)** ALH, head lateral amplitude (µm); **(F)** BCF, beat frequency (Hz). The same 25 males were analyzed in each experimental group (AF, abstinence semen collection frequency; SF, standard semen collection frequency; IFM, intensive semen collection frequency on Monday; and IFF, intensive semen collection frequency on Friday). Graph dots represent individual male values. Means (±SEM) are shown. Different lowercase superscripts letters (a–d) indicate differences ($P \leq 0.05$) among the semen collection frequencies.

ROS production was also observed (43). More interestingly, after a short recovery period (3 days of abstinence during the weekend) in the IF model, apoptosis and mitochondrial activity were significantly improved. The current findings obtained in sperm quality are in accordance with Ihukwumere and Okere observations (51) and could occur because sperm cells may need a minimum storage time in the cauda epididymis. Several epididymal components have collaborated in fertilizing capacity

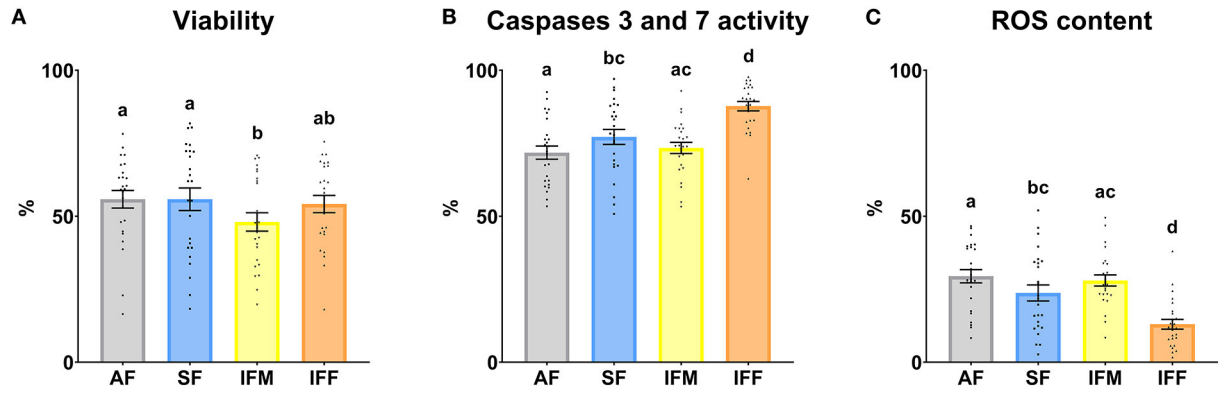

FIGURE 7
Ram sperm functionality according to ejaculate collection frequencies. **(A)** Viability, Zombie Violet™ (%); **(B)** Caspases 3 and 7 activity, CellEvent™ Caspase-3/7 Green (%); **(C)** ROS content, CellROX™ Deep Red (%). The same 25 males were analyzed in each experimental group (AF, abstinence semen collection frequency; SF, standard semen collection frequency; IFM, intensive semen collection frequency on Monday; and IFF, intensive semen collection frequency on Friday). Graph dots represent individual male values. Means (±SEM) are shown. Different lowercase superscripts letters (a–d) indicate differences ($P \leq 0.05$) among the semen collection frequencies.

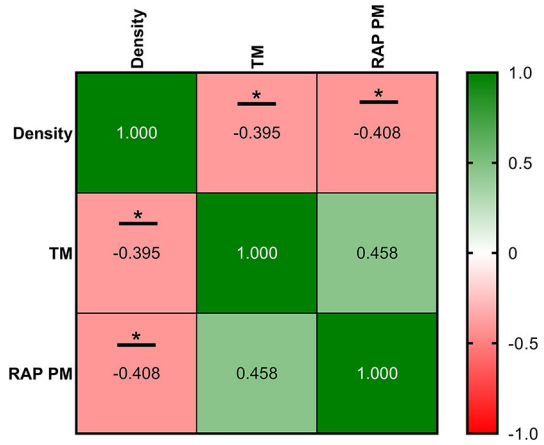

FIGURE 8
Correlation coefficients between ultrasonography measurements and sperm quality parameters in abstinence semen collection frequency (AF). Density, the density of hypoechogenic areas per cm^2 corresponding with the seminiferous tubules; TM, total motility (%); RAP PM, rapid progressive motility (%). The same 25 males were analyzed in each parameter. The *R* squared value between two parameters is represented in each cell and graph. In the correlation matrix, green color indicates positive correlations, and red color indicates negative relationships. The color intensity represents the strength of the correlation between two parameters. Asterisks show significant correlations ($P \leq 0.05$) between ultrasonography measurements and sperm quality parameters.

and motility of sperm cells due to biochemical and biophysical changes and interactions (52). For instance, clusterin, which is the most abundant protein of the cauda epididymal fluid in rams (53), participates in sperm maturation, lipid transport (54), and sperm membrane remodeling; acts as chaperone (55); and prevents peroxidative damage (56).

Traditional methods such as libido and clinical examinations (3, 7, 8) or basic ultrasound evaluation including testicular volume (26) have been used to complete the BSE. In our study, a testicular volume increase ($P \leq 0.05$) was detected in IF in comparison with AF, which could be explained by a high demand for sperm production that provokes a temporal and compensatory testicular hypertrophy (57). Another possible partial explanation to the observed increase of testicular volume is the advance of the breeding season (9) since the duration of the experiment was 2 months. As expected, serum testosterone and libido were higher ($P \leq 0.05$) in IF in relation to the other experimental groups. This could be related to the season and the testicular overexertion, which could trigger the activation of different pathways of the hypothalamus-hypophysis axis, provoking the testosterone increase. Some authors demonstrated this effect when they applied several treatments to improve the reproduction performance, such as buserelin (58) or eCG (59).

In these types of studies, new parameters and integrative studies on ultrasonography assessment could be more predictive and reinforce the optimization of the current ram reproductive handling to obtain high sperm quality and fertility. Consequently, we performed a testicular echotexture test using Ecotext® and a testicular vascularization evaluation using Doppler parameters in combination with some sperm quality analyses, including motility and sperm physiology, to analyze their possible correlations. According to the published literature, this is the first time that a male factor (frequency of semen collection) was used in an integrative way within the ram BSE: from ultrasonographic evaluation, such as ram reproductive ability predictor, to measure to sperm quality analyses.

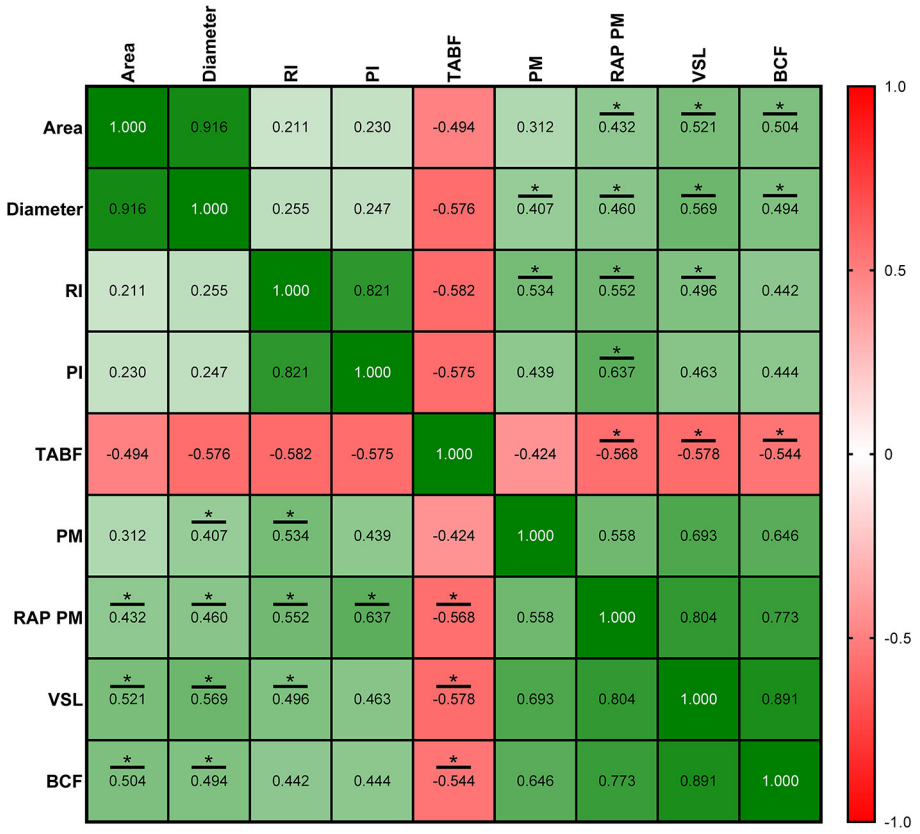

FIGURE 9
Correlation coefficients between ultrasonography measurements and sperm quality parameters in standard semen collection frequency (SF). Area, proportion (%) of the total area corresponding with the lumen of the seminiferous tubules; Diameter, mean diameter (μm) of the lumen of the seminiferous tubules; RI, resistive index; PI, pulsatility index; TABF, total artery blood flow (ml/min); PM, progressive motility (%); RAP PM, rapid progressive motility (%); VSL, straight-line velocity (μm/s); BCF, beat frequency (Hz). The same 25 males were analyzed in each parameter. The *R* squared value between two parameters is represented in each cell and graph. In the correlation matrix, green color indicates positive correlations, and red color indicates negative relationships. The color intensity represents the strength of the correlation between two parameters. Asterisks show significant correlations ($P \leq 0.05$) between ultrasonography measurements and sperm quality parameters.

The echotexture parameters revealed changes in the parenchyma structure, increasing echogenicity as the frequency of semen collection intensifies. We observed a significant decrease in black pixels number (EC1), tubular area, and tubular diameter with the intensification of semen collection; all of them could be related to the lumen of seminiferous tubules. We also observed a significant increase in white pixels number (EC2) and mean gray level of pixels (EC3); both could be due to different lumen cell types, which was demonstrated by Giffin et al. (25). These researchers correlated the testicular echotextural attributes with the predominant cell type (the lower echotexture with the higher cell differentiation degree) in the lumen of the seminiferous tubules in the ovine species. Thus, our findings could be explained by the alteration suffered in the composition of the lumen cells under different semen collection frequencies. Moreover, in a study conducted by Camela et al. (26), peripubertal rams showed lower seminiferous tubules lumen and, therefore, greater testicular echotexture than postpubertal rams. These findings in peripubertal rams could be in accordance with the echotextural changes when the intensity of the semen collection frequency increased, showing less hypoechogenic areas related to seminiferous tubules lumen. On the other hand, the density of hypoechogenic areas per cm^2 did not show changes among regimes. This could be because, in adult males, the relative seminiferous tubule quantity remains stable when males achieve sexual maturity (60). In spite of this, in AF, negative correlations were found between Density and TM and RAP PM. This could suggest that males with more seminiferous tubules have poor sperm motility in an abstinence semen collection frequency. Although a large amount of sperm could be stored in the epididymis in males with more seminiferous tubules, during prolonged abstinence periods, sperm are exposed to several sperm motility inhibiting factors [acidic pH and a high potassium to sodium ratio in

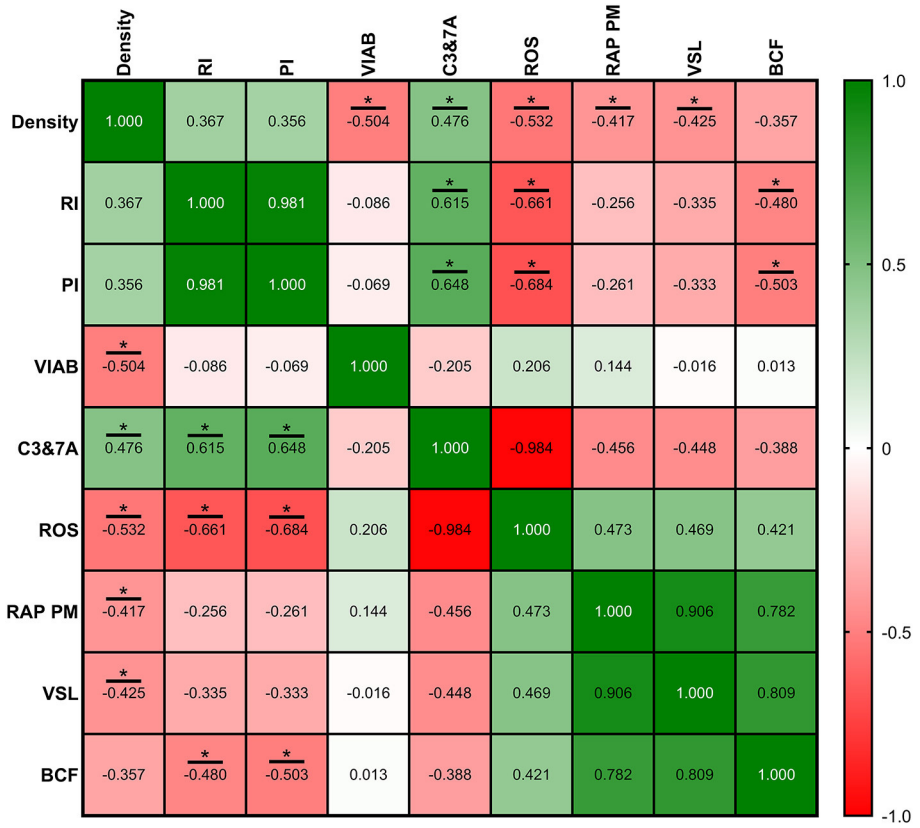

FIGURE 10
Correlation coefficients between ultrasonography measurements and sperm quality parameters in intensive semen collection frequency (IF). Sperm quality parameters used were measured on Friday (corresponding with IRF). Density, density of hypoechogenic areas per cm^2 corresponding with the seminiferous tubules; RI, resistive index; PI, pulsatility index; VIAB, viability (%); C3&7A, caspases 3 and 7 activity (%); ROS, ROS content (%); RAP PM, rapid progressive motility (%); VSL, straight-line velocity (μm/s); BCF, beat frequency (Hz). The same 25 males were analyzed in each parameter. The *R* squared value between two parameters is represented in each cell and graph. In the correlation matrix, green color indicates positive correlations, and red color indicates negative relationships. The color intensity represents the strength of the correlation between two parameters. Asterisks show significant correlations ($P \leq 0.05$) between ultrasonography measurements and sperm quality parameters.

epididymal fluid (61, 62)], which may negatively impact their future motility after ejaculation (63).

Following the integral assessment of the reproductive capacity of rams based on ultrasound evaluation, the Pulse-Doppler mode was described as an indicator of testicular functionality in standard conditions in ram (9, 10), dog (14), stallion (32), or human (64). However, Doppler parameters had not been investigated in different semen collection frequencies correlating these analyses with sperm quality assays. Firstly, PSV and PI increased significantly ($P \leq 0.05$), and RI and TABF did not vary significantly ($P > 0.05$) in SF compared to AF. This could be explained due to PSV and PI may be early predictors of testicular blood perfusion changes, as Jolly et al. (65) described. Moreover, RI is altered when more severe disorders occur (64). Infertile dogs had lower PSV than fertile dogs without varying RI because vascular bed resistance depends on multiple factors such as diameter and tortuosity of the vessels (66). Although it has not been previously described, we observed positive correlations between some ultrasonography (Area, Diameter, RI, and PI) and sperm motility parameters (PM, RAP PM, VSL, and BCF). In this sense, other sperm quality parameters such as live sperm and sperm concentration correlated positively with some seminal plasma antioxidants such as SOD, GPx, and GSH (67). Moreover, Hedia et al. (67) confirmed positive correlations between seminal plasma antioxidants presented in high-quality spermatozoa samples and Doppler indexes, connecting with our positive correlations in SF. On the other hand, all indexes (RI, PI, and TABF) increased significantly in IF with respect to SF. This finding could indicate an increase in resistance to blood flow, pulsatility in the oscillations of the waveform, and blood flow per minute as a consequence of the testicular stress by the intensive semen

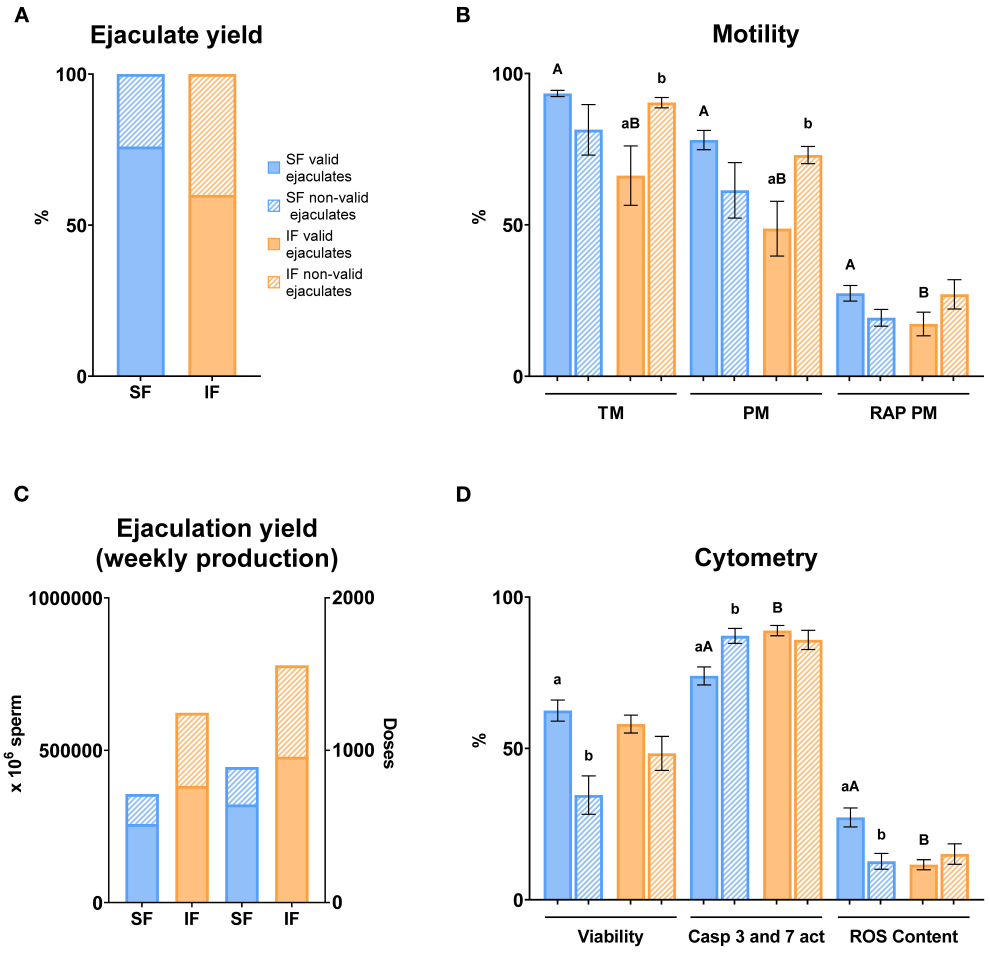

FIGURE 11
Semen yield and sperm quality in valid and non-valid ejaculates. Criteria of valid ejaculate: ejaculate volume >0.5 ml, mass motility >3, sperm concentration > 3,000 × 10^6 sperm/ml. Non-valid ejaculates: some of the criteria previously described below the minimum value. **(A)** Ejaculate yield: % of valid and non-valid ejaculates in two semen collection frequencies (SF, standard semen collection frequency; IF, intensive semen collection frequency corresponding with IFF; Friday); **(B)** Motility: sperm samples of SF (blue columns) and IF (orange columns); TM, total motility (%); PM, progressive motility (%); RAP PM, rapid progressive motility (%); **(C)** Ejaculation yield (weekly production): number of sperm and doses produced weekly in two semen collection frequencies (SF and IF); **(D)** Cytometry: sperm samples of SF (blue columns) and IF (orange columns); Viability, Zombie Violet™ (%); Caspases 3 and 7 activity, CellEvent™ Caspase-3/7 Green (%); ROS content, CellROX™ Deep Red (%). The same 25 males were analyzed in each experimental group. Different lowercase superscripts letters (a,b) indicate differences ($P \leq 0.05$) for each assessment point between valid and non-valid ejaculates. Different lowercase superscripts capital letters (A,B) indicate differences ($P \leq 0.05$) for each assessment point between semen collection frequencies.

collection frequency. Low oxygen tension in the seminiferous tubules is essential for spermatogenesis (68); thus, the poor-quality sperm in IF could be justified by increased blood flow with higher oxygen tension. Recently, Ntemka et al. (29) correlated Doppler indexes (RI and PI) negatively with sperm abnormalities. In our work, we found, for the first time, that these Doppler indexes correlated negatively with functionality sperm parameters measured by flow cytometry in consonance with Hedia et al. (9) and Ntemka et al. (29). Nevertheless, to find these correlations, we hypothesized that it is necessary to overexert the testis. A recent study carried out by Brito et al. (69) comparing young and senile dogs revealed a lesser sperm quality in senile dogs and did not detect significant differences in ultrasonographic B-mode evaluation (70). In this sense, vascular characteristics of the testes may represent the causal factors underlying changes in spermatogenesis and, as a consequence, affecting the sperm quality of donor rams negatively in an intensive semen collection frequency. Based on our results, sophisticated studies of testicular echotexture and vascular evaluation measured by specific software and Doppler mode, respectively, are crucial in the reproductive ultrasonography evaluation of males.

In the second part of the paper, we carried out a field trial to demonstrate the importance of semen collection frequencies

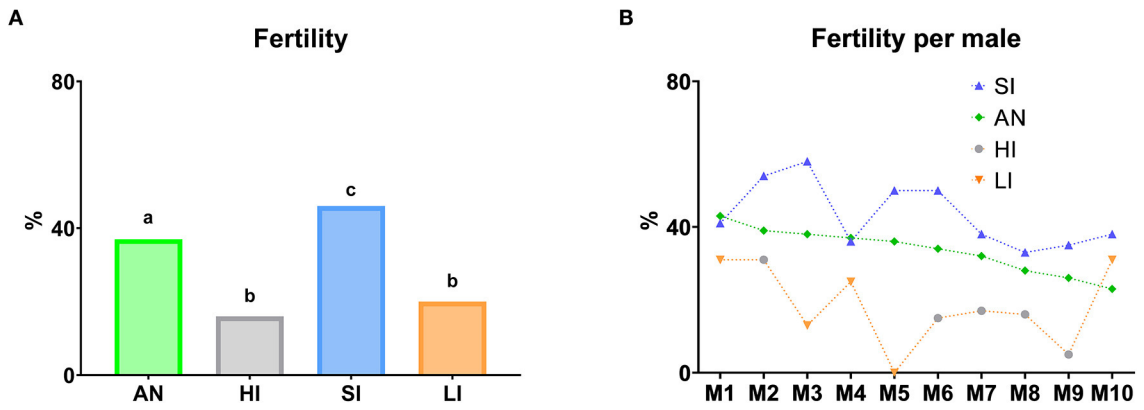

FIGURE 12
Fertility according to different semen collection frequencies. **(A)** Fertility per group according to abstinence days before semen collection for AI. **(B)** Descriptive study of the fertility per male according to abstinence days before semen collection for AI. AN (Annual fertility), annual average of lambing ewes/inseminated ewes (%); HI (Fertility high interval), fertility with seminal doses obtained from males with at least 10 abstinence days; SI (Fertility standard interval), fertility with seminal doses obtained from males with two to five abstinence days; LI (Fertility low interval), fertility with seminal doses obtained from males with semen collection the previous day. In graphic **(A)**, different lowercase superscripts letters (a–c) indicate differences ($P \leq 0.05$) for each assessment point among semen collection ratios due to abstinence days before semen collection for AI. In graphic **(B)**, graph dots represent individual males.

in AI success. It has been demonstrated that fertility is affected by many factors (intrinsic and extrinsic) related to the female such as the age of the ewe, the lambing-AI interval, or the cumulative number of AI/ewe; the farm such as environmental conditions, the sanitary status, or reproductive handling; the technique itself; and the male such as seasonality, sperm quality, or sperm conservation (71–73). Within the male factors, the frequency of semen collection has not been previously related to fertility rates. Although the ejaculates were considered valid according to the criteria of the reproduction centers (33), when we carried out more advanced analyses such as sperm motility and functionality, we detected significant differences in several parameters (TM, PM, RAP PM, Caspases 3 and 7 activity, and ROS content) and, therefore, we think that the fertility could be altered. In our field results, the standard interval of sexual abstinence, from 2 to 5 days, represented the highest fertility rates. Moreover, in a descriptive assay, we could observe the same trend in fertility rates in most evaluated males comparing different abstinence days before semen collection for AI. As we mentioned before, this fact could be due to the influence of the storage time in the cauda epididymis (53). This work paves the way to know what would be the optimal frequency of semen collection for each ram. It would be very beneficial to group males into different frequencies of semen collection to obtain the maximum reproductive performance from each ram and increase their fertility. To achieve this, a complex ultrasonographic evaluation should be included in the ram's BSE to predict the individual ram's reproductive capacity and optimize the reproductive handling of males.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

RM-G: conceptualization, methodology, formal analysis, investigation, writing—original draft, data curation, and visualization. MR: conceptualization, methodology, formal analysis, investigation, supervision, data curation, writing—review and editing, and visualization. LA-L and MA: conceptualization, methodology, investigation, resources, data curation, writing—review and editing, visualization, and funding acquisition. MN-M, CP-M, CO-F, and MH: conceptualization, methodology, and investigation. PP: formal analysis, investigation, resources, data curation, writing—review and editing, visualization, supervision, and funding acquisition. LA: conceptualization, resources, data curation, writing—review and editing, visualization, supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Metabolomic analysis of seminal plasma to identify goat semen freezability markers

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Factors affecting sperm freezability in goat seminal plasma were investigated. Based on the total motility of thawed sperm, goats were divided into a high-freezability (HF) group with >60% total motility ($n = 8$) and a low-freezability (LF) group with <45% total motility ($n = 8$). Sperm and seminal plasma from the HF and LF groups were separated, HF seminal plasma was mixed with LF spermatozoa, LF seminal plasma was mixed with HF sperm, and the products were subjected to a freeze-thaw procedure. Semen from individual goats exhibited differences in freezability. HF semen had higher sperm motility parameters and plasma membrane and acrosome integrity after thawing; this difference could be related to the composition of seminal plasma. Seminal plasma from the HF and LF groups was evaluated using metabolomic analysis, and multivariate statistical analysis revealed a clear separation of metabolic patterns in the seminal plasma of goats with different freezability classifications. Forty-one differential metabolites were identified using the following screening conditions: variable importance in the projection > 1 and 0.05 < P-value < 0.1. Kyoto Encyclopedia of Genes and Genomes pathway analysis revealed significant enrichment of central carbon metabolism in cancer, protein digestion and absorption, aminoacyl-tRNA, and other pathways and significant differences in the abundance of seven differential metabolites, including L-glutamine, L-aspartate, L-arginine, phenylpyruvate, benzoic acid, ketoisocaproic acid, and choline between seminal plasma from the HF and LF groups (P-value < 0.05). These significantly differentially-expressed metabolites may be potential biomarkers for sperm freezability. L-glutamine, L-aspartate, and L-arginine may directly affect sperm freezability. Benzoic acid, ketoisocaproic acid, and choline may regulate sperm freezability by participating in anabolic processes involving phenylalanine, leucine, and phosphatidylcholine in sperm.

KEYWORDS

metabolomic, goat spermatozoa, cryopreservation, freezability, seminal plasma

1. Introduction

Seminal plasma is a mixture of testicular, epididymal, and accessory sexual gland secretions in male animals. Seminal plasma contains many organic and inorganic components required for sperm function, such as amino acids, proteins, ions, sugars, and lipids, and it is an important source of energy and a transport medium for sperm (1, 2).

Seminal plasma is involved in regulating important biological processes in sperm, such as hyperactivation motility, the acrosome reaction, and capacitation (3–6), but its role in semen cryopreservation is difficult to determine because of the complexity of its components.

Several studies have described conflicting views of the effects of seminal plasma on sperm function after cryopreservation. According to a study by Moore et al., exposure of equine spermatozoa to seminal plasma had no significant effect on sperm motility after cryopreservation, but prior to the freezing process, prolonged exposure of spermatozoa to seminal plasma was detrimental (7). Seminal plasma improved the progressive motility and cell structural integrity of boar sperm during cryopreservation (8). The functional variation of seminal plasma after spermatozoa cryopreservation in various species may be attributed to differences in seminal plasma composition caused by the anatomical structure of the accessory sexual glands. However, differences in the contribution of seminal plasma to sperm cryopreservation have also been found in the same species. Rickard found that sperm from low-resilience rams frozen with seminal plasma from high-resilience rams exhibited higher viability after thawing than their own sperm plasma (9). Supplementation of autologous or homologous seminal plasma with high cryoresistance significantly improved sperm fertility and the average number of sperm cells bound to the zona pellucida in stallions (10, 11). These discrepancies were attributed to significant variations in the composition and abundance of small molecular metabolites in seminal plasma.

The metabolome, which is composed of the end products of metabolism within a biological system, determines the current phenotypic state of the cell, and metabolomic analysis is a promising tool for correlating metabolites with cellular biological functions (12, 13). Metabolites have been identified in bovine seminal plasma using metabolomic analysis, and the potential of these metabolites as biomarkers of bovine fertility has been explored (14–16). In this study, we investigated the factors of seminal plasma that affect variation of goat semen freezability and analyzed the metabolites of goat seminal plasma with different freezability classifications using metabolomic analysis. This study aimed to determine whether the seminal plasma metabolome can provide new insights into regulation of the effects of cryopreservation. Identification of potential markers in seminal plasma will provide new suggestions for optimizing existing goat semen cryopreservation strategies.

2. Materials and methods

2.1. Animals and experimental design

All chemicals used in this study, except for antibiotics (Gibco, USA), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Semen samples from twenty bucks were provided by Inner Mongolia JinLai Livestock Technology Co., Ltd. (TuZuo country, Hohhot, Inner Mongolia, China). All bucks with normal ferility, and housed in the same nutrition and management conditions. Semen was collected with an artificial vagina, and the semen was collected three times a week during the reproductive season. To eliminate the influence of the experimental method on the quality of thawed sperm, we applied the same freezing dilution to semen from different individuals and performed a uniform cryopreservation procedure, which ensured maximum accuracy of the experiments. Three experiments were carried out to characterize individual variation of goat semen freezability and to determine whether this variation could be attributed to seminal plasma. First, spermatozoa were assessed to identify bucks with consistently high or low freezability (HF or LF, respectively). Second, mixing of high-freezability seminal plasma (HSP) with LF sperm as EX-LF group and low-freezability seminal plasma (LSP) with HF sperm as EX-HF group, which were performed before a freeze-thaw procedure. Finally, a metabolomic analysis was performed on seminal plasma with different freezability classifications.

2.2. Semen handling

Ejaculates from each buck were centrifuged at 600 × g for 15 mins at 4°C. The supernatant was aspirated and subjected to further centrifugation (10,000 × g, 30 mins) to remove any remaining sperm and cell debris. Then, goat seminal plasma was stored at −80°C; one aliquot was used for remixing semen, and another aliquot was used for further metabolomic analysis.

Ejaculates were slowly diluted with a Tris-citrate-glucose diluent (1:1, semen:diluent, v/v). Centrifugation (200 × g, 27°C, 5 mins) was performed to gently wash the aliquots with the Tris-citrate-glucose diluent. The supernatant, which contained seminal plasma, diluent, and other contaminants, was discarded, and the washed sperm pellets were collected. According to the experimental design described in 2.1, spermatozoa identified as having HF or LF were pooled with HSP or LSP. Washed spermatozoa were diluted (1:10) with a freezing extender that contained 12% seminal plasma. The freezing diluent consisted of 300 mM Tris, 95 mM citric acid, 56 mM glucose, 10% (v/v) egg yolk, 5% glycerol (v/v), and 1% antibiotics (Gibco) (v/v). Ejaculates were slowly diluted (1:10, semen: diluent, v/v) with freezing diluent.

2.3. Semen cryopresevation

The concentration of each sample was determined using a Bovine Accuread Photometer (IMV, France) before being diluted to a concentration of 2×10^8 spermatozoa/mL. Samples were then chilled to 4°C for 3 h, and then 200 mL of the sample was stored in 0.25 mL straws (IMV). The straws were placed at equal intervals on a pre-cooled freezing rack before exposure to liquid nitrogen vapor (4 cm above the liquid nitrogen surface for 7 mins). All straws were then submerged in liquid nitrogen and stored. After a week of storage, the straws were thawed in a water bath at 37°C for 30 s.

2.4. Evaluation of sperm motility variables

Sperm motility was evaluated after thawing. A 3 μL drop of thawed sperm was placed on a pre-warmed Leja slide analysis

chamber, and a computer-assisted sperm analysis system was used (CASA, IVOS II, IMV). A minimum of five fields were evaluated per sample, with a total of 1,000 spermatozoa counted. The sperm motion measurements mainly included total motility (TMOT; %), progressive motility (PMOT; %), curvilinear velocity (VCL; μm/s), straight line velocity (VSL; μm/s); average path velocity (VAP; μm/s), linearity (LIN, %), and amplitude of lateral head displacement (ALH; μm) were evaluated in fresh and frozen/thawed samples. Definitions of these sperm motility parameters can be found in Dorado (17).

2.5. Measurement of sperm cell structural integrity

Flow cytometry was performed using an ACEA NovoCyteTM flow cytometer (ACEA, China). To ensure stability of the results, the flow cytometer was outfitted with a 488-nm blue all-solid-state laser, a 640-nm red all-solid-state laser, and a photomultiplier tube. The fluorescence channels, including FITC, PE, PerCP, and APC, could all be monitored at the same time. NovoExpress software was used to complete data acquisition (ACEA Biosciences, China). The detection parameters included the all-channel area, width, height, and time, which could effectively distinguish adhered cells, cell debris, and single cells. A total of 20,000 events were examined in each sperm sample.

Semen aliquots were diluted in 1 × binding buffer to a concentration of 1.2×10^6 cells/mL. Propidium iodide (PI, 5 μL, 50 μg/mL) was added to identify cell events, excluding cell debris, to assess sperm cells with plasma membrane damage using the protocol described by Bunel (18).

The sperm acrosome status was determined using FITC-peanut (Arachis hypogaea) agglutinin (GENMED SCIENTIFICS, INC., USA). Frozen-thawed sperm samples were diluted to 1.2×10^7 cells/mL, mixed with 150 μL of PNA-FITC and 200 μL of PI (0.4 μg/mL), and incubated in the dark for 15 mins.

2.6. Metabolite extraction

To extract metabolites from 16 seminal plasma samples, 400 μL of cold extraction solvent (methanol/acetonitrile/H_2O, 2:2:1, v/v/v) was added to 100-mg samples, which were then adequately vortexed. After vortexing, the samples were incubated on ice for 20 mins and then centrifuged at 14,000 × g for 20 mins at 4°C. The supernatant was collected and dried in a vacuum centrifuge at 4°C. For liquid chromatography–mass spectrometry (LC-MS) analysis, the samples were re-dissolved in 100 μL acetonitrile/water (1:1, v/v) solvent and transferred to LC vials. Quality control (QC) samples were created by pooling 10 μL of each sample and analyzing them with the other samples to evaluate the stability and repeatability of the instrument. The QC samples were assessed regularly, and one of every eight samples was evaluated.

2.7. LC-MS/MS analysis

The untargeted metabolomics of metabolic extracts were analyzed using a quadrupole time-of-flight mass spectrometer (Sciex TripleTOF 6600) using hydrophilic interaction chromatography *via* electrospray ionization. We used a gradient of solvent A (25 mM ammonium acetate in water and 25 mM ammonium hydroxide in water) and solvent B (acetonitrile) for chromatographic separation *via* an ACQUIY UPLC BEH Amide column (2.1 × 100 × 1.7 mm, Waters, Ireland). After holding at 85% B for 1 min, the gradient was linearly reduced to 65% over 11 mins, then reduced to 40% over 0.1 mins, held for 4 mins, and then increased to 85% over 0.1 mins, with a re-equilibration period of 5 mins. The following settings were used: flow rate of 0.4 mL/min, column temperature of 25°C, autosampler temperature of 5 °C, and injection volume of 2 μL. The mass spectrometer was operated in both negative and positive ionization modes. The electrospray ionization source conditions were set as follows: ion source gas 1: 60, ion source gas 2: 60, curtain gas: 30, source temperature: 600°C, ion spray voltage floating: ± 5,500 V. A rate of 0.20 s/spectra was set as the accumulation time for time-of-flight MS scans, with the instrument set to acquire over an m/z range of 60–1,000 Da. For automatic MS/MS acquisition, sample ion scans were acquired using information-dependent acquisition with high sensitivity mode and an m/z range of 25–1,000 Da. The accumulation time for product ions was set at 0.05 s/spectra. The parameters were set as follows: the collision energy was fixed at 35 V with ± 15 eV; declustering potential, 60 V (+) and −60 V (–); exclusion of isotopes within 4 Da; candidate ions to monitor per cycle, 10.

2.8. Data analysis

ProteoWizard MSConvert was used to convert the raw MS data (wiff.scan files) to MzXML files, which were then imported into the free version of XCMS software. The following parameters were used to select peaks: centWave m/z = 25 ppm and peak width = c (10, 19). The peak grouping parameters were bw = 5, mzwid = 0.025, and minfrac = 0.5. The processed data were uploaded before being imported into SIMCA-P (version 14.1, Umetrics, Umea, Sweden), where they were subjected to multivariate analysis, including Pareto-scaled principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA). Variable importance in projection (VIP) values were calculated for each variable in the OPLS-DA model to determine their role in classification. A *t*-test was used to determine significance.

For Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation, the metabolites were searched against the online database. Fisher's exact test was used to conduct the KEGG pathway enrichment analysis. Pathways with *P*-values < 0.05 were considered significantly altered pathways.

2.9. Statistical analysis

The variables of all samples were analyzed *via* Student's *t*-test using SPSS statistical software (version 22.0; Chicago, IL, USA).

Differences between the HF and LF samples were considered significant at a P-value < 0.05; data are presented as the mean ± SEM.

3. Results

3.1. Sperm quality evaluation

The motion parameters of fresh semen showed no significant differences ($P > 0.05$, Table 1). An expected decline in total motility was observed in spermatozoa from each buck after the freezing-thawing process, but the degree of decline in total motility was different for the 20 goats (Figure 1; Table 1). The goats were divided into an HF group with >60% total motility ($n = 8$) and an LF group with <45% total motility ($n = 8$). CASA was conducted to evaluate various kinetic parameters, including TMOT, PMOT, VAP, VCL, VSL, ALM, and LIN, which were significantly different between the HF and LF groups ($P < 0.001$, Table 1). Spermatozoa from HF goats frozen with LSP displayed a significant decrease in sperm motility performance, and freezing of spermatozoa from LF goats with HSP had a significantly positive effect on the motion parameters ($P < 0.001$, Table 1).

Significant differences in sperm plasma membrane and acrosome integrity between the HF and LF groups are shown in Figures 2A, D ($P < 0.001$). The acrosome integrity of spermatozoa from LF goats frozen with HSP was significantly increased ($P < 0.001$, Figure 2F), but plasma membrane integrity was not significantly affected ($P > 0.05$, Figure 2C). Spermatozoa from HF goats frozen with LSP exhibited a significant decline in plasma membrane and acrosome integrity ($P < 0.001$, Figures 2B, E).

3.2. Metabolic profile analysis

An ultra-high performance liquid chromatography-quadrupole time-of-flight MS system (UHPLC-Q-TOF MS) was used to obtain metabolic profiles for seminal plasma from the HF and LF groups in the positive and negative ion modes. The peak intensity of each feature was obtained using XCMS software. In total, 9,408 and 9,342 molecular features were extracted from each sample in the positive and negative ion modes, respectively. To identify ion peaks that could be used to distinguish between the metabolite profiles of seminal plasma from the HF and LF groups, we used subsequent analytical models to determine the best fit that may reflect changes in categorical identification differences. We plotted the unsupervised PCA scores of all seminal plasma profiles from the HF and LF groups. However, the results showed no intrinsic clustering related to semen freezability in the first two PCs. The PCA score chart showed that QC samples clustered together, indicating that the instrument was stable during the entire sample collection process. Although partial overlap was observed between the HF and LF groups, an overall trend toward separation was noted, both in the positive and negative ion modes (Figures 3A, B). Next, PLS-DA was performed on identified metabolites of seminal plasma from the HF and LF groups under both positive and negative ion modes to determine the significant variables among the different samples (Figures 3C, D). The results indicated a separation of clusters in PLS-DA plots for samples from the two groups. The separation of clusters indicated that significant differences existed among the analyzed samples for both the positive and negative ion modes.

3.3. Screening and identification of differentially expressed metabolites

Based on the successful discrimination of seminal plasma from the HF and LF groups, a search for the specific metabolites that contributed to the metabolomic differences between the two groups was conducted. VIP values were used to evaluate the influence of the expression pattern of each metabolite on the classification and discrimination of samples. Potential differentially-expressed metabolites with a VI P-value > 1 and $0.05 < P$-value < 0.1 were

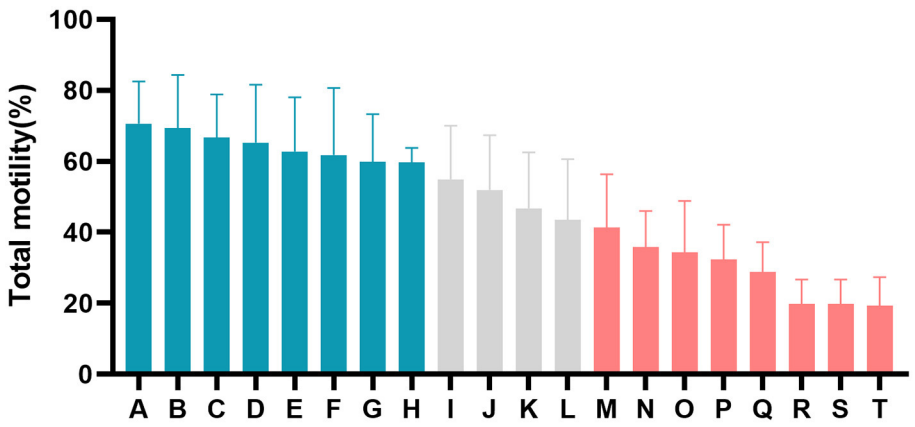

FIGURE 1
Difference in percentage total motility of post-thaw spermatozoa from all goats (A–T). The blue columns represent goats from the high-freezability (HF) group, the red columns represent goats from the low-freezability (LF) group, and the gray columns indicate goats with sperm classified as medium freezability.

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TABLE 1 Differences in the motion kinematics of spermatozoa with high and low cryotolerance among fresh ejaculates, freeze-thaw, and EX-SP freeze-thaw samples.

Characteristic	Fresh		Freeze-thawed		EX-SP freeze-thawed	
	HF	LF	F-HF	F-LF	EX-HF	EX-LF
TM (%)	88.93 ± 0.9^{a}	88.06 ± 1.29^{a}	66.34 ± 5.3^{b}	32.06 ± 2.53^{c}	53.32 ± 6.16^{d}	39.17 ± 4.48^{e}
PM (%)	70.2 ± 4.24^{a}	61.13 ± 5.25^{b}	31.91 ± 3.12^{c}	17.45 ± 1.77^{d}	28.81 ± 3.86^{c}	18.99 ± 6.55^{d}
CLV (μm/s)	156.08 ± 24.7^{a}	136.04 ± 12.08^{b}	74.49 ± 6.20^{c}	41.22 ± 9.75^{d}	64.26 ± 11.01^{c}	43.58 ± 11.73^{d}
SLV (μm/s)	102.82 ± 21.6^{a}	84.15 ± 15.93^{b}	36.98 ± 2.75^{c}	19.35 ± 3.11^{d}	31.71 ± 5.11^{c}	20.87 ± 5.81^{d}
APV (μm/s)	115.30 ± 23.1^{a}	98.49 ± 15.83^{b}	45.21 ± 2.77^{c}	23.76 ± 4.39^{d}	39.16 ± 6.41^{c}	25.53 ± 6.74^{d}
ALH (μm)	5.44 ± 1.04^{a}	6.17 ± 0.93^{b}	3.83 ± 0.35^{c}	2.23 ± 0.53^{d}	3.69 ± 0.74^{c}	2.42 ± 0.46^{d}
LIN (%)	59.43 ± 9.37^{a}	55.41 ± 9.11^{a}	30.74 ± 2.67^{b}	15.75 ± 3.43^{c}	27.44 ± 4.05^{b}	19.78 ± 3.59^{c}

The different superscript letter indicate significant differences between the groups at $P < 0.05$.

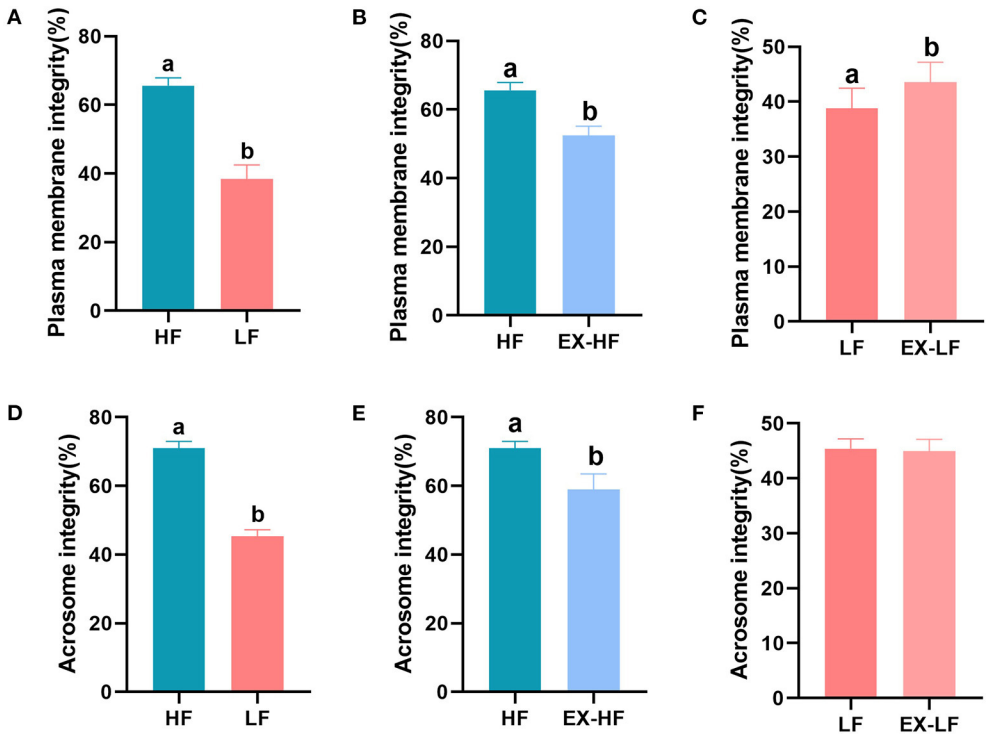

FIGURE 2
Percentage of sperm plasma membrane and acrosome integrity for goats with high and low freezing resilience. **(A–C)** Plasma membrane integrity for HF vs. LF, HF vs. EX-HF, and LF vs. EX-LF and **(D–F)** acrosome integrity for HF vs. LF, HF vs. EX-HF, and LF vs. EX-LF.

selected as differential metabolites, and metabolites with a VI *P*-value > 1 and *P*-value < 0.05 were selected as significant differential metabolites. Then, 38 discriminating differential metabolites were identified in seminal plasma, including 21 in the positive ion mode and 17 in the negative ion mode (Tables 2, 3).

3.4. Analysis of metabolic pathways

Enrichment pathway analysis was performed using Fisher's exact test and the KEGG metabolic pathway database to identify differentially-expressed metabolites. Statistically significant pathways that were overrepresented included central carbon metabolism in cancer; protein digestion and absorption; aminoacyl-tRNA; arginine biosynthesis; ABC transporters; mTOR signaling pathway; alanine, aspartate, and glutamate metabolism; mineral absorption; β-alanine metabolism; proximal tubule bicarbonate reclamation; phenylalanine metabolism; valine, leucine, and isoleucine biosynthesis; pantothenate and CoA biosynthesis; salmonella infection; phenylalanine, tyrosine, and tryptophan biosynthesis; valine, leucine, and isoleucine degradation; and Chagas disease (Figure 4). The enrichment pathway indicated seven differential metabolites, including L-glutamine, L-aspartate, L-arginine, phenylpyruvate, benzoic acid, ketoisocaproic acid, and choline; their abundance was significantly different between seminal plasma from the HF and LF groups (Figures 5–7).

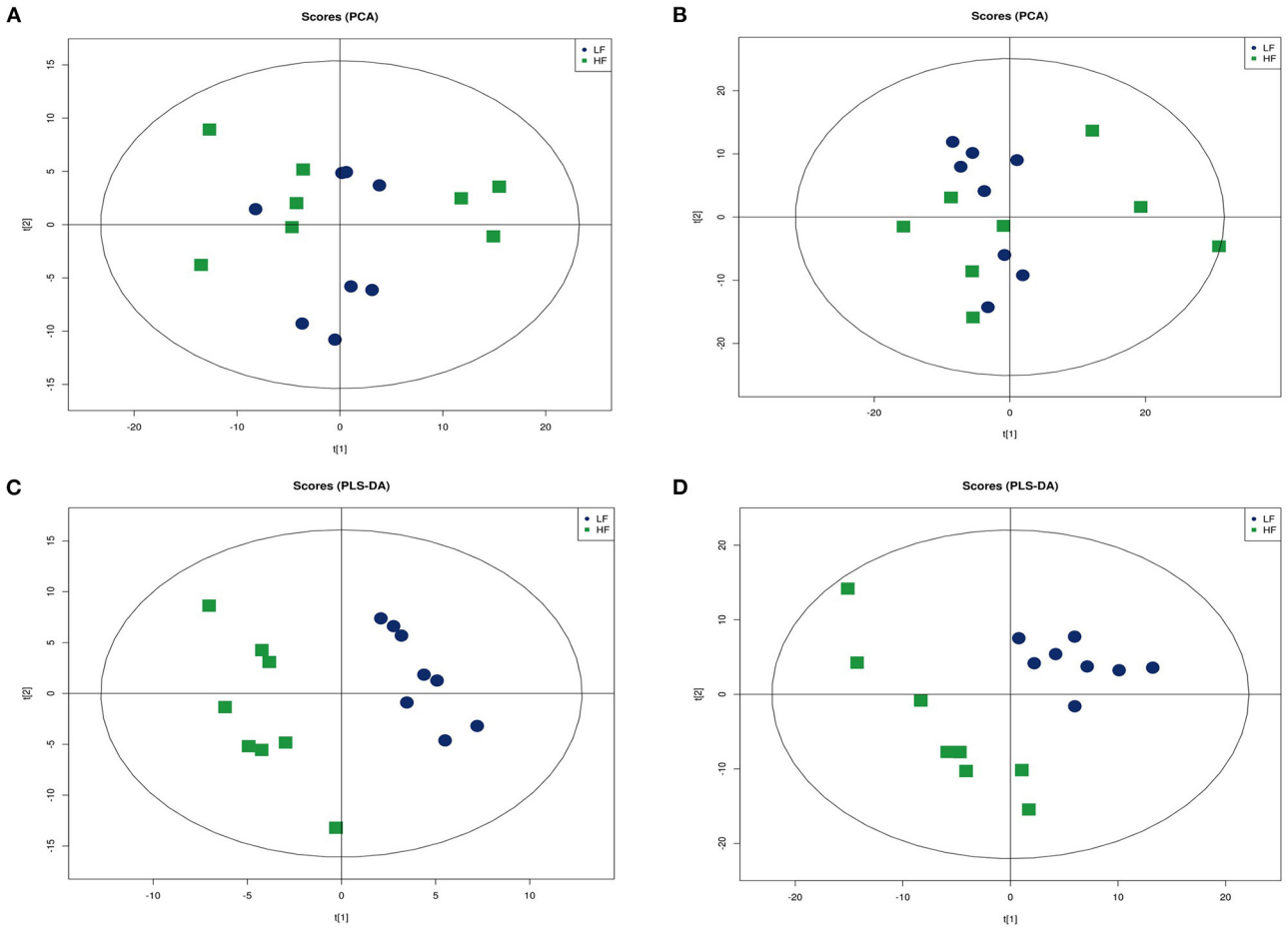

FIGURE 3
Multivariate statistical analysis of seminal plasma metabolic profiling of the HF and LF groups. **(A)** Principal component analysis (PCA) score plots in the positive ion mode. **(B)** PCA score plots in the negative ion mode. **(C)** Partial least squares-discriminant analysis (PLS-DA) score plot in the positive ion mode. **(D)** PLS-DA score plot in the negative ion mode.

4. Discussion

Semen freezability was affected by several factors, including the components of the freezing extender, cryopreservation procedure, lipid composition of sperm, and regulation effect of seminal plasma (20–22). In this study, semen from different individual goats presented diverse degrees of cryodamage, including a remarkable reduction in sperm motility and structural integrity after thawing. Our results demonstrated that seminal plasma makes an important contribution to sperm freezability. Furthermore, HSP significantly improved the post-thaw quality of LF spermatozoa, while LSP decreased the motility parameters and structural integrity of thawed spermatozoa with HF. Several studies have demonstrated the beneficial effects of seminal plasma on sperm cryopreservation. Sperm frozen with HSP exhibited superior motility and cell structural integrity after thawing (9, 23–25). Thus, the variation in freezability of goat semen is associated with differences in seminal plasma composition.

Seminal plasma provides sperm with buffering and energy materials, and small metabolic molecules in seminal plasma are involved in regulating important reproductive events of sperm, such as sperm motility, capacitation, the acrosome reaction, fertilization, and embryo development (26). Metabolomic analysis enables the systematic study of small-molecule metabolites that respond to downstream events in gene expression and protein regulation that are closely related to the functional phenotype of the cell. Additionally, metabolomic analysis of key biomarkers can be used to determine the relationship between metabolite expression patterns and the cellular phenotype (27). In this study, we examined seminal plasma of the HF and LF groups using metabolomic analysis, and PLS-DA revealed a significant separation of the metabolic profiles of the two groups in positive and negative ion patterns. Similar results were found for donkey and pig seminal plasma, i.e., significant differences in the expression patterns of seminal plasma metabolites with different freezing resistance (28, 29). Bioinformatics analysis revealed that the metabolites enriched according to KEGG pathway analysis were significantly different between the HF and LF groups, including L-glutamine, L-aspartate, L-arginine, choline, phenylpyruvate, benzoic acid, and ketoisocaproic acid, which are involved in important physiological functions and amino acid biosynthesis in goat spermatozoa.

TABLE 2 Differential metabolites between the LF and HF groups in the negative ion mode.

HMDB	Description	VIP	FC	*P*-value
HMDB0000094	Citrate	12.43	1.28	0.0842
HMDB0000695	Ketoisocaproic acid	10.54	2.09	0.0083
HMDB0000641	L-Glutamine	7.82	0.64	0.0031
HMDB0004049	20-Hydroxy-PGF2a	4.96	0.45	0.0542
HMDB0001320	15-Keto-PGE1	4.64	0.65	0.0810
HMDB0000210	Pantothenate	3.86	1.66	0.0936
HMDB0000207	Oleic acid	3.53	0.55	0.0464
HMDB0000079	Dihydrothymine	3.34	0.63	0.0025
HMDB0000673	Linoleic acid	2.44	1.62	0.0818
HMDB0000462	Allantoin	1.90	1.35	0.0195
HMDB0002340	2-Methylbenzoic acid	1.67	1.90	0.0236
HMDB0001858	p-Cresol	1.53	2.45	0.0477
HMDB0000205	Phenylpyruvate	1.44	1.71	0.0059
HMDB0001870	Benzoic acid	1.41	1.49	0.0067
HMDB0000191	L-Aspartate	1.25	1.45	0.0225
HMDB0001311	DL-lactate	1.20	0.72	0.0839
HMDB0003345	Alpha-D-Glucose	1.13	1.69	0.0811

TABLE 3 Differential metabolites between the LF and HF groups in the positive ion mode.

HMDB	Description	VIP	FC	*P* value
HMDB0029011	Pro-Arg	9.61	1.30	0.0742
HMDB0000201	Acetylcarnitine	8.26	1.46	0.0147
HMDB0000157	Hypoxanthine	7.73	1.17	0.0891
HMDB0000267	L-Pyroglutamic acid	6.82	0.64	0.0006
HMDB0000687	L-Leucine	4.25	1.36	0.0859
HMDB0000306	Tyramine	4.09	1.43	0.0777
HMDB0000159	L-Phenylalanine	3.48	1.44	0.0713
HMDB0000177	L-Histidine	3.00	1.37	0.0917
HMDB0000641	L-Glutamine	2.97	0.70	0.0002
HMDB0000097	Choline	2.88	0.75	0.0422
HMDB0000062	L-Carnitine	2.77	0.78	0.0260
HMDB0029030	Pro-Val	1.98	1.76	0.0921
HMDB0000848	Stearoylcarnitine	1.86	2.71	0.0022
HMDB0000210	Pantothenate	1.79	1.64	0.0790
HMDB0001403	PGD2	1.44	0.56	0.0051
HMDB0003357	N2-Acetyl-L-ornithine	1.25	0.67	0.0402
HMDB0036458	1-Aminocyclopropane carboxylic acid	1.22	0.66	0.0203
HMDB0000517	L-Arginine	1.12	0.75	0.0741
HMDB0003229	cis-9-Palmitoleic acid	1.09	0.70	0.0054
HMDB0000230	N-Acetylneuraminic acid	1.06	1.53	0.0244
HMDB0000191	L-Aspartate	1.05	1.52	0.0898

The diverse amino acids found in seminal plasma have a variety of biological functions, including reducing free radicals and protecting cells from degeneration (30). The amino acid profiles of bovine seminal plasma with different freezing resistances were clearly distinct and could be used to identify the freezing resistance phenotype (31). Specific amino acids in chicken seminal plasma are associated with sperm viability and DNA integrity after thawing (32). Glutamine is a potent antioxidant in semen, and several studies have found that glutamine protects sperm from reactive oxygen species by enhancing glutathione synthesis (33). Glutamine is a component of sperm cryodilution for several species, such as rabbits (33), boars (34), bucks (35), rams (36), bulls (37), and mouse (38). L-arginine can improve sperm motility and mitochondrial activity after thawing and reduce the structural damage to sperm (39–42). In addition, arginine can increase the proportion of capacitated sperm after thawing. One effective method to produce blastocysts is to add an appropriate amount of arginine during *in vitro* fertilization (43, 44). The addition of aspartate to the medium can protect sperm from oxidative stress damage and plays a positive role in maintaining sperm vitality and reducing lipid peroxidation and DNA fragmentation. Additionally, the developmental ability of bovine sperm embryos treated with aspartic acid was significantly improved (45, 46). Oral aspartate was shown to improve the fertility of young male C5BL/6N mice *in vivo*, increase freeze-thaw sperm quality in sexually immature mice, and play a direct role in the sperm capacitation process and acrosome reaction (47–49).

As a consequence of the extensive protein hydrolysis activity occurring in semen, the concentration of many amino acids increases after ejaculation, and oxidizable substrates capable of acting as energy supplies cause reactions (50, 51). Phenylalanine was identified in bovine seminal plasma and can be considered a biomarker of freezability (31). Moreover, Zhang described the positive effect of leucine on sperm motility in zebrafish (52). Additionally, the concentration of leucine was significantly reduced in the sperm of low-fertility bulls (14). Although a difference in the abundance of phenylalanine and leucine in seminal plasma was found between the two groups in this study, it was not statistically significant. However, the levels of benzoic acid, phenylpyruvate, and ketoisocaproic acid, as intermediate products of phenylalanine and leucine anabolic pathway processes, were significantly different between the HF and LF goat seminal plasma groups in this study, which may indicate a correlation between phenylalanine and leucine anabolism of goat spermatozoa and semen freezability. Menezes compared the metabolic characteristics between bovine spermatozoa with high and low fertility and found remarkable differences in benzoic acid abundance ratios (53). As an intermediate in the anabolic pathway of phenylalanine, the pheA gene encodes a functional enzyme that rearranges chorismate to prephenate and then converts it into phenylpyruvate (54). Benzoic acid is biosynthesized from water-soluble phenylalanine *via two* non-oxidative pathways and a CoA-dependent β-oxidative pathway (55). Spermatogenic cells are equipped to produce ketoisocaproic acid and lactic acid from leucine and glucose using supporting cells (56).

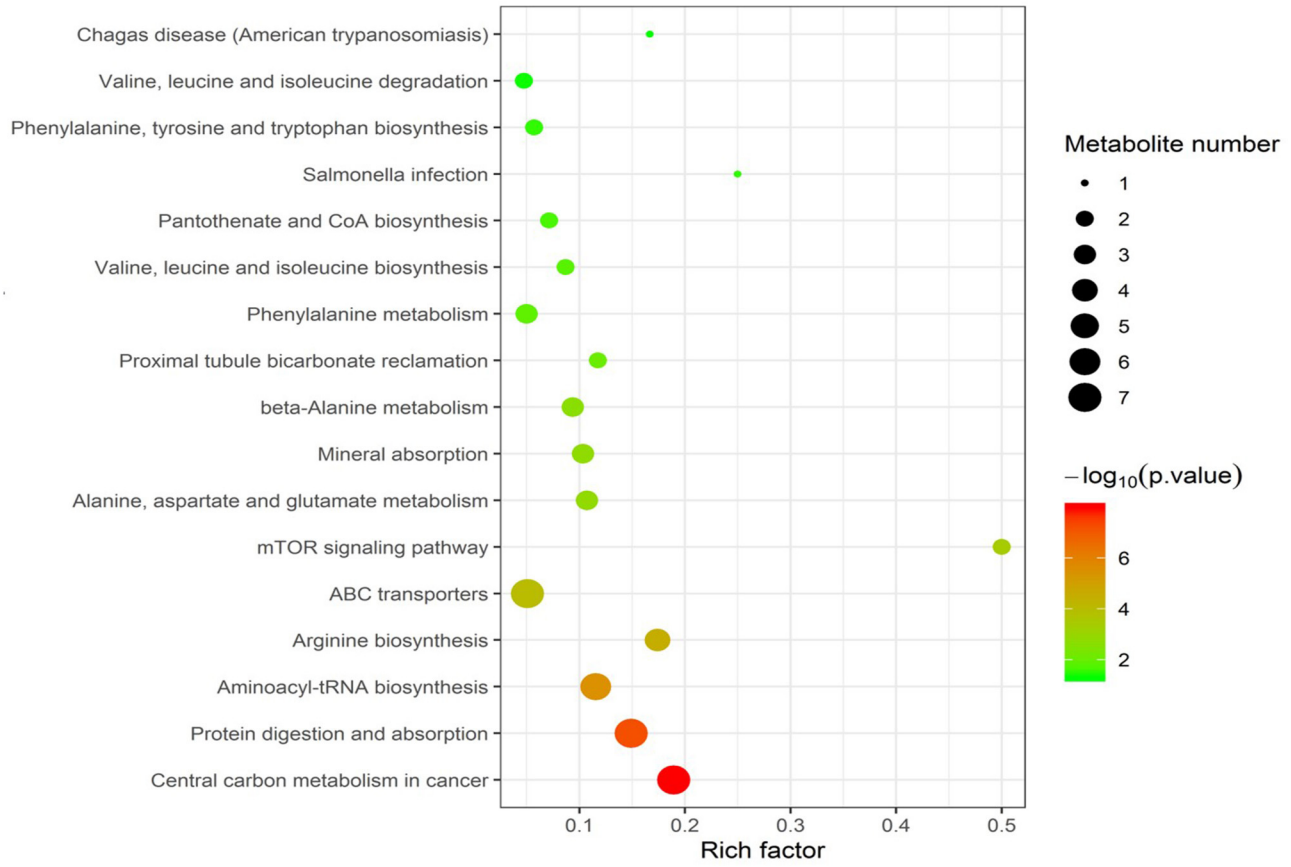

FIGURE 4
Kyoto Encyclopedia of Genes and Genomes enrichment of differential metabolites.

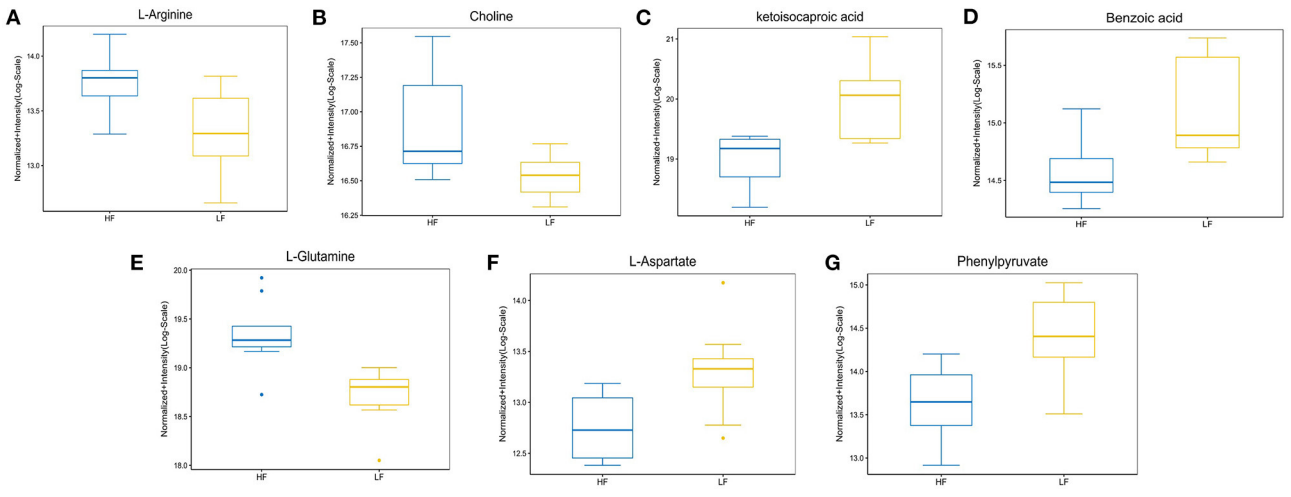

FIGURE 5
The abundance ratio of important metabolites in HF and LF goat seminal plasma. **(A)** L-arginine, **(B)** choline, **(C)** ketoisocaproic acid, **(D)** benzoic acid, **(E)** L-glutamine, **(F)** L-aspartate, and **(G)** phenylpyruvate showed significantly different abundance between HF and LF goat seminal plasma.

Choline is an important precursor for the synthesis of phosphatidylcholine (PC), which is produced in animal cells *via* the CDP-choline pathway. Phosphatidylethanolamine can be converted into PC in cells, which is then catabolized to choline (57). PPC is an essential phospholipid in mammalian cells and tissues, and several studies have illustrated the importance of PC for sperm

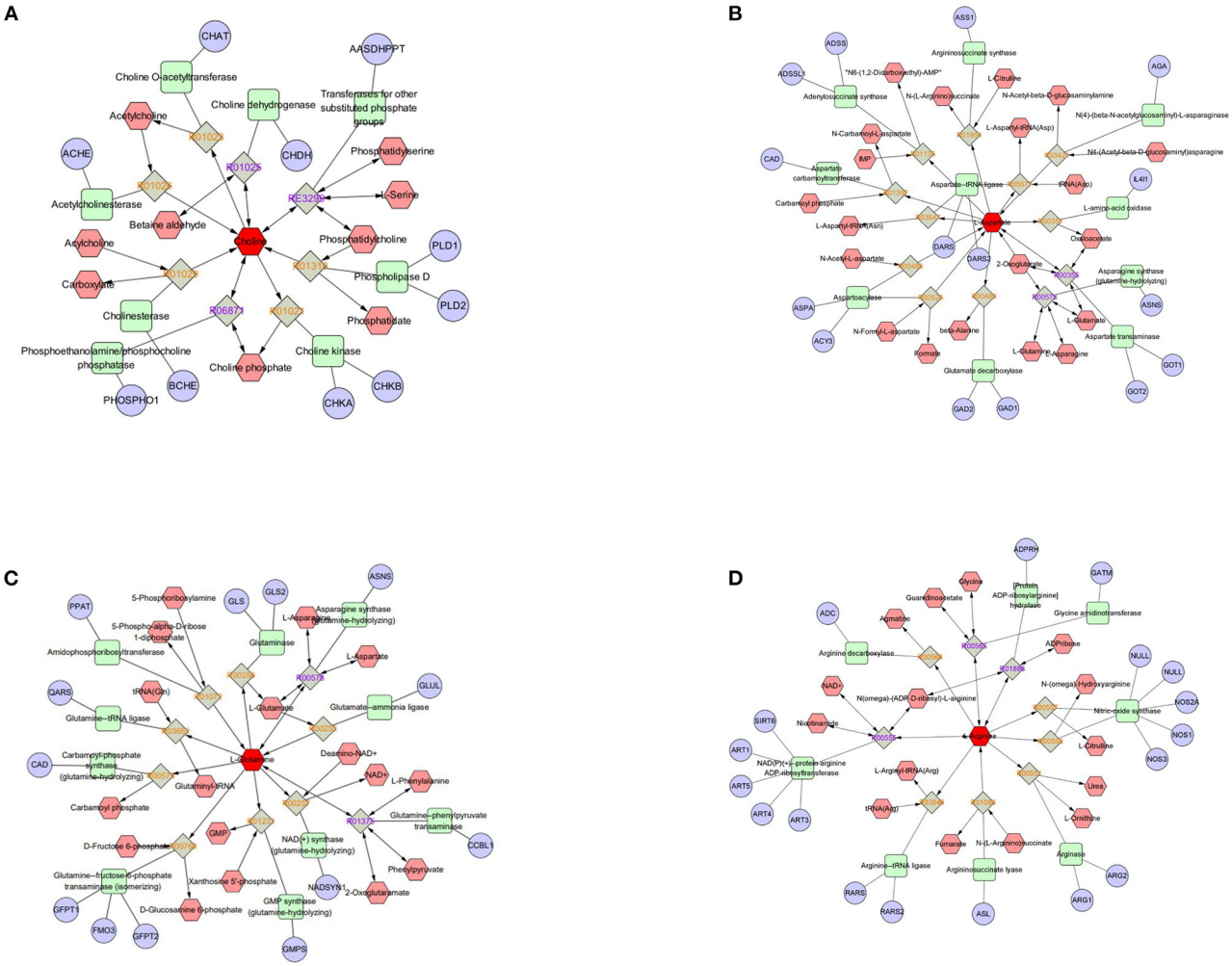

FIGURE 6
Compound-reaction-enzyme-gene networks of important differential metabolite biomarkers de-termined using Metscape. **(A)** Choline, **(B)** L-aspartate, **(C)** L-glutamine, and **(D)** L-arginine. The metabolites are shown in red hexagons. Gray squares: reaction nodes with reaction ID; pale red hexagons: compound nodes; green squares: enzyme nodes; blue circles: gene nodes.

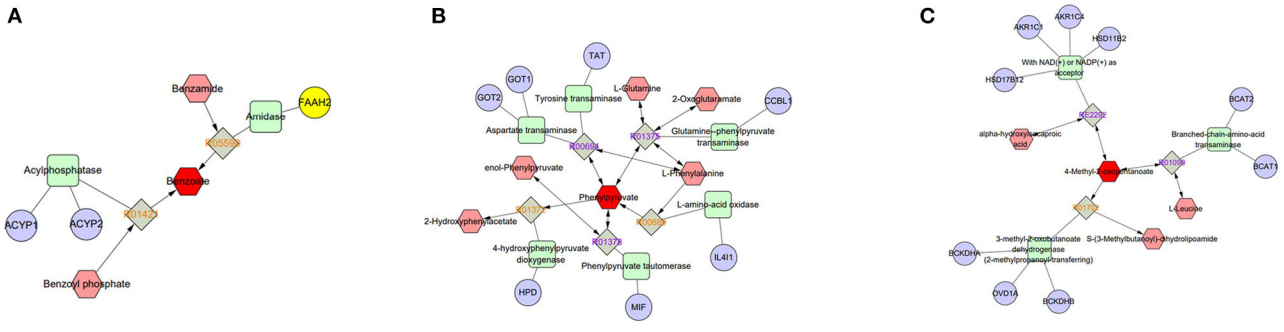

FIGURE 7
Compound-reaction-enzyme-gene networks of important differential metabolite biomarkers de-termined using Metscape. **(A)** Benzoic acid, **(B)** phenylpyruvate, and **(C)** 4-methyl-2-oxopentanoate (ketoisocaproic acid). The metabolites are shown in red hexagons. Gray squares: reaction nodes with reaction ID; pale red hexagons: compound nodes; green squares: enzyme nodes; blue circles: gene nodes.

freezability, including the ability of PC to affect sperm motility and fertilization ability (20, 58, 59). PC is a major component of cryoprotectants during the semen cryopreservation process of several species, and substances such as egg yolk and soy lecithin prevent decreases in sperm viability and structural integrity after thawing (19, 60).

5. Conclusions

In summary, wide individual variability was observed in goat semen freezability, and the composition of seminal plasma may affect sperm freezability. In this study, 41 differential metabolites were identified between goat seminal plasma with high and low semen freezability by metabolomic analysis. Therefore, sperm freezability may be directly affected by amino acids in goat seminal plasma, such as L-glutamine, L-aspartate, and L-arginine. In addition, intermediate metabolites in the anabolic processes of phenylalanine, leucine, and phosphatidylcholine, including phenylpyruvate, benzoic acid, ketoisocaproic acid, and choline, may indirectly regulate the cryotolerance of sperm, and these metabolites may serve as potential biomarkers of goat sperm freezability.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

The animal study was reviewed and approved by the Academic Ethics Committee of Inner Mongolia Agricultural University [Approval No: (2020) 056]. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

BX conceptualized the study and wrote the manuscript. BX, JL, and JiaxZ conceptualized, designed, and carried out the investigations. BX, XB, JianZ, and BL performed the experiments. BX, YZ, RS, RW, ZW, and QL analyzed the data. JiaxZ and JL critically revised the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

JL was employed by Inner Mongolia Jinlai Animal Husbandry Technology Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Methylprednisolone improves the quality of liquid preserved boar spermatozoa *in vitro* and reduces polymorphonuclear neutrophil chemotaxis and phagocytosis

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After artificial insemination, immune cells such as polymorphonuclear neutrophils will be recruited into the genital tract and induce endometrial inflammation, adversely affecting the spermatozoa. This study aimed to analyze the effect of methylprednisolone (MPS) on boar spermatozoa quality of *in vitro* liquid preservation and chemotaxis and phagocytosis of polymorphonuclear neutrophils toward boar spermatozoa. Various concentrations of MPS were added to the extender and analyzed for their effects on spermatozoa motility, kinetic parameters, abnormality rate, total antioxidant capacity (T-AOC) levels, H_2O_2 content, mitochondrial membrane potential and acrosome integrity. Testing of MPS on chemotaxis and phagocytosis of polymorphonuclear neutrophils toward spermatozoa induced by lipopolysaccharide (LPS). The results showed that an extender containing 2×10^{-7}mol/mL MPS was the most effective for preserving boar spermatozoa during *in vitro* liquid preservation at 17°C. It effectively improved spermatozoa motility, kinetic parameters, T-AOC levels, mitochondrial membrane potential and acrosome integrity, reducing the abnormality rate and H_2O_2 content. Meanwhile, the chemotaxis and phagocytosis of polymorphonuclear neutrophils toward spermatozoa under LPS induction were inhibited in a concentration-dependent manner. In conclusion, MPS has positive implications for improving *in vitro* liquid preserved boar spermatozoa quality, inhibiting chemotaxis and phagocytosis of polymorphonuclear neutrophils toward spermatozoa.

KEYWORDS

methylprednisolone, spermatozoa quality, polymorphonuclear neutrophils, chemotaxis, phagocytosis

1. Introduction

In modern swine farms, artificial insemination (AI) is generally used as a fundamental and necessary means of reproduction to maintain normal production conditions (1). After AI, spermatozoa will induce an innate immune response in the uterus after entering the sow's genital tract, and polymorphonuclear neutrophils (PMNs), as one of the most important cellular components of uterine immunity, will be recruited to the uterus in large numbers 1–2 h after

insemination (2–4). As a foreign cell, the spermatozoa are antigenic and the immune cells in the swine's uterus phagocytosis most of the spermatozoa so that only a small percentage of spermatozoa can reach the fallopian tube and have a chance to unite with the egg (5–7).

In addition to spermatozoa that can induce uterine immunity, bacterial invasion can also cause the onset of the uterine immune response. This is mainly due to mechanical damage and continuous manipulation in AI, which can lead to sterility in the sow and affect the reproductive cycle (8). The bacteria that cause uterine immunity are mainly Gram-negative bacteria such as Enterobacter, Burkholderia, Serratia and Parvimonas (9). Lipopolysaccharide (LPS) released by Gram-negative bacteria affects the endometrial epithelium of the sow. It induces uterine inflammation, so this substance is often used to mimic the inflammatory response *in vitro* (10). This cytotoxicity not only affects sows, but also reduces the quality of boar spermatozoa under *in vitro* liquid preservation. However, substances such as antibiotics have been used to contain the harmful effects of bacteria in the extender (11).

Although the results of boar spermatozoa under *in vitro* liquid preservation have been partially satisfactory, limitations such as short storage time, microbial colonization in extenders and induction of uterine immune response still need improvement (12–14). Therefore, how to improve the quality of *in vitro* liquid preservation of boar spermatozoa and reduce the loss of spermatozoa due to uterine immunity to enhance the fertility rate has become one of the crucial directions of current research on swine AI technology. We have demonstrated in a study that dexamethasone (DEX) improves the quality of *in vitro* liquid preservation boar spermatozoa and inhibits chemotaxis and phagocytosis of PMNs towards spermatozoa (15). Methylprednisolone (MPS), another common glucocorticoid, has a better biological effect compared to DEX, thus completing immunosuppression faster (16). However, few studies have reported the effects of MPS on the quality of boar semen at *in vitro* liquid preservation (17°C) or the chemotaxis and phagocytosis of PMNs toward spermatozoa.

Given this, we believe that adding MPS to extenders may also have a beneficial effect. Glucocorticoids are thought to act directly on cell membrane receptors and modulate the immune system through non-genomic mechanisms to exert anti-inflammatory effects (17). Therefore, our study aimed to assess whether extenders containing MPS are beneficial for *in vitro* liquid preservation of boar spermatozoa by measuring spermatozoa motility, abnormality rate, kinetic parameters, plasma membrane integrity, mitochondrial activity, total antioxidant capacity (T-AOC) activity and H_2O_2 content by a computer-assisted spermatozoa analysis (CASA) system or spectrophotometer. The effects of MPS on the chemotaxis and phagocytosis of PMNs induced by LPS were analyzed by blind well chemotaxis chamber test and phagocytosis test.

2. Materials and methods

2.1. Chemical sources and preparation

Unless otherwise stated, all chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA). The modified Modena extender consists of 152.64 mmol/L glucose, 46.64 mmol/L Tris, 26.74 mmol/L sodium citrate, 15.09 mmol/L citric acid, 11.90 mmol/L sodium bicarbonate, 6.98 mmol/mL EDTA-2Na·H_2O, 1 million U/L streptomycin, 1 million U/L penicillin and 4.00 g/L BSA (prepared just before use). Erythrocyte lysis buffer consists of 150.00 mmol/L NH_4Cl, 12.00 mmol/L $KHCO_3$ and 0.12 mmol/L EDTA. Processed on a sterile bench through a 0.22 μm filter to ensure no bacterial contamination and placed at 4°C. These chemicals were dissolved using pure water and adjusted to the appropriate concentration.

2.2. Semen collection and treatment

Six large white boars aged 2–3 years were selected for semen collection from a commercial insemination station (Jingyu Animal Husbandry, Daqing, China). The boars were kept in separate pens with natural light and free access to water and food. Semen was collected once a week for a total of 4 times to obtain the final 24 samples. Samples were stored at 17°C and sent to the laboratory within 2 h. Assessment of spermatozoa viability using CASA (Songjingtianlun Biotechnology, Nanning, China), only if the quality of the spermatozoa meets the requirements (milky white, and slightly smelly, with viability >80%), we will conduct follow-up experiments. Then, the fresh semen was centrifuged at 750 × *g* for 3 min at 17°C (18). The supernatant was then removed, and the spermatozoa pellet was resuspended at a concentration of 1×10^8 cells/mL in modified Modena extender containing different concentrations of MPS (control (0 mol/mL), 1×10^{-7} mol/mL, 2×10^{-7} mol/mL, 4×10^{-7} mol/mL and 6×10^{-7} mol/mL). Finally, extended spermatozoa samples were all stored in the incubator, and the temperature was set at 17°C. Spermatozoa quality parameters were detected at each time point (0, 1, 2, 3, 4, and 5) days. Each concentration of MPS samples was set to be repeated four times. Furthermore, fresh semen was used for the chemotaxis and phagocytosis assay of PMNs.

2.3. Spermatozoa motility and kinetic parameters evaluation

CASA was used to analyze spermatozoa motility and kinetic parameters. Pipette 10 μL of sample onto a chamber slide (chamber depth 10 μm) with coverslip and preheat for 15 min at 37°C. The standard parameters were set at 30 frames/s. The measured spermatozoa kinetic parameters included average path velocity (VAP), average straight-line velocity (VSL) and average curvilinear velocity (VCL). We defined motility as the percentage of spermatozoa with straightness of path (STR) > 75% and VSL > 25 μm/s. The sample was analyzed using accompanying software, with 5 observation fields randomly selected for each sample and at least 150 spermatozoa recorded per field. This evaluation was technically repeated four times.

2.4. Spermatozoa morphology evaluation, T-AOC activity and H_2O_2 content determination

Spermatozoa morphology evaluation was assessed by Williams staining as reported by Kavak et al. (19) with appropriate modifications. Briefly, 5 μL aliquots of spermatozoa were placed on a slide and naturally dried. The slides were fixed in absolute ethanol for 2–3 min, naturally dried, then immersed in 0.5% chloramine T for

1–2 min, washed with pure water for 1–2 min, dehydrated quickly in 96% ethanol, naturally dried, then rehydrated with carbonic acid solution for 10–15 min, washed twice, stained with merine, naturally dried. A positive phase-contrast microscope (TS100F; Nikon, Tokyo, Japan) was used for observing the samples at 1000 × magnification (Nikon 20 × 0.40 PLAN objective). Spermatozoa morphology was assessed subjectively by counting 200 spermatozoa, differentiating spermatozoa head deformities and spermatozoa tail deformities (coiled tails, or tails folded at the neck and midpiece). This evaluation was technically repeated four times.

T-AOC activity and hydrogen peroxide (H_2O_2) content were determined using the assay kit according to the manufacturer's operating instructions (Jiancheng Bioengineering Institute, Nanjing, China). Sample preparation was carried out according to the operating instructions. A spectrophotometer (PERSEE, Beijing, China) measured T-AOC activity at 520 nm and H_2O_2 content at 405 nm. This evaluation was technically repeated four times.

2.5. Mitochondrial membrane potential evaluation

Mitochondrial membrane potential was assessed by JC-1 and propidium iodide (PI) as reported by Ma et al. (20) with appropriate modifications. JC-1 is a fluorescent probe widely used to detect mitochondrial membrane potential. When the mitochondrial membrane potential is at a high level, the probe aggregates in the mitochondrial matrix to form a polymer; when the mitochondrial membrane potential is at a low level, the probe cannot aggregate in the mitochondrial matrix, and the probe is monomeric (21). Briefly, add 100 µL of sample to 400 µL of isotonic buffer diluent containing 1 mmol/L JC-1 and 5 mmol/L PI, mix well and incubate for 30 min at 37°C. Add 15 µL of sample dropwise onto a slide with coverslip, observe by inverted fluorescence microscope (Mshot photoelectric technology, Guangzhou, China) and analyze the sample using the accessory software. Each view was not less than 200 spermatozoa. The heads of spermatozoa with high mitochondrial membrane potential (hMMP) show red fluorescence, while the heads of spermatozoa with medium and low mitochondrial membrane potential show green fluorescence. This evaluation was technically repeated four times.

2.6. Spermatozoa acrosome integrity evaluation

Spermatozoa acrosome integrity was assessed by fluorescein peanut agglutinin isothiocyanate (FITC-PNA) as reported by Aboagla et al. (22) with appropriate modifications. Briefly, 30 µL aliquots of spermatozoa were placed on a slide and naturally dried, fixed using methanol for 10 min at 22–25°C. Then 30 µL FITC-PNA solution (100 µg/mL) in phosphate-buffered saline (PBS) was spread over each slide. The slides were incubated in an incubator for 10 min at 37°C, protected from light. The slides were then gently rinsed with PBS and naturally dried. Mounted with 10 µL of an antifade solution to preserve fluorescence. The slide surface is covered with coverslip and sealed with colorless varnish. The acrosome status of the spermatozoa was examined using an epifluorescence microscope (Ti2-U; Nikon, Tokyo, Japan). Each view was not less than 200 spermatozoa. This evaluation was technically repeated four times.

2.7. Swine PMNs preparation

Depending on the estrus of the Large White sows, 10–20 mL of peripheral venous blood was collected during the luteal phase using a heparinized vacuum blood collection tube. Delivered to the laboratory within 2 h at 4°C and then processed to collect PMNs. Centrifuge the samples at 1000 × *g* for 10 min at 4°C using a refrigerated centrifuge (SIGMA Laborzentrifugen, Osterode am Harz, Germany), and the buffy coat in the pooled blood plasma from 2 to 4 sows was collected and mixed with 10 mL of PBS in a screw-capped polypropylene centrifuge tube. The buffy coat was washed twice with PBS by centrifugation with 320 × *g* for 10 min at 4°C. The pellet was re-suspended in 4 mL of PBS, carefully layered over 3 mL Histopaque®-1077, and centrifuged at 400 × *g* for 30 min. The supernatant including the mononuclear cells at the interface was removed. Resuspend the particles in 4 mL of erythrocyte lysis buffer. Three minutes later, the solution was centrifuged at 400 × *g* for 10 min, and the lysis procedure was repeated on the subsequent pellet. The PMNs pellet was washed twice at 400 × *g* for 10 min with 5 mL of PBS, resuspended at 1×10^8 cells/mL in 1 mL of PBS, and stored at 4°C until use. The PMNs suspension was used for each replicate within 24 h after preparation. For phagocytosis assay and chemotaxis assay of PMNs, PMNs were also collected from peripheral venous blood of mature boars to ensure that the phagocytotic activities of PMNs are from sows.

2.7.1. PMNs chemotaxis assay

The chemotaxis of PMNs from venous blood to spermatozoa was determined by blind well chemotaxis chamber (BW100; Neuro Probe, Gaithersburg, MD, USA) (23). Briefly, fill the lower chamber with 100 µL of resuspended sample, and place the polycarbonate film (PFA8; Neuro Probe, Gaithersburg, MD, USA) with holes (Ø8 µm) on the upper part of the lower chamber. Connecting tubular assemblies to construct upper chambers and fill with 100 µL of 1×10^7/mL neutrophil TL-HEPES suspension (with different concentrations of MPS). The blind well chemotaxis chamber was incubated at 38.5°C for 90 min, after which the polycarbonate membrane was removed, turned over and placed on a slide and fixed using a clamp. After 15 min of methanol fixation, the polycarbonate membrane was stained with Giemsa solution for 10–15 min. Washing with pure water and treatment with 90% xylene, the polycarbonate membrane was exposed to glacial acetic acid and covered with coverslips. Under a positive phase-contrast microscope with 400× magnification, the number of PMNs passing through the filter was counted at three different areas (0.1520 mm^2 per area) of each filter. The mean number of PMNs per mm^2 in three different filter areas was recorded as an observed chemotaxis index of PMNs. This evaluation was technically repeated four times.

2.7.2. PMNs phagocytosis assay

The phagocytosis of PMNs to spermatozoa assay was referred to Matthijs et al. (24) with appropriate modifications. Briefly, 80 µL aliquots of PMNs suspension in TL-HEPES containing various supplements (1×10^{-6} g/mL LPS + different concentrations of MPS)

were transferred to a polystyrene culture dish. PMNs suspension was mixed with 20 µL spermatozoa suspension and incubated for 60 min at 38.5°C and 5% CO_2. The final concentrations of PMNs and spermatozoa were 8×10^6 cells/mL and 2×10^6 cells/mL, respectively. After incubation, an equal volume of heparin (40 mg/mL in TL-HEPES) was added to the solution of PMNs and spermatozoa to facilitate the dissociation of agglutinated PMNs. The samples were mixed thoroughly, left for 15 min, and mixed again. Subsamples of 75 µL were fixed by adding 25 µL of 2% (v/v) glutaraldehyde. The fixed samples were mounted on the glass slides and examined under positive phase-contrast microscope at 400× magnification. A minimum of 200 PMNs were counted in each area of the specimen. The percentage of PMNs with phagocytized spermatozoa was recorded as phagocytosis rate. This evaluation was technically repeated four times.

2.8. Statistical analysis

All data from each experiment were tested for normality with one-sample Kolmogorov–Smirnov's test. If some data did not show normality, we arcsine-transformed the variable before the analysis and again checked the normality using one sample Kolmogorove-Smirnov's test for this parameter. Furthermore, all data from each experiment were checked for the homogeneity of variances using Levene's test. The test results showed that the replicated data from each experiment were homogeneous. Then, all data were analyzed by one-way Analysis of variance using STATVIEW 5.0 (Abacus Concepts, Berkeley, CA, USA). If the ANOVA *P*-value was less than 0.05, a Bonferroni/Dunn's HSD test was carried out using the same program. All data were expressed as mean ± SD. Findings were considered significantly different at $P < 0.05$.

3. Results

3.1. Effects of different concentrations of MPS on boar spermatozoa motility and kinetic parameters

Effects of different concentrations of MPS on boar spermatozoa motility as shown in Table 1. On day 2 of spermatozoa preservation, motility was significantly higher in 1×10^{-7} mol/mL MPS, 2×10^{-7} mol/mL MPS and 4×10^{-7} mol/mL MPS samples than in control samples ($P < 0.05$). On the day 3 of spermatozoa preservation, motility was significantly higher in 1×10^{-7} mol/mL MPS and 2×10^{-7} mol/mL MPS samples than in 6×10^{-7} mol/mL MPS and control samples ($P < 0.05$). On the day 4 of spermatozoa preservation, motility was significantly higher in 1×10^{-7} mol/mL MPS, 2×10^{-7} mol/mL MPS and 4×10^{-7} mol/mL MPS samples than in 6×10^{-7} mol/mL MPS and control samples ($P < 0.05$). On the day 5 of spermatozoa preservation, motility was significantly higher in 1×10^{-7} mol/mL MPS and 2×10^{-7} mol/mL MPS samples than in 6×10^{-7} mol/mL MPS and control samples ($P < 0.05$).

Effects of different concentrations of MPS on boar spermatozoa VAP as shown in Table 2. On the day 2 and 3 of spermatozoa preservation, VAP was significantly higher in 2×10^{-7} mol/mL MPS samples than in control samples ($P < 0.05$). On the day 5 of spermatozoa preservation, VAP was significantly higher in 1×10^{-7} mol/mL MPS, 2×10^{-7} mol/mL MPS and 4×10^{-7} mol/mL MPS samples than in 6×10^{-7} mol/mL MPS and control samples ($P < 0.05$), with 2×10^{-7} mol/mL MPS samples having the highest VAP.

Effects of different concentrations of MPS on boar spermatozoa VSL as shown in Table 3. On the day 3 and 4 of spermatozoa preservation, VSL was significantly higher in 1×10^{-7} mol/mL MPS, 2×10^{-7} mol/mL MPS and 4×10^{-7} mol/mL MPS samples than in 6×10^{-7} mol/mL MPS and control samples ($P < 0.05$). On the day 5 of spermatozoa preservation, VSL of each MPS samples was significantly higher than in control sample ($P < 0.05$), with 2×10^{-7} mol/mL MPS samples having the highest VSL.

Effects of different concentrations of MPS on boar spermatozoa VCL as shown in Table 4. On the day 5 of spermatozoa preservation, VCL of each MPS samples was significantly higher than in control sample ($P < 0.05$), with 2×10^{-7} mol/mL MPS samples having the highest VCL.

3.2. Effects of different concentrations of MPS on boar spermatozoa morphology, T-AOC activity and H_2O_2 content

Effects of different concentrations of MPS on boar spermatozoa morphology as shown in Table 5. On the day 4 of spermatozoa preservation, abnormality rate was significantly lower in 1×10^{-7} mol/mL MPS, 2×10^{-7} mol/mL MPS and 4×10^{-7} mol/mL MPS samples than in 6×10^{-7} mol/mL MPS and control samples ($P < 0.05$). On the day 5 of spermatozoa preservation, abnormality rate was significantly lower in 1×10^{-7} mol/mL MPS, 2×10^{-7} mol/mL MPS and 4×10^{-7} mol/mL MPS samples than in control samples ($P < 0.05$), with 2×10^{-7} mol/mL MPS samples having the lowest abnormality rate. Compared with other MPS samples, the effect of

TABLE 1 Effects of different concentrations of methylprednisolone (MPS) on boar spermatozoa motility (%).

Treatments	Storage time (day)					
	0	1	2	3	4	5
Control	94.17 ± 1.36^a	89.49 ± 2.15^a	75.43 ± 2.13^b	64.71 ± 2.19^c	55.57 ± 2.10^c	46.87 ± 1.74^c
1×10^{-7} mol/mL MPS	94.17 ± 1.36^a	89.26 ± 2.88^a	78.71 ± 2.06^a	70.91 ± 2.06^a	65.26 ± 2.04^a	54.56 ± 1.99^a
2×10^{-7} mol/mL MPS	94.17 ± 1.36^a	90.98 ± 1.17^a	79.71 ± 1.85^a	72.68 ± 2.75^a	67.83 ± 2.68^a	57.33 ± 1.56^a
4×10^{-7} mol/mL MPS	94.17 ± 1.36^a	90.46 ± 1.46^a	79.13 ± 1.49^a	70.44 ± 2.03^{ab}	61.40 ± 2.12^b	51.15 ± 2.33^b
6×10^{-7} mol/mL MPS	94.17 ± 1.36^a	90.75 ± 3.55^a	76.87 ± 1.84^{ab}	67.22 ± 2.02^{bc}	57.91 ± 2.12^c	49.18 ± 2.02^{bc}

In the same column, values with different letter superscripts mean a significant difference ($P < 0.05$). Similarly here in after.

TABLE 2 Effects of different concentrations of methylprednisolone (MPS) on boar spermatozoa average path velocity (VAP; μm/s).

Treatments	Storage time (day)					
	0	1	2	3	4	5
Control	45.49 ± 0.77^{a}	42.72 ± 2.04^{a}	34.55 ± 2.25^{b}	28.07 ± 1.74^{c}	23.16 ± 1.38^{c}	18.19 ± 2.54^{c}
1×10^{-7} mol/mL MPS	45.49 ± 0.77^{a}	44.02 ± 0.99^{a}	36.10 ± 1.34^{ab}	31.90 ± 2.83^{ab}	26.77 ± 1.71^{b}	23.21 ± 1.52^{b}
2×10^{-7} mol/mL MPS	45.49 ± 0.77^{a}	43.86 ± 2.03^{a}	37.90 ± 2.60^{a}	33.72 ± 1.78^{a}	31.09 ± 2.41^{a}	27.48 ± 1.44^{a}
4×10^{-7} mol/mL MPS	45.49 ± 0.77^{a}	43.94 ± 0.97^{a}	35.60 ± 1.42^{ab}	30.40 ± 3.06^{abc}	26.13 ± 1.72^{bc}	22.15 ± 1.88^{b}
6×10^{-7} mol/mL MPS	45.49 ± 0.77^{a}	41.36 ± 2.34^{a}	34.94 ± 0.88^{b}	28.44 ± 4.57^{bc}	24.16 ± 2.97^{bc}	18.49 ± 1.86^{c}

TABLE 3 Effects of different concentrations of methylprednisolone (MPS) on boar spermatozoa average straight-line velocity (VSL; μm/s).

Treatments	Storage time (day)					
	0	1	2	3	4	5
Control	45.19 ± 1.44^{a}	38.46 ± 2.34^{a}	34.67 ± 1.44^{a}	27.70 ± 0.83^{c}	19.92 ± 1.75^{c}	14.61 ± 1.77^{c}
1×10^{-7} mol/mL MPS	45.19 ± 1.44^{a}	41.37 ± 2.00^{a}	36.58 ± 2.80^{a}	31.05 ± 0.32^{b}	26.64 ± 1.17^{b}	20.18 ± 1.32^{b}
2×10^{-7} mol/mL MPS	45.19 ± 1.44^{a}	41.38 ± 4.52^{a}	37.40 ± 2.31^{a}	33.46 ± 1.59^{a}	29.53 ± 2.27^{a}	24.76 ± 1.57^{a}
4×10^{-7} mol/mL MPS	45.19 ± 1.44^{a}	40.03 ± 3.34^{a}	35.93 ± 2.08^{a}	30.25 ± 1.59^{b}	26.02 ± 1.76^{b}	19.69 ± 2.13^{b}
6×10^{-7} mol/mL MPS	45.19 ± 1.44^{a}	39.04 ± 1.67^{a}	35.42 ± 1.83^{a}	27.90 ± 1.92^{c}	22.33 ± 1.42^{c}	17.82 ± 1.79^{b}

TABLE 4 Effects of different concentrations of methylprednisolone (MPS) on boar spermatozoa average curvilinear velocity (VCL; μm/s).

Treatments	Storage time (day)					
	0	1	2	3	4	5
Control	64.85 ± 1.60^{a}	55.27 ± 1.72^{a}	44.40 ± 1.66^{b}	38.15 ± 1.47^{b}	30.43 ± 1.69^{c}	23.93 ± 1.06^{d}
1×10^{-7} mol/mL MPS	64.85 ± 1.60^{a}	56.02 ± 2.79^{a}	46.10 ± 1.66^{ab}	41.64 ± 1.30^{ab}	34.52 ± 1.52^{ab}	30.74 ± 1.03^{b}
2×10^{-7} mol/mL MPS	64.85 ± 1.60^{a}	57.08 ± 1.99^{a}	47.59 ± 1.89^{a}	42.97 ± 2.68^{a}	36.49 ± 2.07^{a}	34.68 ± 2.08^{a}
4×10^{-7} mol/mL MPS	64.85 ± 1.60^{a}	56.89 ± 2.00^{a}	45.51 ± 2.10^{ab}	39.37 ± 2.54^{ab}	32.61 ± 1.17^{bc}	29.08 ± 1.43^{bc}
6×10^{-7} mol/mL MPS	64.85 ± 1.60^{a}	56.71 ± 3.03^{a}	45.20 ± 1.12^{ab}	39.94 ± 2.66^{ab}	32.83 ± 2.42^{bc}	27.47 ± 1.97^{c}

TABLE 5 Effects of different concentrations of methylprednisolone (MPS) on boar spermatozoa abnormality rate (%).

Treatments	Storage time (day)					
	0	1	2	3	4	5
Control	4.16 ± 0.46^{a}	6.48 ± 0.78^{a}	8.48 ± 0.69^{a}	10.96 ± 1.82^{a}	13.14 ± 1.25^{a}	17.48 ± 1.57^{a}
1×10^{-7} mol/mL MPS	4.16 ± 0.46^{a}	5.46 ± 0.69^{a}	7.59 ± 0.69^{ab}	8.70 ± 1.20^{bc}	8.80 ± 0.85^{c}	14.46 ± 1.46^{b}
2×10^{-7} mol/mL MPS	4.16 ± 0.46^{a}	5.67 ± 0.69^{a}	6.64 ± 0.70^{b}	7.74 ± 1.41^{c}	8.27 ± 0.59^{c}	11.61 ± 2.21^{c}
4×10^{-7} mol/mL MPS	4.16 ± 0.46^{a}	5.96 ± 0.91^{a}	7.91 ± 0.54^{ab}	9.30 ± 1.00^{abc}	10.42 ± 1.73^{b}	14.31 ± 1.24^{b}
6×10^{-7} mol/mL MPS	4.16 ± 0.46^{a}	6.41 ± 0.41^{a}	8.85 ± 1.53^{a}	10.04 ± 1.27^{ab}	12.55 ± 0.36^{a}	16.75 ± 1.34^{ab}

6×10^{-7} mol/mL MPS samples on abnormality rate during the whole storage period shows the opposite result, although the difference is not statistically significant.

Effects of different concentrations of MPS on boar spermatozoa T-AOC activity and H_2O_2 content as shown in Table 6. T-AOC activity was significantly higher in 1×10^{-6} g/mL LPS + 2×10^{-7} mol/mL MPS samples and 2×10^{-7} mol/mL MPS samples than in 1×10^{-6} g/mL LPS and control samples at all three test points ($P < 0.05$). T-AOC activity was lower in 1×10^{-6} g/mL LPS samples than in control samples, but this difference was only significant at day 3 ($P < 0.05$). The difference in H_2O_2 content between 1×10^{-6} g/mL LPS + 2×10^{-7} mol/mL MPS and 2×10^{-7} mol/mL MPS samples was not significant at all three test points ($P > 0.05$). On the day 3 and 5 of spermatozoa preservation, H_2O_2 content was significantly lower in 1×10^{-6} g/mL LPS + 2×10^{-7} mol/mL MPS and 2×10^{-7} mol/mL MPS samples than in 1×10^{-6} g/mL LPS samples ($P < 0.05$).

3.3. Effect of different concentrations of MPS on mitochondrial membrane potential

Effects of different concentrations of MPS on mitochondrial membrane potential as shown in Figure 1. On the day 3 of spermatozoa preservation, hMMP levels of each MPS samples were

TABLE 6 Effects of different concentrations of methylprednisolone (MPS) on boar spermatozoa total antioxidant capacity (T-AOC) activity and H_2O_2 content.

Items	Treatments	Storage time (day)		
		1	3	5
T-AOC activity (U/mL)	Control	5.37 ± 1.08^{b}	3.55 ± 0.76^{b}	1.97 ± 0.50^{b}
	1×10^{-6} g/mL LPS	4.63 ± 0.42^{b}	2.56 ± 0.88^{c}	0.99 ± 0.14^{b}
	1×10^{-6} g/mL LPS + 2×10^{-7} mol/mL MPS	6.60 ± 0.68^{a}	5.30 ± 0.37^{a}	3.95 ± 1.16^{a}
	2×10^{-7} mol/mL MPS	7.24 ± 0.56^{a}	5.61 ± 0.29^{a}	3.73 ± 0.91^{a}
H_2O_2 content (mmol/L)	Control	13.21 ± 1.72^{ab}	20.49 ± 2.86^{ab}	33.01 ± 1.90^{b}
	1×10^{-6} g/mL LPS	14.82 ± 1.07^{a}	22.91 ± 2.16^{a}	36.17 ± 1.43^{a}
	1×10^{-6} g/mL LPS + 2×10^{-7} mol/mL MPS	13.05 ± 1.80^{ab}	18.65 ± 2.65^{b}	29.03 ± 1.71^{c}
	2×10^{-7} mol/mL MPS	12.42 ± 2.02^{ab}	17.22 ± 2.43^{b}	27.74 ± 1.54^{c}

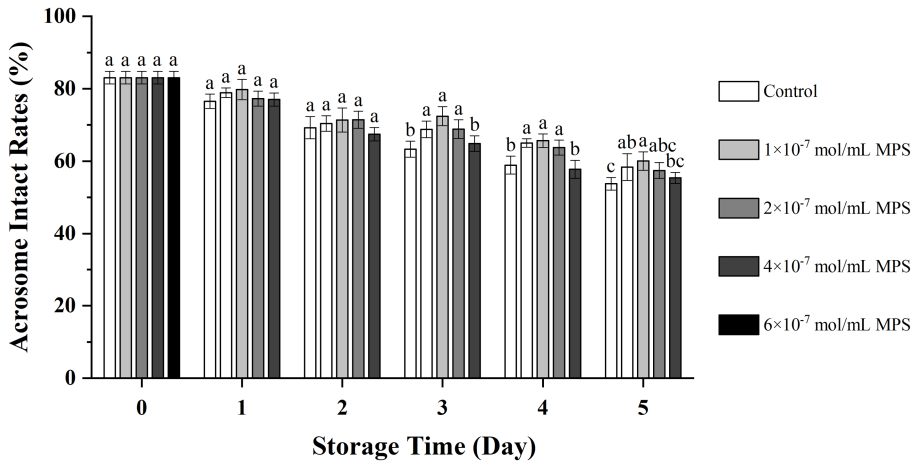

FIGURE 1
Effects of different concentrations of methylprednisolone (MPS) on mitochondrial membrane potential. In the figure, values with different letters mean a significant difference (P<0.05). Similarly here in after.

significantly higher than in control sample ($P < 0.05$). On day 4 of spermatozoa preservation, hMMP levels was significantly higher in 1×10^{-7} mol/mL MPS, 2×10^{-7} mol/mL MPS and 4×10^{-7} mol/mL MPS samples than in 6×10^{-7} mol/mL MPS and control samples ($P < 0.05$). On day 5 of spermatozoa preservation, hMMP levels was significantly higher in 2×10^{-7} mol/mL MPS samples than in other MPS samples ($P < 0.05$).

3.4. Effect of different concentrations of MPS on acrosome integrity

Effects of different concentrations of MPS on acrosome integrity as shown in Figure 2. On the day 3 and 4 of spermatozoa preservation, acrosome integrity was significantly higher in 1×10^{-7} mol/mL MPS, 2×10^{-7} mol/mL MPS and 4×10^{-7} mol/mL MPS samples than in 6×10^{-7} mol/mL MPS and control samples ($P < 0.05$). On day 5 of spermatozoa preservation, acrosome integrity was significantly higher in 2×10^{-7} mol/mL MPS samples than in 6×10^{-7} mol/mL MPS and control samples ($P < 0.05$).

3.5. Effects of MPS on the chemotaxis of PMNs toward spermatozoa

Effects of MPS on the chemotaxis of PMNs toward spermatozoa as shown in Figure 3. The chemotaxis index of PMNs to spermatozoa of each MPS sample is significantly lower than in control sample ($P < 0.05$). And chemotaxis index was reduced in a MPS concentration-dependent manner. In addition, there was no significant difference in the chemotaxis index between the 4×10^{-7} mol/mL MPS and 6×10^{-7} mol/mL MPS samples ($P > 0.05$).

3.6. Effects of MPS on the phagocytosis of PMNs toward spermatozoa

Effects of MPS on the phagocytosis of PMNs toward spermatozoa as shown in Figure 4. The phagocytosis percentage of PMNs to spermatozoa of each MPS sample is significantly lower than in control sample ($P < 0.05$). And phagocytosis percentage was also reduced in a MPS concentration-dependent manner. The difference between the

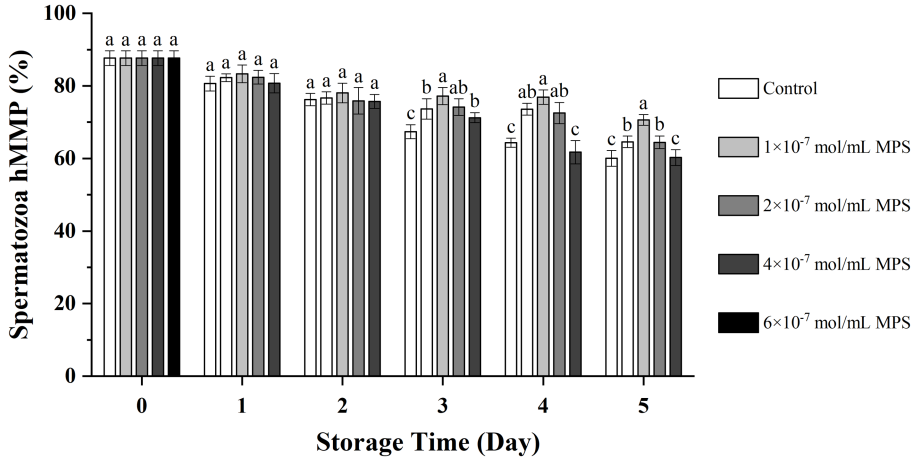

FIGURE 2
Effect of different concentrations of methylprednisolone (MPS) on acrosome integrity.

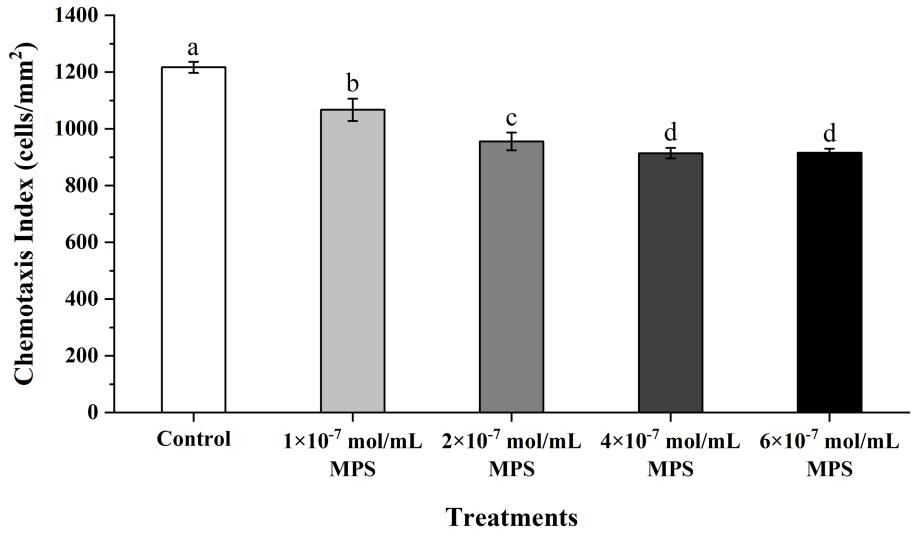

FIGURE 3
Effects of methylprednisolone (MPS) on the chemotaxis of polymorphonuclear neutrophils (PMNs) toward spermatozoa.

1×10^{-7} mol/mL MPS and 2×10^{-7} mol/mL MPS samples was not significant, and the same was true for the 4×10^{-7} mol/mL MPS and 6×10^{-7} mol/mL MPS samples ($P>0.05$).

4. Discussion

The activity of spermatozoa in the female genital tract is influenced by several factors, the main favorable ones being the increase in temperature and the activation of certain chemicals that increase their motility, thus their ability to reach the site of fertilization more rapidly. However, the boar sperm motility depending on the swine breed, individual differences, reproductive capacity, epidemics and conditions of spermatozoa storage. The higher the spermatozoa motility, the higher the number of spermatozoa reaching the fertilization site and the better the fertilization rate. Our results showed that the spermatozoa motility of the 2×10^{-7} mol/mL MPS sample was optimal under 17°C storage conditions, maintaining 57.33% motility at day 5. MPS is based on the addition of double bonds and methyl groups to the molecular structure of prednisone. This further improvement in structure allows MPS to act more swiftly on the cells (25), thus providing faster protection to boar spermatozoa.

The motility pattern of mammalian spermatozoa is generally divided into regular and hyperactivation (26). The hyperactivation of spermatozoa depends mainly on the length of the tail and the thickness of the flagellum (27), so damage to the spermatozoa structure during *in vitro* preservation will directly lead to a reduction in the ability of the spermatozoa to penetrate the egg. Our results show that the spermatozoa kinetic parameters of the 2×10^{-7} mol/mL MPS samples were significantly higher than the other samples under *in vitro* liquid storage (17°C).

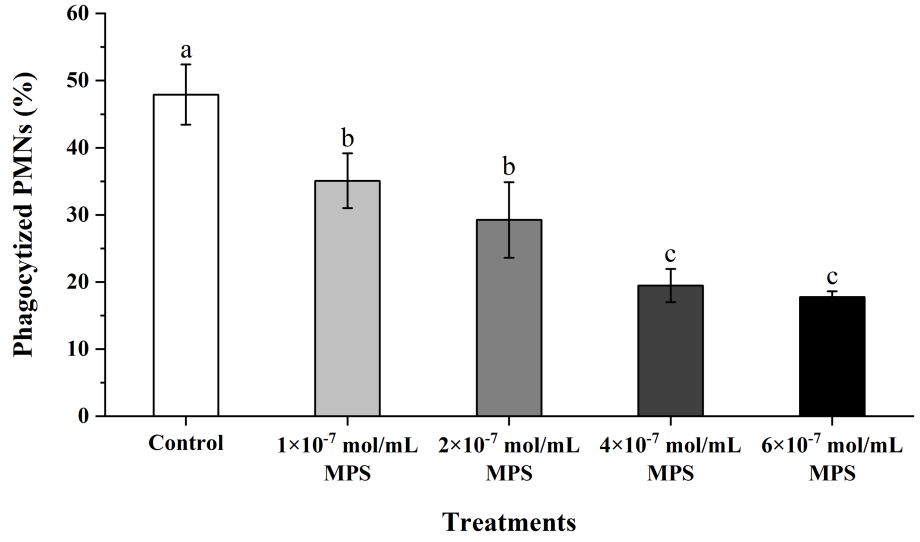

FIGURE 4
Effects of methylprednisolone (MPS) on the phagocytosis of polymorphonuclear neutrophils (PMNs) toward spermatozoa.

Spermatozoa morphology is an important predictor of fertilization capacity in semen quality analysis. The morphological abnormalities of spermatozoa can be caused by defects in spermatogenesis and maturation, or by changes in the external environment during spermatozoa preservation. Spermatozoa malformation is generally classified into four types: head defects, neck and mid-section defects, tail defects and excessive cytoplasmic residues (28). Our results showed that at day 5 of spermatozoa preservation, the abnormality rate of 2×10^{-7} mol/mL MPS samples was still significantly lower than all other groups, but the high concentration of MPS samples (6×10^{-7} mol/mL) instead increased the degree of spermatozoa malformation, which suggested to us the biological toxicity of MPS. Xu et al. (29) showed that MPS protects cell membranes, scavenges free radicals and stabilizes lysosomal membranes.

Total antioxidant capacity activity reflects the total antioxidant capacity of the antioxidants in a system and provides some indication of the overall ability of a system to scavenge reactive oxygen species (ROS). The most common forms of ROS are H_2O_2 and O_2^-. Under steady-state conditions, superoxide dismutase (SOD) in the mitochondria rapidly converts excess O_2^- to the less reactive H_2O_2. H_2O_2 is less active but is non-polar and can penetrate the cell membrane environment, directly attacking polyunsaturated fatty acids (PUFA) (30). H_2O_2 is a kind of ROS with the most extended half-life, so it is regarded as a beacon of ROS production level (31). Our results show that MPS alleviates the decrease in T-AOC activity and increase in H_2O_2 content during *in vitro* liquid preservation under LPS induction.

An early study based on Munich-Wistar rats showed that MPS reduced the level of lipid peroxidation in glomerular tissue and the extent of ROS-mediated kidney injury by enhancing glomerular antioxidant enzyme activity (32). Lee et al. showed that the MPS preserved the rabbit endothelium-dependent vasorelaxation against the attack of ROS in a dose-related manner (33). Although excessive accumulation of ROS can cause irreversible damage to spermatozoa, vital physiological processes such as spermatozoa capacitation and acrosome reaction require the involvement of ROS. Usually, spermatozoa can neutralize ROS through their antioxidant system to avoid oxidative damage. However, in spermatozoa *in vitro* liquid storage, changes in the external environment and temperature will lead to the accelerated production of ROS (34).

Spermatozoa motile by their whiplash tails, and the maintenance of this motility depends on the energy released from ATP hydrolysis (35). A mature spermatozoon typically contains 72–80 mitochondria, the ATP produced is used to maintain intracellular environment stability and provide energy for relevant physiological processes (36, 37). When the ROS produced by the mitochondria increases, the mitochondrial membrane potential will also decrease. Our results showed that the mitochondrial hMMP of boar spermatozoa in the 2×10^{-7} mol/mL MPS was higher than other samples under *in vitro* 17°C storage. The mitochondrial hMMP remained at 70.64% at day 5. The mitochondrial membrane potential is closely related to ATP content, and hMMP indicates that the energy metabolism of spermatozoa typically proceeds, whereas this energy metabolism requires oxidative phosphorylation to produce ATP for its maintenance, with a positive correlation (38).

When the spermatozoa enter the oviduct and encounter the egg, the spermatozoa with intact and normal acrosome can generally produce the acrosome reaction and release acrosomal enzymes to cross the zona pellucida into the oocyte and complete fertilization (39). Glucocorticoids can effectively resist free radicals and inhibit calcium influx from stabilizing lysosomes in cells further, thus attenuating the harmful oxidative damage to the cell membrane and more effectively maintaining the complete structure of the cell membrane (40). Therefore, the effect of MPS on acrosome integrity might be similar to that of.

In females during estrus, due to regulating reproductive hormones such as progesterone, many immunocytes migrate into the endometrium to remove foreign cells or substances such as spermatozoa, bacteria and antigens that may subsequently enter the genital tract. After entering the genital tract, spermatozoa will have activated the complement cascade in uterine secretions, after which complement will have induced the chemotaxis with neutrophils and eventually lead to phagocytosis (41). The persistence of this immune response is detrimental to spermatozoa-egg binding after AI, so it is crucial to prevent spermatozoa chemotaxis and phagocytosis by blocking PMNs.

Li et al. (3) reported that heparin could inhibit neutrophil chemotaxis and phagocytosis of spermatozoa by binding ligands and blocking the cascade reaction. Alghamdi et al. found by AI of semen with or without seminal plasma (SP) in mares with uterine inflammation, 0.5% (1/22) of mares conceived without SP and 77% (17/22) with SP (42). The beneficial effect of DEX on the inhibition of PMNs chemotaxis and phagocytosis towards spermatozoa has been demonstrated in one of our previous reports (15), while the duration of action of MPS (12–36 h) was shorter compared to dexamethasone (36–54 h) (43), this facilitates faster restoration of the immune barrier function of the sow's uterus. MPS has been widely reported for its anti-inflammatory role and potent inhibitory effects on immune cells such as PMNs, and it helps regulate the body's immune system (44). Although chronic or excessive exogenous glucocorticoid exposure is considered a risk to the human fetus, the potential physiologic benefits make short-term or one-time treatment acceptable (45). Our results show that MPS positively affects the inhibition of PMNs chemotaxis and phagocytosis of spermatozoa, which is more substantial with increasing concentrations of MPS.

5. Conclusion

In conclusion, our results indicate that MPS enhances the motility and kinetic parameters of boar spermatozoa under *in vitro* liquid preservation (17°C) and inhibits the effects of environmental variations on spermatozoa malformations. Adding MPS to the modified Modena extender reduced radical oxidative damage to spermatozoa, enhanced T-AOC activity and reduced H_2O_2 content, which is essential for maintaining boar spermatozoa's normal physiological functions. Meanwhile, MPS also positively affected spermatozoa hMMP levels and acrosome integrity, essential for spermatozoa insemination ability. In the chemotaxis and phagocytosis assays of PMNs, MPS showed a positive concentration-dependent inhibition of the adverse effects of LPS-induced PMNs on spermatozoa.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by The Animal Experiments Committee of Heilongjiang Bayi Agricultural University. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

YL: writing—original draft, conceptualization and methodology. HW: writing—original draft and visualization. SW: writing—review and editing and validation. QZ: writing—review and editing and data curation. HZ: investigation. TL: resources and formal analysis. QW: data curation. MG: investigation and software. HF: resources and investigation. YS: formal analysis. GW: funding acquisition and supervision. JL: project administration and writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Myo-inositol improves the viability of boar sperm during liquid storage

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Introduction: Liquid preservation of boar semen is a highly preferred method for semen preservation in pig production. However, oxidative stress is the main challenge during the liquid preservation of boar semen in a time dependent manner. Therefore, supplementation of sperm with antioxidants during storage to protect them from oxidative stress has been the focus of recent research. Myo-inositol (Myo-Ins), the most active form of inositol, which belongs to the vitamin (Vit.) (B1 group has been shown to improve semen quality) (1). This study aimed to investigate whether Myo-Ins supplementation protects boar sperm in liquid preservation against oxidative stress and determine the appropriate concentration of Myo-Ins to be used in this regard.

Methods: Boar sperm was diluted with a semen extender with different concentrations of Myo-Ins (2, 4, 6, and 8 mg/mL) depending on the previous studies (1, 24). Sperm motility and viability, plasma membrane and acrosome integrity, mitochondrial membrane potential (MMP), semen time survival, and gene expression were measured and analyzed on days 0, 1, 3, 5, and 7 for the different samples.

Results: Different concentrations of Myo-Ins exerted different protective effects on the boar sperm quality. The addition of 2 mg/mL Myo-Ins resulted in higher sperm motility and viability, plasma membrane and acrosome integrity, MMP, and effective survival time. Investigation of mRNA expression patterns via qRT-PCR suggested that the 2 mg/mL Myo-Ins sample had increased expression of antioxidative genes.

Conclusion: The addition of Myo-Ins to semen extender improved the boar semen quality by decreasing the effects of oxidative stress during liquid preservation at 17°C. Additionally, 2 mg/mL is the optimum inclusion concentration of Myo-Ins for semen preservation.

KEYWORDS

Myo-inositol, viability, boar, sperm, storage

1. Introduction

Artificial insemination (AI) is the deliberate introduction of sperm into the cervix or uterine cavity of a female to achieve pregnancy (*in vivo* fertilization) through means other than sexual intercourse or *in vitro* fertilization (1). It is a fertility treatment for humans and a common practice in animal breeding, including pigs (1). Cryopreservation and liquid preservation are important methods for preserving the boar semen (1). Cryopreservation has been the main method for preserving semen for a long time. It is estimated that approximately 40% to 50% of boar spermatozoa do not survive after cryopreservation as they are highly sensitive to cold shock due to the plasma membrane composition having high unsaturated phospholipids levels and low cholesterol levels As a result of this, boar spermatozoa have a short lifespan when subjected to the freezing/thawing process because of their low survivability rates compared to other mammals (2). Therefore, an alternative method, liquid preservation of semen, is used to preserve boar semen for AI. Liquid preservation, is a simple and easy method to maintain high quality semen for a short duration, and is commonly used in pig production practices. However, the quality of boar semen declines rapidly; therefore, fresh boar semen cannot be stored for a long duration. The investigation of novel semen storage techniques may aid in extending the time and geographic boundaries of AI (2).

In the common boar sperm storage technique, sperm is stored at 17°C, which inhibits the movement of sperm and causes a reduction in the energy consumption so that the sperm can survive *in vitro* for longer (3). Despite the longer storage time, fertility and quality of boar sperm decline with time (4). Boar spermatozoa have high polyunsaturated fatty acid concentration in their plasma membranes and a low cholesterol/phospholipid ratio compared to the other animals semen making it more prone to oxidative stress (5). Therefore, boar spermatozoa are more prone to attack by reactive oxygen species (ROS), resulting in lipid peroxidation (6). *In vitro* storage of sperm leads to an increase in ROS production when certain conditions such as temperature are changed (7). Excessive ROS production reduces sperm motility, impairs cell integrity, and inhibits sperm-oocyte fusion (8). Previous studies have reported that adding antioxidants to semen extenders is an efficient way to protect boar sperm from oxidative stress *in vitro* (9).

Myo-inositol (Myo-Ins), the most active naturally existing form of inositol, and belongs to vitamin B1 complexes and is mainly produced in the human body by glucose-6-phosphate (1). In humans, supplementation of Myo-Ins *in vitro* protects sperm against oxidative stress to DNA and increases sperm motility and vitality in cryopreservation (10, 11). Myo-Ins play an essential role in various cellular activities such as cytogenesis, cell membrane formation, cell growth, morphogenesis, and lipid synthesis (12). Myo-Ins modulate intracellular calcium levels in the cellular signal transduction system by acting as a precursor for secondary messengers (13). Therefore, it plays a vital role in alterations in metabolism, sensitization of insulin, and especially in reproduction (14, 15). Myo-Ins levels in the seminiferous tubules were substantially higher than those in the serum (16). In human testicles, Sertoli cells are mainly involved in the production of Myo-Ins in response to follicle-stimulating hormone (FSH) (17). In addition, high concentrations of Myo-Ins monophosphatase-1 and Myo-Ins phosphate synthase in male reproductive organs promote the synthesis of Myo-Ins (18). Previous research was performed by Hinton and his collaborators in which he found out the concentration of Myo-Ins in luminal fluid of the testes and epididymis of different mammalian species in which boar sperm has a concentration of Myo-Ins 1.0–2.0 mM (19). Myo-Ins regulates many processes, including maturation, motility, capacitation, and the acrosomal reaction of spermatozoa (17), and is involved in the osmoregulation of seminal plasma (16). Furthermore, Myo-Ins is involved in improving the mitochondrial functions of sperm via Ca^{+2} ion influx that promotes the oxidation and generation of adenosine triphosphate (ATP), thereby condensing and preventing chromatin from apoptosis (18).

Myo-Ins was previously studied as an antioxidant agent *in vitro*/*in vivo* to treat infertility in men by improving spermatozoa quality and ultimately fertilization (20, 21). *In vitro* supplementation of Myo-Ins improved the sperm quality in oligoteratoasthenozoospermia (OAT) (18, 22) and the rate of fertilization with the embryo quality (21). Inositol plays a vital role in the protection of enzyme systems from lipid peroxidation and cryodamage and preserves the acrosomal integrity of ram post-thawed spermatozoa (23). *In vitro* supplementation of Myo-Ins in the freezing extender of frozen-thawed bull sperm improved sperm motility and other sperm parameters (24). Myo-Ins supplementation in the freezing extender protected cryopreserved dog sperm, attenuated sperm motility, kinematic parameters, membrane integrity, and curtailed chromatin damage and apoptosis (1). To date, there is a paucity of studies regarding the effects of Myo-Ins supplementation on boar semen during liquid preservation.

Therefore, this study aimed to investigate the effect of Myo-Ins supplementation on the motion characteristics (total motility, progressive motility and kinematic parameters), quality parameters [sperm viability, plasma membrane integrity, acrosome integrity, and mitochondrial membrane potential (MMP)], and gene expression (antioxidant genes) of boar semen during liquid preservation.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, all chemicals used in this study were purchased from Sigma Aldrich Co. (St. Louis, MO, United States). The LIVE/DEAD sperm viability kit and rhodamine 123 (Rd123) were purchased from Thermofischer Co. (Eugene, OR, United States). Semen extender (Duragen) was obtained from Darby Co. (Anseong, Gyeonggi-do, Republic of Korea).

2.2. Semen collection and transportation

The semen used in this experiment was commercial boar semen purchased from a semen sales company (Darby Co., Anseong, Gyeonggi-do, Republic of Korea) with no significant difference in vigor during all the seasons (25). Semen samples were packed in 90 mL commercial doses at a concentration of 3×10^9 spermatozoa/dose and shipped to our laboratory within 1 h. After that high-quality sperm with >80% spermatozoa with normal morphology,

approximately 80% motility, 60% progressive motility, 80% sperm viability, and 80% non-reacted acrosomes were kept at 17°C and used for further experiments. The fridge temperature was regularly monitored with a thermometer installed insides it. Thirty ejaculates were used in this study. The quality of spermatozoa was analyzed on day 0 (collection day) and on days 1, 3, 5, and 7 of preservation to evaluate total sperm motility, forward progressive motility, kinematic parameters, viability, membrane integrity, acrosome integrity, MMP, and gene expression.

2.3. Extender preparation

Duragen (Darby Co. Anseong, Gyeonggi-do, Republic of Korea), the long-term extender used in the present study, is composed of glucose 79.83 g, sodium citrate 7.07 g, diphasic potassium phosphate 3.54 g, tris 3.91 g, sodium bicarbonate 4.24 g, apramycin 0.59 g, and ampicillin 0.82 g/L in deionized water. In the treatment samples, we added Myo-Ins with the concentration of 2, 4, 6, and 8 mg/mL. Semen of the control group was stored in Duragen extender solution without adding Myo-Ins.

2.4. Semen processing

After collecting the fresh boar semen, the quality of the semen samples was evaluated, and all the semen samples were divided into five equal fractions. The control sample fraction was diluted with Duragen and the other fractions were diluted with 2, 4, 6, and 8 mg/mL of Myo-Ins. Lastly, all the semen samples were stored in a refrigerator at 17°C. All experiments were performed with at least four replicates for all the samples.

2.5. Sperm motility

Sperm motility was determined on days 0, 1, 3, 5, and 7 using a computer-assisted semen analysis system (CASA) (Sperm Class Analyzer, Microptic, Barcelona, Spain). In short, 2 µL of diluted semen was placed in a counting chamber (GoldCyto, Microptic, Spain) on a heated stage at 38°C. For each analysis, five fields were analyzed and at least 500 spermatozoa were counted. Motility patterns, including total sperm motility (TM), rapid progressive motility (RPM), medium progressive motility (MPM), immotility (IM), curvilinear velocity (VCL µm/s), straight line velocity (VSL µm/s), average path velocity (VAP µm/s), linearity [LIN = (VSL/VCL) × 100%], and straightness [STR = (VSL/VAP) × 100%], were measured as indicated in Figure 1.

2.6. Sperm viability

The viability of spermatozoa was assessed using the LIVE/DEAD sperm viability kit, as described by Yu and Leibo (26) with slight modifications. This was evaluated on days 0, 1, 3, 5, and 7. Basically, 5 µL of SYBR14 was added to (2.5×10^6/mL)of spermatozoa, and 5 µL of propidium iodide (PI), and incubated in the dark for 5 min each at 37°C. Two smears were prepared from each sample, and smear-stained semen was used, air-dried, and observed under an epifluorescence microscope at 400× magnification. Approximately 300 spermatozoa were counted from each sample and classified as dead (red-stained) or live (green-stained) Spermatozoa with red and green fluorescence were recorded as percentages.

2.7. Plasma membrane integrity

The hypoosmotic swelling test (HOST) was performed to evaluate the functional membrane integrity of the sperm using the method described by Osinowo et al. (27). This was evaluated on the days 0, 1, 3, 5, and 7. In this assay, 30 µL of semen was mixed with a 300 µL hypo-osmotic solution (7.35 g of sodium citrate and 13.51 g of fructose in 1000 mL of distilled water with 150 mOsm/kg) (2). Sperm swelling was assessed after incubation at 37°C for 1 h, in which 10 µL of sperm was placed on a glass slide and observed at 400× magnification using a phase contrast microscope. A total of 300 spermatozoa were counted in at least five microscopic fields. Spermatozoa were classified as

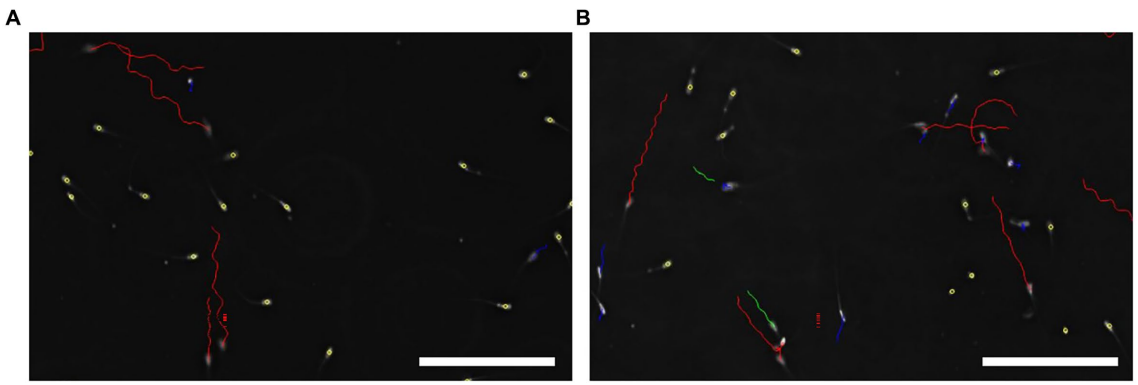

FIGURE 1
Representative image of sperm motility and trajectory paths captured with computer assisted semen analysis system (CASA). **(A)** Boar sperm motility in the control group. **(B)** Sperm motility in the treatment group (2 mg/mL) on day 7. Rapid, medium and slow movements are illustrated with red, green and blue colors, respectively. Immotile spermatozoa are indicated with yellow color. Scale bar = 100 µm.

positive or negative based on the absence or presence of coiled tails spermatozoa with curled or swollen tails were recorded as percentages.

2.8. Acrosome integrity

The acrosome integrity of spermatozoa was assessed using a fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) staining using a slightly modified procedure described by Yu and Leibo (26). This was evaluated on days 0, 1, 3, 5, and 7. Additionally, a glass slide smear using 10 µL of sperm was prepared and air dried for each sample. The smears were then fixed with absolute methanol at room temperature, stained with a FITC-PNA solution (100 µg/mL), and then spread with PBS. Subsequently, all stained smears were covered with parafilm for 20 min, dipped in distilled water for 15 min, and then air-dried. For each replicate, two slides from each sample were examined under an epifluorescence microscope. The percentage of fluorescent intact acrosomal spermatozoa with bright green fluorescence in the acrosomal region was counted with at least 300 spermatozoa per slide.

2.9. MMP assay

The mitochondrial activity of spermatozoa was assessed by Rd123 and PI staining using a previously described method (28). In this assay, 5 µL PI and 5 µL of Rd123 were added to the (1.25×10^7/mL) spermatozoa and kept it in a dark dry heat block at 37°C for 15 min. At least 200 stained sperm cells were analyzed on each slide under a fluorescence microscope at 400× magnification. The sperm cells displaying red fluorescence in the head region were counted as dead, and the sperm cells showing a green mid-piece with no red heads were counted as viable sperm exhibiting functional mitochondria.

2.10. Quantitative reverse transcription-polymerase chain reaction

Expression of mRNA was analyzed using qRT-PCR for five specific genes with antioxidant functions, including nuclear erythroid factor 2 related factor 2 *(NRF2),* NAD(P)H dehydrogenase (quinone) 1 *(NQO1),* glutathione disulfide reductase *(GSR),* glutamate-cysteine ligase catalytic subunit *(GCLC),* and Kelch-like-ECH associated protein 1 *(KEAP1).* The primer sequences are listed in Table 1.

On day 7, control and Myo-Ins supplemented at 2 mg/mL sperm samples were stored at −80°C after washing with PBS. Trizol (TaKaRa Bio, Inc., Otsu, Shiga, Japan) was used for RNA extraction using the manufacturer's instructions. SuperScript IV VILO Master Mix (Thermo Fisher Scientific, MA, United States) was used to convert the extracted RNA (1 µg of total RNA) into complementary DNA (cDNA). The synthesized cDNA was 2× SYBR Premix Ex Taq (TaKaRa Bio, Inc. Otsu, Shiga, Japan), and 5 pmol of specific primers (Macrogen, Inc., Seoul, Republic of Korea) were used for qRT-PCR. The mRNA expression was analyzed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, United States). The cycling parameters were performed consecutively as follows: 95°C for 5 min, 40 cycles at 95°C for 15 s, 56°C for 15 s, and 72°C for 30 s. Relative quantification was performed by comparing the threshold cycle (Ct) at constant fluorescence intensity. Relative mRNA expression (R) was calculated using the equation $R = 2^{-[\Delta Ct\,sample\ -\ \Delta Ct\,control]}$ (29). The R values were normalized to those of *RN18S* in the semen samples from each group.

2.11. Statistical analysis

All experiments in this study were conducted at least three times. Statistical analysis of data was done using GraphPad Prism (GraphPad Software, San Diego, CA, United States) and SPSS (version 12.0; SPSS, IBM, Armonk, NY, United States). Following the one-way analysis of variance test, Duncan's multiple range test was used to examine

TABLE 1 Primers used for gene expression analysis.

mRNA	Primer sequences	Product size (bp)	Genbank accession number
RN18S	F: 5′-CGCGGTTCTATTTTGTTGGT-3′	219	NR_046261
	R: 5′-AGTCGGCATCGTTTATGGTC-3′		
NRF2	F: 5′-CCCATTCACAAAAGACAAACATTC-3′	75	XM_021075133
	R: 5′-GCTTTTGCCCTTAGCTCATCTC-3′		
KEAP1	F: 5′-AGCTGGGATGCCTCAGTGTT-3	100	NM_001114671
	R: 5′-AGGCAAGTTCTCCCAGACATTC-3′		
GCLC	F: 5′-GTTTTGTGAATCAGGACCCTA-3′	212	XM_003483635_4
	R: 5′-GCTTAGCTGAAGCTTTATTGC-3′		
GSR	F: 5′-TGGGCTCTAAGACGTCACTG-3′	106	XM_003483635
	R: 5′-TCTATGCCAGCATTCTCCAG-3′		
NQO1	F: 5′-ATGAACTTCAATCCCGTCAT-3′	191	NM_0011596
	R: 5′-CTCGGCAGGATACTGWGT-3′		

TABLE 2 Effect of Myo-Ins on boar sperm total motility and progressive motility.

Parameter	Treatment (mg/mL)	Time of storage (days)				
		0	1	3	5	7
Total motility (%)	0	87.4 ± 0.5	82.8 ± 1.8	62.8 ± 3.1^{ab}	50.2 ± 2.3^{b}	38.4 ± 1.5^{b}
	2	87.4 ± 0.5	84.1 ± 1.0	70.5 ± 4.3^{a}	58.6 ± 1.2^{a}	48.9 ± 2.5^{a}
	4	87.4 ± 0.5	83.2 ± 0.6	66.0 ± 2.4^{ab}	55.9 ± 2.3^{ab}	45.5 ± 3.7^{ab}
	6	87.4 ± 0.5	84.5 ± 1.4	64.5 ± 1.5^{ab}	52.5 ± 3.3^{ab}	44.7 ± 4.2^{ab}
	8	87.4 ± 0.5	83.8 ± 1.9	60.1 ± 1.6^{b}	50.9 ± 2.7^{ab}	42.9 ± 1.8^{ab}
Forward progressive motility (%)	0	72.9 ± 1.0	70.0 ± 2.8	48.5 ± 1.8^{b}	37.3 ± 2.6	27.4 ± 1.9
	2	72.9 ± 1.0	68.3 ± 2.3	55.7 ± 3.6^{a}	44.5 ± 1.8	33.4 ± 2.4
	4	72.9 ± 1.0	68.9 ± 1.7	48.3 ± 1.1^{b}	41.8 ± 3.6	30.8 ± 3.9
	6	72.9 ± 1.0	64.7 ± 2.8	45.3 ± 2.5^{bc}	39.9 ± 4.1	27.9 ± 3.2
	8	72.9 ± 1.0	63.3 ± 2.2	40.5 ± 1.6^{c}	37.9 ± 2.9	30.9 ± 1.9

$^{a-c}$Values in the column with different superscript letters differed significantly ($p < 0.05$).

percentage data (total motility, forward progressive motility, kinematic parameters, sperm viability, plasma membrane integrity, acrosome integrity, and MMP). An unpaired two-tailed student's *t*-test was used to examine the average data (qPCR of day 7 sperm) from the two groups. Data are presented as mean ± standard error of the mean (SEM). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effect of Myo-Ins on the total motility and forward progressive motility

The effects of different concentrations of Myo-Ins on boar sperm total motility (TM) and forward progressive motility (FPM) are shown in Table 2. Total motility of sperm was decreased from day 0 (87.4 ± 0.5%) with an increase in the preservation time day 7 (38.4 ± 1.5%) *in vitro*. Meanwhile, total motility of the sperm treated with 2 mg/mL Myo-Ins was higher than the other samples, and significantly ($p < 0.05$) improved on day 5 (58.6 ± 1.2%) and day 7 (48.9 ± 2.5%) as compared to the control sample. These results indicated that supplementation with 2 mg/mL Myo-Ins in the semen extender improved total sperm motility (Figure 1). Forward progressive motility of sperm gradually decreased from day 0 (72.9 ± 1.0%) to day 7 (27.4 ± 1.9%) in the control sample compared to the treatment sample. On day 3, in the 2 mg/mL treated sample, forward progressive motility was (55.7 ± 3.6%) compared with the control group which was (48.5 ± 1.8%) increased significantly ($p < 0.05$). However, on days 5 and 7, the FPM in the control group was (37.3 ± 2.6%) and (27.4 ± 1.9%) compared with the 2 mg/mL treated sample in which the FPM was (44.5 ± 1.8%) and (33.4 ± 2.4%). FPM on days 5 and 7 improved in the 2 mg/mL treated sample but not significantly compared with the control sample.

3.2. Effect of Myo-Ins on kinematic parameters

Evaluation of spermatozoa kinematic parameters showed that Myo-Ins supplementation significantly improved VSL (μm/s) and STR (%) parameters (Table 3). On days 1 and 7, VSL kinematic parameter significantly improved in the 8 mg/mL treated sample (79.0 ± 5.4) (42.7 ± 2.8) compared with the control group (57.2 ± 2.4) (28.8 ± 3.7). VSL significantly increased in 6 mg/mL treated sample (45.2 ± 4.9) compared with the control sample (35.6 ± 1.8) on day 5. Additionally, Myo-Ins supplementation in the 8 mg/mL sample on days 1, 3, 7 significantly improved the STR (61.8 ± 2.3), (61.9 ± 3.2), (75.7 ± 1.4) compared with the control sample (51.1 ± 3.5), (53.2 ± 1.8) and (63.5 ± 3.8). Moreover, STR also increased significantly in the 6 mg/mL on days 3 (63.0 ± 0.9) and 7 (76.1 ± 4.9) compared with the control sample (53.2 ± 1.8) (63.5 ± 3.8). On day 1, VCL (μm/s) parameter was higher at the 4 mg/mL sample (163.8 ± 3.7) compared to the control sample (142.4 ± 9.8). On day 5, 6 mg/mL sample (73.9 ± 8.6) showed the highest VCL compared to control sample (64.7 ± 5.7); however, on day 7, 8 mg/mL sample (64.7 ± 4.0) had the highest VCL kinematic parameter compared to control group (53.4 ± 2.1). Myo-Ins supplementation improved the other kinematic parameters assessed by CASA; however, there were no significant differences between the treated and control samples (Table 3).

3.3. Effect of Myo-Ins on the sperm viability

The effects of different concentrations of Myo-Ins on boar sperm viability during liquid preservation are presented in Figure 2. On day 1, sperm viability of the samples treated with 2 (84.5 ± 0.9%), 6 (84.9 ± 1.3%), and 8 mg/mL (85.1 ± 1.5%) Myo-Ins treated samples increased significantly compared to the control sample (80.8 ± 0.9%). On day 3 (88.0 ± 1.6%) and 7 (81.6 ± 1.6%), 2 mg/mL treated sample showed significantly higher sperm viability compared to the control sample (80.9 ± 1.6%) and (70.2 ± 2.7%). However, no significant differences were observed at 4, 6, and 8 mg/mL on days 3, 5, and 7 as shown in Figure 2.

3.4. Effect of Myo-Ins on the sperm plasma membrane integrity

The effects of different Myo-Ins concentrations on boar sperm plasma membrane integrity during liquid preservation are shown in Figure 3. All treatment samples (2, 4, and 8 mg/mL) showed higher

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TABLE 3 Effect of Myo-Ins on boar sperm kinetic parameters.

Parameter	Treatment (mg/mL)	Time of storage (days)				
		0	1	3	5	7
VAP (μm/s)	0	122.1 ± 3.2	113.9 ± 7.9	87.1 ± 17.1	53.7 ± 4.4	44.1 ± 3.7
	2	122.1 ± 3.2	115.9 ± 8.8	91.6 ± 18.2	58.3 ± 3.7	48.4 ± 3.7
	4	122.1 ± 3.2	135.2 ± 3.2	86.7 ± 16.9	59.6 ± 5.7	47.6 ± 3.4
	6	122.1 ± 3.2	119.2 ± 7.4	76.3 ± 9.5	59.9 ± 6.4	42.4 ± 4.2
	8	122.1 ± 3.2	119.7 ± 5.7	75.3 ± 11.8	54.5 ± 3.7	51.9 ± 3.4
VCL (μm/s)	0	149.3 ± 4.0	142.4 ± 9.8	109.8 ± 19.9	64.7 ± 5.7	53.4 ± 2.1^{ab}
	2	149.3 ± 4.0	145.3 ± 11.1	108.2 ± 18.7	71.0 ± 5.0	61.3 ± 3.9^{ab}
	4	149.3 ± 4.0	163.8 ± 3.7	104.0 ± 19.1	71.4 ± 7.2	60.4 ± 3.6^{ab}
	6	149.3 ± 4.0	147.2 ± 7.9	93.5 ± 9.6	73.9 ± 8.6	52.1 ± 4.0^{b}
	8	149.3 ± 4.0	139.8 ± 3.5	89.8 ± 13.5	64.5 ± 3.7	64.7 ± 4.0^{a}
VSL (μm/s)	0	60.0 ± 1.7	57.2 ± 2.4^{c}	47.2 ± 10.2	35.6 ± 1.8^{b}	28.8 ± 3.7^{b}
	2	60.0 ± 1.7	58.5 ± 3.6^{c}	50.5 ± 13.2	36.8 ± 1.7^{ab}	33.2 ± 1.5^{ab}
	4	60.0 ± 1.7	73.1 ± 1.0^{ab}	54.0 ± 12.9	42.9 ± 1.3^{ab}	36.7 ± 4.7^{ab}
	6	60.0 ± 1.7	67.0 ± 4.1^{bc}	53.6 ± 7.9	45.2 ± 4.9^{a}	34.9 ± 5.0^{ab}
	8	60.0 ± 1.7	79.0 ± 5.4^{a}	50.2 ± 7.3	43.2 ± 2.8^{ab}	42.7 ± 2.8^{a}
STR (%)	0	50.2 ± 1.1	51.1 ± 3.5^{b}	53.2 ± 1.8^{b}	65.7 ± 3.8	63.5 ± 3.8^{b}
	2	50.2 ± 1.1	51.9 ± 3.5^{b}	51.9 ± 2.4^{b}	61.7 ± 2.3	66.9 ± 1.8^{ab}
	4	50.2 ± 1.1	51.4 ± 0.6^{b}	56.6 ± 1.4^{ab}	70.1 ± 5.2	69.2 ± 4.3^{ab}
	6	50.2 ± 1.1	57.3 ± 2.2^{ab}	63.0 ± 0.9^{a}	70.3 ± 3.9	76.1 ± 4.9^{a}
	8	50.2 ± 1.1	61.8 ± 2.3^{a}	61.9 ± 3.2^{a}	73.3 ± 2.0	75.7 ± 1.4^{a}
LIN (%)	0	41.8 ± 1.1	41.5 ± 3.5	45.5 ± 2.1	57.4 ± 4.1	54.3 ± 4.4
	2	41.8 ± 1.1	41.5 ± 3.1	45.4 ± 2.9	52.2 ± 2.5	55.8 ± 1.9
	4	41.8 ± 1.1	42.2 ± 0.8	48.3 ± 1.4	59.1 ± 5.0	55.9 ± 4.5
	6	41.8 ± 1.1	46.5 ± 2.1	53.9 ± 3.9	57.9 ± 4.6	63.8 ± 5.4
	8	41.8 ± 1.1	50.9 ± 3.7	52.5 ± 5.4	62.2 ± 2.2	61.9 ± 1.6

[a–c]Values in the column with different superscript letters differed significantly ($p < 0.05$). All the experiments are conducted with at least four replicates. VAP, average path velocity (μm/s); VSL, straight linear velocity (μm/s); VCL, curvilinear velocity (μm/s); STR, straightness [(VSL/VAP) × 100]; LIN, linearity [(VSL/VCL) × 100].

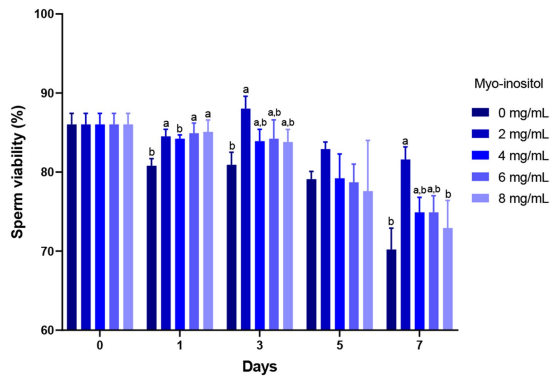

FIGURE 2
Effects of different concentrations of Myo-Ins on sperm viability sperm viability of boar semen during the liquid preservation. Within each endpoint, bars with different letters (a,b) indicate significant differences ($p < 0.05$) at various Myo-Ins concentrations For graph, all the values represent mean ± SEM. The experiments are replicated six times.

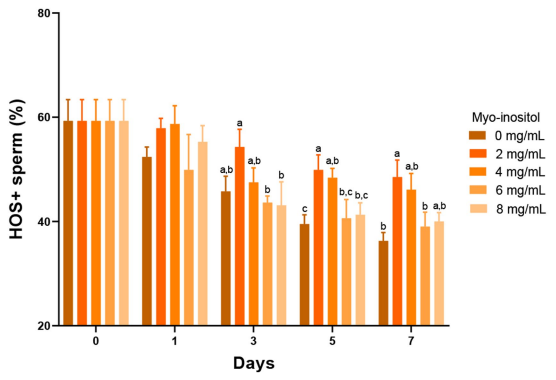

FIGURE 3
Effects of different concentrations of Myo-Ins on plasma membrane integrity Plasma membrane integrity of boar semen during liquid preservation. Within each endpoint, bars with different letters (a,b,c) indicate significant differences ($p < 0.05$) at various Myo-Ins concentrations For graph, all the values represent mean ± SEM. The experiments are replicated six times.

plasma membrane integrity than the control sample. After 3 days of preservation, significant results were observed on day 5 (49.9 ± 2.9%) (48.4 ± 1.8%) in the samples treated with 2 and 4 mg/mL compared to the control sample (39.5 ± 1.8%). In addition, on day 7 in the sample treated with 2 mg/mL plasma membrane integrity was significantly improved (49.9 ± 4.3%) as compared to the control sample (38.6 ± 0.8%). However, plasma membrane integrity did not improve significantly in the other treatment samples as illustrated in the Figure 3.

3.5. Effect of Myo-Ins on the sperm acrosome integrity

The effects of different Myo-Ins concentrations on boar sperm acrosome integrity during liquid preservation are shown in Figure 4. Acrosome integrity gradually decreased in all samples from day 0 to day 7; however, no significant difference was observed in the treated samples compared to the control sample. After 7 days of preservation, 2 mg/mL treated sample (91.9 ± 0.5%) showed significantly ($p < 0.05$) higher acrosome integrity compared to the control sample (85.2 ± 1.6%) as indicated in Figure 4.

3.6. Effect of Myo-Ins on the sperm mitochondrial membrane potential

The effects of different concentrations of Myo-Ins on the mitochondrial activity of boar sperm during liquid preservation are shown in Figure 5. Rd123 was used to assess the mitochondrial activity of sperm cells and PI was used to stain dead spermatozoa. The Myo-Ins-treated samples showed higher MMP compared to the control sample in a time-dependent manner. In 2 (89.5 ± 1.4%) and 6 mg/mL (88.6 ± 1.2%) samples, the MMP was significantly higher ($p < 0.05$) on the day 7 compared to the control sample (83.4 ± 1.4%). In the 4 and 8 mg/mL samples, MMP tended to increase; however, it did not significantly increase with storage time (Figure 5).

3.7. Effect of Myo-Ins on the gene expression

After determining that the optimal Myo-Ins supplementation concentration during the liquid preservation of boar semen was 2 mg/mL, we conducted qRT-PCR using the control sample and 2 mg/mL on day 7 samples. The expression levels of *NRF2* and *GCLC* were significantly increased ($p < 0.05$) in the 2 mg/mL sample compared to those in the control sample. In contrast, the expression levels of *NQO1*, *KEAP1*, and *GSR* were not significantly different from those in the control sample (Figure 6).

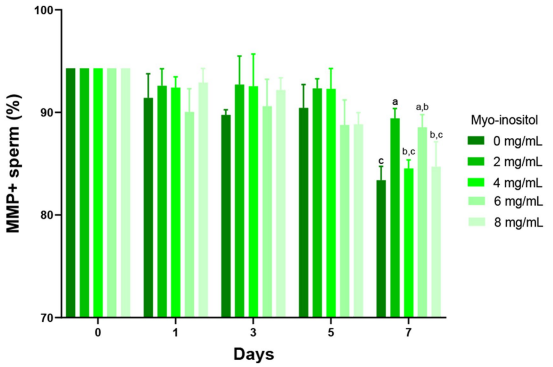

FIGURE 5
Effects of different concentrations of Myo-Ins on mitochondrial membrane potential (MMP) MMP of boar semen during liquid preservation. Within each endpoint, bars with different letters (a,b,c) indicate significant differences ($p < 0.05$) at various Myo-Ins concentrations. For graph, all the values represent mean ± SEM. The experiments are replicated four times.

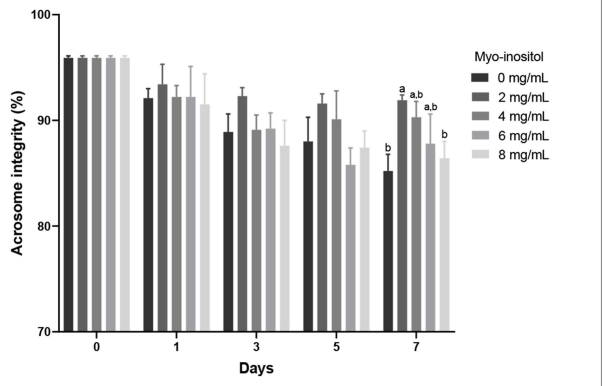

FIGURE 4
Effects of different concentrations of Myo-Ins on acrosome integrity Acrosome integrity of boar semen during liquid preservation. Within each endpoint, bars with different letters (a,b) indicate significant differences ($p < 0.05$) at various Myo-Ins concentrations For graph, all the values represent mean ± SEM. The experiments are replicated six times.

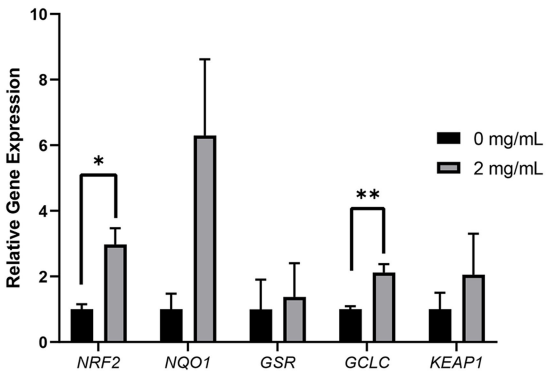

FIGURE 6
Relative mRNA expression levels of genes associated with oxidative stress. The mRNA expression levels of oxidative stress-related genes (*NRF2, NQO1, GSR, GCLC* and *KEAP1*) in sperm from the control and treatment (2 mg/mL) group. Data are normalized to the *RN18S* gene expression. Asterisks indicate statistical significance (*$p < 0.05$ and **$p < 0.01$). For all graphs, the value represents mean ± SEM. The experiments are replicated three or four times.

4. Discussion

During liquid preservation of boar semen, spermatozoa are susceptible to oxidative stress, and antioxidants are added to the extender to decrease the detrimental effects of ROS levels on sperm quality (30, 31). Myo-inositol is a type of vitamin B1 acting as a strong antioxidant, and its concentration in the boar reproductive tract is 1–2 mM (19); however, previous studies demonstrated that the *in vitro* optimal concentration of Myo-Ins in humans is 2–20 mg/mL (32), in bulls (3 mg/mL) (24), and in dogs (1 mg/mL) (1) therefore, in this study, we used 2, 4, 6, and 8 mg/mL Myo-Ins. To date, the impact of Myo-Ins supplementation on boar semen quality during liquid preservation has not been investigated. Therefore, this study aimed to evaluate the effect of different concentrations of Myo-Ins supplementation in extender on boar semen quality during liquid preservation by examining sperm function quality parameters such as kinematic parameters (33, 34), acrosome integrity, plasma membrane integrity, viability (35, 36), MMP (37, 38), and gene expression.

Sperm motility is a crucial factor in determining the effects of semen storage, and declines with longer storage duration (39). Sperm motility is an indicator of normal metabolism and intact membranes (39). Oxidative stress is known to reduce sperm motility and impair sperm function (40). Previous studies have shown that antioxidants protect sperm cells and support the maintenance of sperm motility during the preservation phase (31, 41). In previous studies, the beneficial effects of Myo-Ins on the motility of spermatozoa were elucidated by its ROS scavenging effect, thus controlling oxidative stress in mammalian sperm cells (42). In this study, sperm motility was evaluated on days 0, 1, 3, 5, and 7 in all the samples. These results indicated that sperm motility in the Myo-Ins-treated samples was higher than that in the control sample. Sperm kinematics parameters such as VCL, VAP, and VSL were higher at 4 mg/mL compared to the control group. In contrast, total motility and progressive motility were higher in the 2 mg/mL treated group compared to the 4 mg/mL group. The results of the present study also suggest that the treatment samples had a higher effective survival time than the control samples. These findings were similar to those of previous studies in which Myo-Ins was added to the freezing extender for sperm cryopreservation in dogs (1) and bulls (24). Furthermore, the optimum concentration for Myo-Ins was 2 mg/mL in the semen extender, in contrast to previous studies (1, 24). This might be due to the different animals, types of extenders, and preservation procedures. Concurrently, different temperatures for semen preservation may have also led to varying optimum concentrations of Myo-Ins, resulting in a variation from previous studies. These findings, along with the present data, suggest that a Myo-Ins supplemented extender may improve boar semen motility by reducing the negative effects of ROS produced by sperm cells during liquid preservation (1, 24).

The structure and functional integrity of the plasmalemma, which is the outer membrane of sperm, is important for the metabolism of sperm, ova binding, capacitation, and acrosome reaction (43). The integrity of the plasma membrane of sperm is pivotal for its survival inside the female reproductive tract (43). Damage to the plasmalemma can cause cellular death and loss of homeostasis (44). In the present study, HOST was used to investigate sperm plasma membrane integrity in the control and Myo-Ins-treated samples during semen preservation. The number of HOS-positive spermatozoa was higher on days 5 and 7 at 2 mg/mL samples compared to the control samples, which is similar to the plasma membrane integrity result of dog cryopreserved sperm, in which a high intact plasma membrane was observed in the treatment samples (1). On the other hand, sperm viability, a vital sperm quality parameter, was assessed in the control and treatment samples, and showed more viable spermatozoa in the treated samples, which is in accordance with previous studies on human and dog sperm where high levels of viable spermatozoa were seen in Myo-Ins-treated samples (1, 45). The plasma membrane integrity and sperm viability were higher at 4 mg/mL on day 1 compared to the 2 mg/mL sample. Although the result of sperm viability on day 3 is numerically higher than that of day1, it is difficult to say that it has increased because it is within the error range.

Acrosome integrity is a key indicator of sperm quality (46). The acrosome is an essential organelle that makes it easier for spermatozoa to pass through the oocyte's zona pellucida before fertilization (46). In this study, Myo-Ins supplementation improved sperm motility, membrane integrity, and acrosome integrity, but there was a difference between high and low dose levels that resulted in these parameters being lower than in the control group. A Myo-Ins addition of 2 mg/mL proved to be the optimal concentration in boar semen liquid preservation. Therefore, we assume that Myo-Ins is a dose-dependent chemical and high concentration may reduce the sperm quality parameters in boar. Our results also showed that 2 mg/mL Myo-Ins treatment of extender exerted a protective effect on acrosome integrity in boar semen liquid preservation. These findings are similar to those of Qamar et al. (1) where 1 mg/mL Myo-Ins resulted in a higher percentage of intact sperm acrosomes in dogs after freeze-thawing; similarly, in the present study, we found that the percentage of intact acrosomes was higher at 2 mg/mL Myo-Ins on day 7 compared to the control group. This result indicates that the long-term storage of boar semen with Myo-Ins may improve the quality of sperm cells and cause less damage to the acrosome.

Oxidative stress plays a major role in the deterioration of boar semen quality during liquid preservation (47). Previous studies have shown that the sperm plasma membrane of mammals is rich in unsaturated fatty acids and thus susceptible to peroxide damage (48). During preservation, sperm-generated ROS (lipid hydroperoxides, hydrogen peroxides, and peroxide anion free radicals) induce damage to spermatozoa, resulting in decreased sperm quality (49). In this study, we observed that the accumulation of sperm ROS during liquid preservation occurred and subsequently led to oxidative stress in sperm resulting in decreased sperm motility, acrosome integrity and viability which are considered as the most sensitive indicators of ROS (6, 50). On the basis of these sperm ROS indicators, we assume that oxidative stress decreased during the *in vitro* storage of sperm Therefore, in order to improve the semen quality antioxidants are added to improve the semen quality and decrease the oxidative stress. It has been recommended that Myo-Ins is a strong antioxidant and ROS scavenger, which consequently improves sperm quality. Therefore, adding Myo-Ins to the extender improved semen quality by attenuating ROS stress, which is consistent with previous studies on human and dog sperm (1, 42, 51). Correspondingly, we analyzed

the relative expression of antioxidant related genes (*NRF2, NQO1, GCLC, GSR,* and *KEAP1*) in the control and 2 mg/mL Myo-Ins treatments. Since all the sperm quality parameters were best seen in the 2 mg/mL of Myo-Ins therefore, we selected 2 mg/mL as the optimal concentration to perform gene expression, which is similar to the previous studies on humans, improving and enhancing the sperm quality parameters with antioxidant capacity (12, 52). The activity of antioxidant genes, such as *NRF2* and *GCLC* significantly increased with the addition of an optimal concentration of Myo-Ins, which is similar to previous findings in fish (53). In contrast, the expression levels of *NQO1, GSR* and *KEAP1* were increased but not significantly compared to those in the treatment sample. Taken together, these findings suggest that the addition of Myo-Ins to the extender may improve boar semen quality by ameliorating ROS levels during storage.

Mitochondria are vital organelles and involved in redox regulation, ATP synthesis, and apoptosis (54). Interestingly, Condorelli et al. (18) investigated the role of Myo-Ins in sperm mitochondria, where they found an increased number of high membrane potential spermatozoa in OAT patients, which is in line with this study in which high membrane potential spermatozoa were found in Myo-Ins treated samples in a time-dependent manner during liquid preservation. Myo-Ins acts specifically at the level of mitochondria exerting positive effects on the ATP production via oxidative phosphorylation process subsequently results in high sperm motility, viability and MMP In this study, we assumed that Myo-Ins might protect boar semen against ROS by inhibiting apoptosis and inducing protection of sperm mitochondria; however, further studies are required to evaluate the specific mechanisms of Myo-Ins protection against mitochondria (18). We may hypothesize that Myo-Ins acts as an antioxidant and improves sperm motility and assesses plasma membrane integrity, which is related to the antioxidant content and high sperm viability.

The present study has some limitations in that the level of ROS was not determined using commercial kits such as MDA, ROS, and total antioxidant capacity kits (55). Secondly, we chose only one dose, i.e., 2 mg/mL since all the sperm parameters were best observed and the control group to investigate the antioxidant gene expression. Further study is required to investigate the effect of Myo-Ins on boar semen during liquid preservation to overcome these limitations.

5. Conclusion

The present study shows that the addition of Myo-Ins to the semen extender improved sperm motility, viability, plasma membrane integrity, acrosome integrity, and MMP and significantly improved the antioxidant related genes (*NRF2, GCLC*) in pigs. Additionally, 2 mg/mL was determined to be the optimal of Myo-Ins concentration for liquid preservation. These data could facilitate the development of a strategy for liquid storage of boar semen.

Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

Author contributions

AJ, JL, and S-HH: conceptualization, validation, writing—(original draft preparation), and writing—(review and editing). AJ, DO, HC, MK, and LC: methodology, investigation, and formal analysis. JL and S-HH: funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Mito-Tempo improves acrosome integrity of frozen-thawed epididymal spermatozoa in tomcats

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Introduction: In tomcats, epididymal spermatozoa provide an additional source of male gametes available for cryopreservation. While this procedure is feasible, the survival rate and motility of epididymal cat spermatozoa are both low after thawing. Cryopreservation is known to induce oxidative stress in spermatozoa, with mitochondria and the plasma membrane being the two major generation sites, and an imbalanced presence of free radicals is a possible cause for this low survival rate. Different antioxidants have been tested before for their effect on cryopreserved cat spermatozoa quality, with varying results. Here, we used Mito-Tempo, which is a synthetic mitochondria-targeted antioxidant and a specific scavenger of the mitochondrial superoxide system. By supplementing Mito-Tempo with the freezing extender, we aimed to improve the sperm quality of frozen-thawed cat epididymal spermatozoa.

Methods: Epididymal spermatozoa obtained from twelve tomcats were assessed for motility and concentration. Prior to freezing, samples were diluted in TRIS buffered extender with egg yolk and glycerol and divided into five aliquots supplemented with 0 (control), 0.5, 5, 50, and 1005M of Mito-Tempo. After thawing, sperm motility, concentration, morphology, plasma membrane integrity, acrosome integrity, and mitochondrial membrane potential were evaluated. A Friedman rank sum test with a Bonferroni post-hoc test was used to determine statistical in-between group differences in post-thaw semen parameters.

Results and discussion: The results indicated a slight improvement in acrosome integrity across all groups that were supplemented with Mito-Tempo, with the group that received 55M of Mito-Tempo showing the greatest improvement [(median of 67.99%, IQR of 5.55) compared to the control group (median of 65.33%, IQR of 7.75; $P = 0.05$)]. For all other sperm parameters, no significant differences ($P > 0.05$) were detected between different Mito-Tempo concentrations. These findings highlight the protective effect of Mito-Tempo on acrosome integrity and suggest that 55M is the most effective concentration for maintaining acrosome integrity. Since Mito-Tempo has shown a positive effect on multiple sperm parameters in other species, such as men, boars, roosters, rams, and bulls, we need to conclude that species-specificity may play a role here.

KEYWORDS

cat, epididymal spermatozoa, antioxidant, Mito-Tempo, reactive oxygen species

1. Introduction

Over 60% of the Felidae are classified as endangered and vulnerable or near threatened by the International Union for the Conservation of Nature (1). The fragmentation of Felidae populations into smaller, isolated groups is, among other factors, leading to an increased risk of extinction (2–4). This fragmentation is reducing gene flow, leading to a reduction in genetic diversity that results in a decrease in fertility (2, 5). Studies have shown that limited genetic variability is associated with higher production of malformed spermatozoa (5). For this reason, it is extremely important to encourage genetic variability by preserving gametes from a wider number of animals. To this aim, deceased animals can represent a convenient source for the collection of gametes to enrich genetic banks. The optimization of collection and preservation protocols is the key to support endangered species preservation and the domestic cat (*Felis catus*) represents an excellent model, providing an accessible and more abundant source of gametes.

Cryopreservation is widely used to preserve animal and human gametes (6), but the process can result in significant damage to various aspects of sperm parameters, such as the plasma membrane integrity, acrosome integrity, sperm motility, and DNA integrity (7). This degradation is primarily caused by the formation of free radicals. Free radicals can be categorized into reactive oxygen species (ROS), reactive nitrogen species, and other non-radical reactive species (6). Among these, the most commonly encountered free radicals are those belonging to the ROS family. An imbalance in the oxidant-antioxidant system caused by an overproduction of oxidants results in oxidative stress. The resulting oxidative stress can lead to lipid peroxidation, where polyunsaturated fatty acids in the plasma membrane are attacked by ROS, resulting in the formation of lipid peroxide molecules that cause physical and functional alterations to the plasma membrane, such as increased permeability, decreased fluidity, and changes in membrane protein function (6). Reactive Oxygen Species that enter the spermatozoa pose a significant threat to the genetic material. They cause destruction of the mitochondrial DNA, leading to a reduction in intracellular ATP production (8, 9). This reduction is affecting both sperm function and motility (10).

Antioxidants employed in cryopreservation extenders may serve as a safeguard against the formation of ROS and the onset of oxidative stress. These antioxidants can be classified into enzymatic activity antioxidants (such as glutathione peroxidase, superoxide dismutase (SOD), and catalase) or non-enzymatic activity antioxidants (such as vitamin C, vitamin E, vitamin B12, melatonine, resveratrol, and glutathione) (11). For this reason, several antioxidants have been tested as additives to the freezing extender in different species to improve sperm cryosurvival (12, 13). Mito-Tempo (MT) is a synthesized mitochondria-targeted antioxidant derived from piperidine nitroxide, TEMPO, conjugated with a lipophilic triphenylphosphonium cation (TPP+) and functions as a specific scavenger of mitochondrial superoxide. TEMPO works as a superoxide dismutase mimetic in the catalytic cycle of superoxide (Figure 1). TPP^+ is a membrane permeant cation that, driven by membrane potential, rapidly passes the lipid membrane and massively accumulates in energized mitochondria. The combination of TEMPO and TPP^+ creates a chemical with superoxide scavenging properties which specifically targets the mitochondria (8, 9, 14).

The effect of MT supplementation to semen extender has been investigated in various species such as humans (15, 16), boars (17), roosters (18, 19), rams (20), and bulls (21, 22). In these studies, sperm quality was revamped upon supplementation of MT when compared to the control group. To our knowledge, the effect of MT supplementation to semen extender has never been investigated in felids. The present research aimed therefore to assess the impact of this specific antioxidant on the post-thaw quality of feline epididymal spermatozoa.

2. Materials and methods

All products were purchased from Sigma (Sigma, St. Louis, USA) except if stated otherwise.

2.1. Animal samples

Testes and epididymides from twelve tomcats were collected from multiple first-line veterinary clinics across Wroclaw, Poland between April and May 2022. All tomcats were clinically healthy stray cats and underwent elective orchiectomy in a trap, neuter and release programme. After collection, samples were immediately placed into 50 mL falcon tubes containing 0.9% NaCl and transported inside a portable fridge (4°C with controlled temperature system) to the laboratory of the Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Science. Samples were processed within 1–4 h following orchiectomy. Briefly, after being removed from the physiological saline solution, the samples were washed with phosphate buffer saline (PBS; P4474) and each epididymis was carefully dissected away from the testis. Epididymal mincing was performed for spermatozoa collection. Specifically, the cauda from both epididymides, along with the ductus deferens, were placed into a Petri dish containing 5 mL of semen collection medium [Ham's F-10 medium (N6013) supplemented with 2 mM of L-glutamine (G7513), and 5% of Fetal Bovine Serum (F9665)]. A sterile scalpel blade was used to perform multiple cuts, avoiding blood vessels, on both the cauda and the ductus deferens, allowing the spermatozoa to swim out into the semen collection medium. After 10 mins of incubation at 37°C, the semen collection medium containing the spermatozoa was collected, filtered (CellTrics 30 mm, Partec) and placed into a pre-warmed 15 mL falcon tube.

2.2. Pre-freezing sperm assessment

2.2.1. Sperm motility

Sperm motility was evaluated immediately following collection of the spermatozoa. Briefly, 10 μL of the sperm suspension was placed on a pre-warmed microscopic slide, covered with a pre-warmed glass cover slip, and assessed subjectively by two equally experienced operators under a phase-contrast microscope

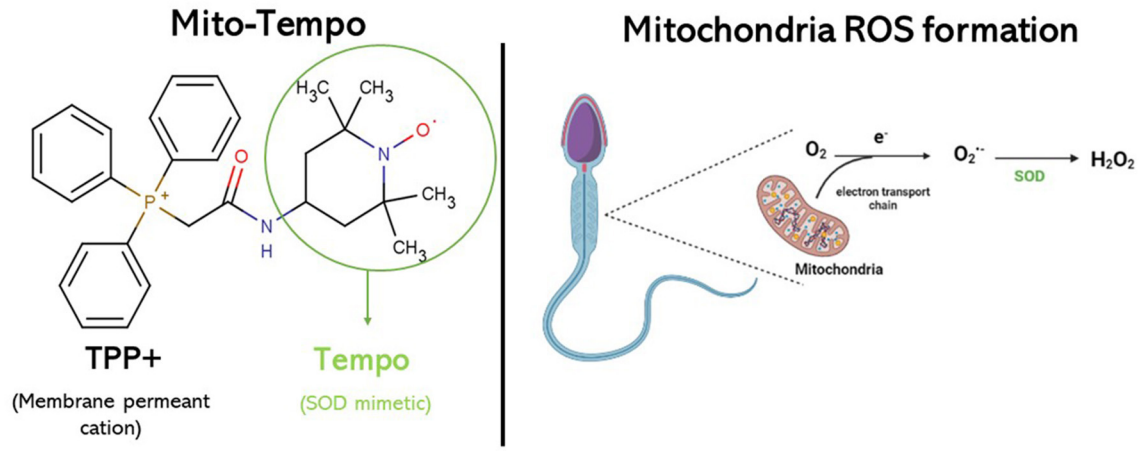

FIGURE 1
Mito-Tempo molecular formula. Acting as SOD mimetic, it can decrease ROS generation in the inner mitochondrial membrane.

equipped with a warming stage (37°C) (Nikon Eclipse E200). Total sperm motility was evaluated under five different fields by both operators and the mean was calculated and recorded.

2.2.2. Sperm concentration

Sperm concentration was measured using a Thoma counting chamber. For this procedure, 10 μL of the sperm suspension were diluted into 190 μL of water. After delicately mixing the suspension, both grids of the Thoma chamber were filled with 10 μL of the suspension. Spermatozoa were counted using a phase-contrast microscope (magnification x40) (Nikon Eclipse E200) and the concentration was calculated.

2.3. Cryopreservation and thawing

Each sperm sample was centrifuged at 620 × *g* for 5 mins at 22°C (23). The supernatant was removed, and the pellet was resuspended into semen extender I, containing 3% glycerol and 20% egg yolk in Tris buffer [3.025 g Tris(hydroxymethyl)aminomethane (T6066), 1.7 g citric acid (C7129), 1.25 g fructose (F3510), and 0.1 g streptomycin (S9137) in 100 ml distilled water] to reach a concentration of 16 × 10^6 spermatozoa/ml (24). The extended sample was then divided into four equal volumes and placed into 2 ml Eppendorf tubes (Eppendorf, Germany).

Each aliquot was supplemented with a specific concentration of Mito-Tempo (MT; SML0737) (Group A control: no MT; Group B: 5 μM MT; Group C: 50 μM MT; and Group D: 100 μM MT). The MT volume to reach the desired concentration in each aliquot was calculated and half of it was immediately added into each Eppendorf tube. Afterwards, samples were placed in a warm water bath (37°C) and cooled to 4°C. After 90 mins, the second semen extender (extender II containing 7% glycerol, 1% Equex paste, and 20% egg yolk in Tris buffer) was added alongside the other half of the calculated amount of MT to reach an final concentration of 8 × 10^6 spermatozoa/ml (24). The samples were then directly loaded into precooled 0.25 mL straws, placed 5 cm above the surface of liquid nitrogen for 10 mins before being plunged into liquid nitrogen (23). Straws were then stored at-−196°C for at least 3 days before further analyses (23).

Thawing was obtained by submerging the straws in a 37°C warm water bath for 30 secs and post-thaw sperm parameters were immediately evaluated (23).

2.4. Post-thaw sperm evaluation

2.4.1. Sperm motility and morphology

Sperm motility was evaluated as previously described for fresh samples. Sperm morphology was assessed after eosin nigrosin staining. Briefly, 10 μL of eosin nigrosin solution (v:v) were added to 10 μL of thawed sperm suspension and smeared onto a microscopic slide. After the slide was air dried, two hundred sperm cells were assessed under a bright-field microscopy (Olympus BX51TF, Tokyo, Japan) at 1,000x magnification under oil immersion. The percentages of morphologically normal sperm cells and sperm cells with abnormal heads, abnormal tails, proximal cytoplasmic droplets, and distal cytoplasmic droplets were then recorded.

2.4.2. Flow cytometer analysis

The post-thaw semen samples were diluted with TRIS buffer (3.02% (w/v) TRIS, 1.35% (w/v) citric acid, 1.25% (w / v) fructose, in distilled water; pH 6.5, all reagents purchased from Merck, Poland) to obtain a concentration of 5 × 10^6 spermatozoa/mL. Each diluted sample was then divided into three aliquots of 300 μL each to assess plasma membrane integrity, acrosome integrity, and mitochondrial activity by flowcytometry Guava EasyCyte 5 (Merck KGaA, Darmstadt, Germany) cytometer. The fluorescent probes were excited by an Argon ion 488-nm laser. Detection of green fluorescence was set with an FL1 band-pass filter (525 nm / 30 nm), orange fluorescence was measured using FL2 filter (583/26 nm) and red fluorescence was measured using an FL3 filter (695/50 nm).The

non-sperm events were gated out based on scatter properties and excluded from the analysis. A total of 10,000 events were analyzed per parameter for each sample. Gametes acquisitions were analyzed with the GuavaSoft™ 3.1.1 software (Merck KGaA, Darmstadt, Germany).

2.4.2.1. Plasma membrane integrity

Plasma membrane integrity was assessed using SYBR-14 and propidium iodide (PI) (Live/Dead Sperm Viability Kit; L7011, Life Technologies Ltd, Carlsbad, CA, USA) according to Prochowska et al. (23). SYBR-14 is a membrane permeable fluorescent dye that binds to DNA in live sperm cells and emits green fluorescence. SYBR-14 is used in combination with propidium iodide (PI), a DNA-specific stain that cannot enter the intact plasma membrane, as a dead-marker counterstain (25). Briefly, 5 μL of 0.02 mM of SYBR-14 was added to 300 μL of sperm suspension and incubated at room temperature in the dark for 10 mins. Afterwards, 1.8 μL of 2.4 mM PI was added and the sample was immediately analyzed by flow cytometry. The percentage of sperm cells with an intact plasma membrane was recorded.

2.4.2.2. Acrosome integrity

Acrosome status was evaluated using lectin PNA (PNA from *Arachis hypogaea*, Alexa Fluor 488 conjugate; L21409, LifeTechnologies Ltd, Carlsbad, CA, USA) and PI according to Prochowska et al. (23). PNA is a lectin conjugated with Alexa Fluor 488 (green-fluorescent dye) as a fluorescent probe. It specifically binds to β-galactose on the outer acrosomal membrane of sperm cells (26). Briefly, 6 μL of 0.1 mg/mL PNA was added to 300 μL of sperm suspension and incubated at room temperature in the dark for 5 mins. Afterwards, the sample was centrifuged at 620 × *g* for 5 mins and the pellet was resuspended with 300 μL of TRIS. Finally, 1.8 μL of 2.4 mM PI was added and the sample was immediately analyzed by flow cytometry. The percentage of sperm cells with an intact acrosome was recorded.

2.4.2.3. Sperm mitochondrial membrane potential

Evaluation of the sperm mitochondrial membrane potential was performed using JC-1 staining (T3168, Life Technologies Ltd, Carlsbad, CA, USA) according to Prochowska et al. (23). JC-1 is a lipophilic cationic fluorescent dye that specifically targets mitochondria. When the mitochondrial potential is low, JC-1 emits green fluorescence in its monomer form, while in high mitochondrial potential, JC-1 emits orange fluorescence in its J-aggregate form (23). Briefly, 0.4 μL of JC-1 solution (2 mg/ml JC-1 in DMSO) was added to 300 μL of the semen sample. Samples were incubated at 37°C in the dark for 20 mins. Afterwards, 1.8 μL of 2.4 mM PI was added and the sample was immediately analyzed by flow cytometry. The percentage of live sperm cells with high mitochondrial activity was recorded.

2.5. Statistical analysis

The statistical analysis was conducted using R software version 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria). Normality of the fresh and post-thaw semen parameters was assessed using Shapiro-Wilk test ($P \leq 0.05$). Friedman rank sum test with Bonferroni post-hoc test was used to determine statistical in-between group differences in post-thaw semen parameters. Specifically, differences in the amounts of motile spermatozoa (%), morphologically normal spermatozoa (%), abnormal sperm heads (%), abnormal sperm tails (%), proximal (%) and distal cytoplasmatic droplets (%), plasma membrane integrity (%), acrosome integrity (%), and mitochondrial membrane potential (%) were assessed. Significance was considered for *p*-values ≤ 0.05.

3. Results

Acrosome integrity was significantly improved in all samples treated with MT in comparison to untreated samples. Specifically, group B (5 μM of MT) appeared to exert the most protective effect on acrosome integrity compared to the control group (median 67.90%, IQR 5.55 and median 65.33%, IQR 7.75, respectively; $P = 0.05$), whereas group C and group D only showed a tendency to improve acrosome integrity in comparison to the control group ($P = 0.07$ for both concentrations). No significant differences in the percentages of motile, morphologically normal spermatozoa, or morphologically abnormal sperm cells were found between the different groups investigated. Nor were any differences found between the investigated groups in terms of plasma membrane integrity and mitochondrial membrane potential. Results (median, IQR, and overall p-values) are reported in Table 1.

4. Discussion

In the present study, we found a small increase in acrosome integrity in frozen-thawed cat epididymal semen supplemented with 5 μM of MT during freezing. All the other investigated parameters, such as sperm motility, morphology, plasma membrane integrity, and mitochondrial membrane potential, were not significantly improved. This finding was in contrast with previous studies conducted in other species, which demonstrated improvement in sperm parameters such as motility, membrane functionality, mitochondrial active potential, acrosome integrity, and viability, as well as a decrease in lipid peroxidation and DNA fragmentation (18–21). It should be noted that the assessment of sperm motility in this study was conducted subjectively. Although two operators with equal experience evaluated motility in five different fields and calculated the mean after counting spermatozoa, a significant limitation arises from the absence of more advanced techniques such as computer-assisted sperm analysis (CASA). Subjective motility assessment may not adequately capture subtle variations in motility patterns or minor changes over time, which can restrict the detection of more nuanced alterations in sperm motility. Furthermore, CASA offers the opportunity to evaluate additional characteristics of sperm kinematics that could be influenced by the supplementation of Mito-Tempo. For instance, higher straight-line velocity and amplitude of lateral head displacement are frequently associated with capacitation. Considering that Mito-Tempo exhibited a

TABLE 1 Effect of Mito-Tempo on post-thaw sperm parameters in treatment groups.

	Group A (control)		Group B (5 μM)		Group C (50 μM)		Group D (100 μM)		Overall *P*-value
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	
Motile spermatozoa (%)	33.75	25.00	27.50	11.87	30.00	23.12	30.00	16.25	0.84
Morphologically normal spermatozoa (%)	61.00	16.75	63.00	23.75	65.00	17.25	61.00	16.00	0.77
Abnormal sperm head (%)	10.50	7.50	10.50	7.50	9.00	17.00	10.00	4.50	0.91
Abnormal sperm tail (%)	15.50	20.00	15.00	18.00	17.50	14.00	17.00	15.00	0.66
Proximal cytoplasmic droplet (%)	4.00	2.25	4.50	3.25	4.00	1.75	3.00	3.25	0.10
Distal cytoplasmic droplet (%)	5.00	4.50	6.50	6.25	5.00	3.50	4.50	1.75	0.13
Plasma membrane integrity (%)	42.45	15.84	35.39	12.74	37.42	15.42	41.68	12.39	0.37
Acrosome integrity (%)	65.33[a]	7.75	67.90[b]	5.55	66.67	6.55	66.46	8.17	0.07
Mitochondrial membrane potential (%)	30.25	36.52	25.94	25.34	57.95	69.78	23.82	21.16	0.99

[a,b]Indicate significant differences ($P \leq 0.05$).

mild enhancement in acrosome integrity, it would be beneficial to assess these parameters by CASA as well to gain a more comprehensive understanding of the effects of Mito-Tempo supplementation.

Although the exact cause for this disparity remains unclear, Len et al. (27) has suggested before that antioxidants might act in a species-specific manner. For instance, the supplementation of 200 IU/ml catalase (CAT), a hydrogen peroxide targeted enzymatic antioxidant, to Tris egg yolk glycerol (TEY) extender reduced the motility of feline sperm cells (28), although supplementation of CAT at the same concentration increased the same parameter in cryopreserved bovine spermatozoa (29). Unexpectedly, adding double the concentration of CAT (400 IU/ml) did not improve the motility, viability, or acrosomal integrity of frozen-thawed cat spermatozoa (30). Therefore, an interspecies difference between cats and previously studied species that were supplemented with MT (15–22) may be a potential explanation. Epididymal spermatozoa are believed to have lower levels of antioxidant enzymes due to the lack of exposure to seminal plasma, making them more vulnerable to oxidative stress. However, in the case of cat semen, there is conflicting evidence as it is thought to be more resilient to lipid peroxidation (23). Various studies have supported this claim. In comparison, post-thaw epididymal cat spermatozoa did not experience an increase in lipid peroxidation after 6 h of incubation at 37°C (31), while frozen-thawed human spermatozoa showed increased lipid peroxidation after being incubated for 15 to 60 mins at the same temperature (32). Additionally, cold storage of equine spermatozoa for 48 h led to a significant increase in lipid peroxidation (33), whereas cat epididymal sperm stored at 5°C maintained high quality for up to 48 h of cooling and were not exposed to oxidative stress until after 72 h of cooling (34). It can be hypothesized that high levels of endogenous antioxidant activity may be present in cat spermatozoa and/or epididymal fluid that may neutralize excessive ROS concentrations during semen processing and cryopreservation. Indirect evidence for such endogenous antioxidant activity was demonstrated by Thuwanut et al. (31) who found that lipid peroxidation was only detected after 6 h post-thaw incubation with lipid peroxidation promoter [100 mM ferrous sulfate (FeSO4)] in epididymal cat spermatozoa.

Our study detected a mild protective effect of MT on acrosome integrity in epididymal cat spermatozoa after thawing when supplemented with a 5 μM concentration. Cryocapacitation, a capacitation-like change, occurs in spermatozoa during the freeze-thaw process, but its mechanism is not wellunderstood (35). Mito-Tempo is an antioxidant that acts as a mimetic of SOD, a substance that helps to preserve normal acrosome integrity and prevents premature hyperactivation and capacitation by superoxide radicals before ejaculation (36). Although the enhancement of acrosome integrity in our study was not substantial, it is an important factor that correlates directly with fertility rate. Compared to sperm motility and morphology, previous research has indicated that assessing the integrity of the plasma membrane and acrosome is a more dependable indicator for predicting *in vitro* fertility rate (37, 38). Verstegen et al. (39) reported that high levels of sperm with acrosome defects are associated with fertilization difficulties. Also, Tanghe et al. (40) demonstrated a moderate correlation between acrosome integrity and pronuclei formation after *in vitro* fertilization in bovine.

Life is a balance of opposing forces such as oxidant and antioxidant. A proper balance between these two elements is maintained through equalization, with any disequilibrium leading to potential damage. Thus antioxidants should only be used as a supplement when oxidant overproduction is expected or/and when antioxidant defense system is weakened. Consequently when antioxidant is used without a rationale, the supplementation of these additives to the semen extender might even be detrimental (41). An example of this phenomenon is the effect of catalase supplementation to the extender of chilled ram semen. Concentrations over 200 U/mL decreased the sperm quality, whereas lower concentrations have a positive effect (42). The choice of testing concentrations ranging from 0 to 100 μM was based on research on other species. Significant results were obtained upon supplementation of 5 μM and/or 50 μM of MT to the extender in several species (15, 17–22), whereas another study reported an improvement in human semen parameters, and an enhancement of antioxidant enzymes activity upon supplementation of 10 μM and 100 μM of MT (16). Nevertheless, none of the concentration chosen in the present study suggested that increasing the concentration would be neither beneficial nor detrimental for feline sperm cells. Therefore, combining two antioxidants with different functional properties, may have an additive positive effect, mitigating the cryo-stress instead of increasing the concentration of an antioxidant. For instance, when catalase and SOD were both added to an extender for boar semen, a greater improvement in post-thaw sperm parameters was achieved compared to the supplementation of either of them alone (43). Mito-tempo is a SOD mimetic antioxidant that can be targeted to mitochondria in protecting against the selective mitochondrial oxidant stress, scavenging the superoxide anion (14). On the other hand, glutathione peroxidase can then breakdown hydrogen peroxide into oxygen and water. Since the positive effect of glutathione peroxidase on feline spermatozoa has been proven (35), it might be worth investigating if combining MT or SOD with glutathione peroxidase could further enhance their protective performance on feline sperm parameters.

5. Conclusion

Our results showed that Mito-Tempo supplementation improved acrosome integrity with no positive effect on all other semen parameters investigated in this study. Previous studies have not examined the use of Mito-Tempo as a supplement to semen extenders for both ejaculated and epididymal spermatozoa in cats, so it is not possible to compare our findings to other similar studies. However, in recent years, the effect of supplementing various antioxidants to freezing extenders on sperm quality has been widely studied in various species, with inconsistent improvement of sperm quality amongst species, including felids (13, 27, 28, 44). Further research should be performed to investigate another and/or larger feline population. In addition to investigate the effect of MT on ROS production which may reveal potential combinations of MT with antioxidants.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical review and approval was not required for the animal study because the study analyzed epididymal spermatozoa from cats that had undergone castration either as a part of a spay program or based on the owner's request. The castration was not performed for the purpose of the study, but the samples were utilized to prevent their wastage. It is important to note that none of the cryopreserved sperm was used for insemination or *in vitro* fertilization. Instead, all post-thaw samples were thawed and analyzed to evaluate the effect of Mito-Tempo supplementation on various sperm parameters.

Author contributions

HA and AV: conceptualization. HA, SP, and LV: methodology and investigation. PB: statistical analysis. HA and SP: resources. HA and PB: writing—original draft preparation and visualization. SP, GD, and AV: writing—review and editing. SP, AV, and WN: supervision. AV and WN: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Reviving vacuum-dried encapsulated ram spermatozoa via ICSI after 2 years of storage

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Introduction: Freeze-drying techniques give alternative preservation mammalian spermatozoa without liquid nitrogen. However, most of the work has been conducted in the laboratory mouse, while little information has been gathered on large animals that could also benefit from this kind of storage.

Methods: This work adapted a technique known as vacuum-drying encapsulation (VDE), originally developed for nucleic acid conservation in anhydrous state, to ram spermatozoa, and compared it to canonical lyophilization (FD), testing long-term storage at room temperature (RT) and 4°C.

Results and discussion: The results demonstrated better structural stability, namely lipid composition and DNA integrity, in VDE spermatozoa than FD ones, with outcomes at RT storage comparable to 4°C. Likewise, in VDE the embryonic development was higher than in FD samples (12.8% vs. 8.7%, $p < 0.001$, respectively). Our findings indicated that in large mammals, it is important to consider dehydration-related changes in sperm polyunsaturated fatty acids coupled with DNA alterations, given their crucial role in embryonic development.

KEYWORDS

encapsulation, lipidomics, polyunsaturated fatty acids, ICSI, ovine

Introduction

Cryobiobanking in liquid nitrogen is a universally-applied and well-mastered methodology, but it is costly, energy-expensive, and has a sizeable carbon footprint (1), thus restricted to wealthy countries that have the facilities to produce liquid nitrogen and deliver uninterrupted energy supply. An alternative way for biobanking was first successfully presented in 1998 when Wakayama and Yanagimachi (2) produced offspring using freeze-dry mouse spermatozoa delivered by intracytoplasmic sperm injection (ICSI). Initially treated with indifference by the scientific community, dry storage of male gametes started to gain attention when repeated in other species. We believe that developing alternative and low-cost storage procedures is highly desirable for two main reasons. First, every undertaking that cuts the carbon footprint, like establishing and maintaining dry biobanks with storage at room temperature, is welcome and in line with global policies. Second, semen storage in the anhydrous state, besides being user-friendly and low-cost, simplifies transportation enormously; this is crucial in the era of space

travel, as already demonstrated by dry mouse spermatozoa being able to retain fertilizing capacity following exposure to cosmic radiation (3).

However, while most knowledge in the field was gained on laboratory animals such as rabbits (4), rats (5), and hamsters (6), very little research was done in farm animals such as pigs (7), cattle (8), horses (9), and sheep (10).

It is generally suggested to store vials with freeze-dried spermatozoa at sub-zero temperatures (−20°C, or even better, at −80°C), especially if long-term storage is intended (11), distancing the technique from the ideal and decisive energy-cheap room temperature storage. Full sustainable shipping and storage of spermatozoa in the dry state were demonstrated only in the ovine (12) and murine model (13). Efforts should concentrate on large mammal models for future applications in farm animals and, subsequently, in human assisted reproduction. The current state-of-the-art in drying male gametes is ill-defined, very fluid, and uncertain in terms of whether freezing should be applied, the methods chosen for water removal, and the rehydration approach. A recent review highlighted the availability of 10 other methods for water subtraction; however, only canonical lyophilization has been extensively explored, with the others receiving only marginal attention (14). This is a major shortcoming. If we observe how anhydrobiotic organisms survive desiccation, freezing and water sublimation should have received more attention.

Following an exhaustive literature review, we found a nucleic acid preservation method for long-term storage in the anhydrous state at room temperature, vacuum-drying followed by encapsulation (15), and decided to apply it to desiccating ram spermatozoa.

This study aimed to explore this alternative water extraction and packaging strategy to preserve structural integrity at the membrane and DNA levels and, consequently, the sperm fertilizing capacity using ram spermatozoa as a model. Furthermore, the effects of long-term storage temperature were analyzed.

Materials and methods

Ethics approval

The animal experiment (semen collection) has been approved by the Italian Ministry of Health (No. 200/2017-PR) based on the research description prepared by the ethics committee of the Istituto Zooprofilattico Sperimentale di Teramo (Prot. 944F0.1 del 04/11/2016). All methods were performed following the relevant guidelines and regulations of the Italian Minister of Health.

Chemicals

Unless otherwise stated, all materials used were purchased from Sigma Aldrich (St Louis, MO, USA).

Semen collection

Semen of a fertile Sardinian ram whose reproductive ability was previously confirmed (10, 16) was collected using an artificial vagina filled with warm water (40–44°C) and connected to a 15-mL sterile tube. Sperm motility was evaluated using a stereomicroscope immediately after collection. The sample was diluted 1:1 with Basic Medium (300 mM TRIS base, 105 mM citric acid, 82 mM fructose, 150,000 IU penicillin G, 2 mM streptomycin in 67.2 mL bi-distilled water) and transported to the laboratory in a transportable incubator (INC-RB1 Biotherm, Cryologic, Blackburn, Australia) at 32–35°C.

Upon arrival, the ejaculate was split in two and was processed at two institutions by two drying techniques (FD and VDE; Figure 1 and the following subsections).

All experiments described herein were performed using a single ejaculate to reduce variability and produce the most objective observations of the tested variables.

Sperm cryopreservation

Semen cryopreservation was conducted following the established protocol outlined in (16). In brief, the freezing medium was prepared as follows: a Basic Medium was created by dissolving 2.42 g of TRIS base, 1.36 g of citric acid, 1.00 g of fructose, 100,000 IU of penicillin G, and 0.1 g of streptomycin in 67.20 mL of bi-distilled water, with the pH adjusted to a range of 6.7–6.8. Next, the Basic Medium was divided into two equal volumes, resulting in Medium A (maintained at 30°C) and Medium B (kept at 4°C). Medium A contained 20% egg yolk and 12.8% ddH2O, while Medium B contained 20% egg yolk and 12.8% glycerol. An equal volume of each of these two mediums was added to the ejaculate, achieving a final concentration of 400×10^6 spermatozoa/ml. The process involved adding Medium A (at 30°C) to the ejaculate, which was then maintained at 4°C for a duration of 2 h. Subsequently, Medium B (at 4°C) was introduced and maintained for an additional 2 h at the same temperature. Throughout this process, tubes were agitated every 30 min. Finally, 250 µL straws were filled with the semen and allowed to equilibrate on liquid nitrogen vapors for 20 min. Following this, the straws were sealed and immediately submerged in liquid nitrogen.

Sperm drying and samples storage

Sperm freeze-drying

Sperm FD was performed at the Faculty of Veterinary Medicine, University of Teramo (Italy), as previously described (10). Briefly, on arrival at the laboratory, the motile component was isolated by swim-up. Ejaculated semen (20 µL) was placed at the bottom of a 15-mL sterile tube containing 1.5 mL Basic Medium and left inclined at 45° for 20 min at 37°C. Subsequently, the top 750 µL containing the after swim-up spermatozoa was collected, and the concentration was corrected to 20×10^6 spermatozoa/mL in the lyophilization medium (1 mL 0.5 M TRIS, 5 mL 0.5 M EGTA, 2.5 mL 1 M NaCl, all dissolved in bi-distilled water). Aliquots of 100 µL were placed in glass vials (Ø 8 mm), loaded into Mister Frosty (Thermo Fisher Scientific, Waltham, MA, USA), and progressively cooled to −50°C at a cooling rate of −1°C/min.

After freezing, the glass vials with the frozen samples were inserted into larger glass vials (Ø 20 mm), partially covered with stoppers, and placed inside the freeze-drying apparatus (VirTis BenchTop 2.0, SP Scientific, Gardiner, NY, USA) with the condenser temperature set at −58°C and the freeze-drying chamber at −12°C. As soon as the vials were placed in the lyophilizer, the vacuum pump was started, and drying proceeded for 16 h. The vials were sealed under a vacuum (pressure of 15 µbar). The freeze-drying chamber's temperature reached −30°C.

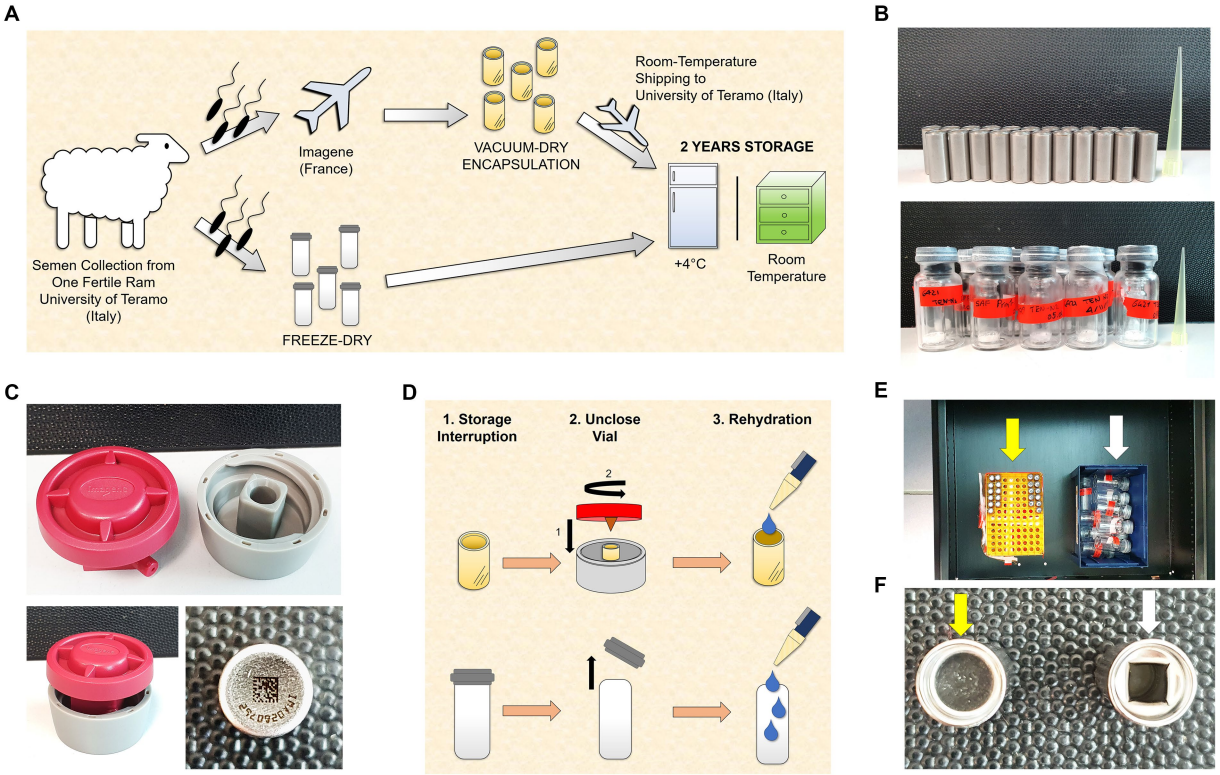

FIGURE 1
Experimental design. **(A)** Semen collection was carried out at the University of Teramo (Italy). An aliquot was freeze-dried (FD) immediately, and another aliquot was sent to Imagene (France) to perform the vacuum drying followed by encapsulation (VDE). The VDE spermatozoa were returned to the University of Teramo at room temperature and stored with the FD samples for 2 years at 4°C (4C) and in a drawer at room temperature (RT). **(B)** Stainless steel capsules containing the VDE spermatozoa sealed by laser under an inert gas atmosphere in the top panel. Glass vials [small (Ø 8 mm) in large (Ø 20 mm)] with the FD spermatozoa sealed under vacuum in the bottom panel. **(C)** The *ad-hoc* shellOpener was developed to open the VDE capsules (top and bottom left pictures). The sample number and QR code on the bottom of the capsule label the VDE samples (bottom right image). **(D)** The FD and VDE samples, stored for 2 years, were opened and rehydrated after two years of storage. VDE minicapsules (upper row) were opened using the *ad-hoc* shellOpener. **(E)** FD (white arrow) and VDE (yellow arrow) spermatozoa stored for 2 years in a drawer. **(F)** Top view of a sealed VDE (yellow arrow) and opened (white arrow) minicapsule.

Sperm vacuum drying encapsulation

The spermatozoa, suspended in the Basic Medium, were transferred at 4°C to Imagene (France). Upon arrival, the semen's motility was assessed, yielding a motility rate of 80%. Subsequently, the tubes were centrifuged, and the pellet was resuspended in the lyophilization medium and the concentration was corrected to 20×10^6 spermatozoa/mL. Aliquots (50 µL) were placed in stainless steel minicapsules and dried under vacuum for 55 min in an evapo-concentrator (HT4, Genevac, Ipswich, UK). The minicapsules were then transferred into a glove box and maintained for 72 h under an anoxic and anhydrous argon/helium atmosphere for further desiccation. At the end of the process, the minicapsules were capped with stainless steel caps and sealed by laser welding. Finally, the minicapsules were checked for leakage by mass spectrometry.

Storage condition and international room-temperature sample shipping

The ready minicapsules were shipped from France to Italy (University of Teramo) at room temperature. All samples (freeze-dried and vacuum-dried) were stored for 2 years, 30 ($n = 15$ each) at 4°C in the dark (FD_4C and VDE_4C, respectively) and 30 ($n = 15$ each) at room temperature (RT) in the dark (FD_RT and VDE_RT, respectively). The samples stored at RT were kept in a controlled environment with a temperature range between 20 and 25°C. At the end of the 2-years storage, the samples were used for *in vitro* fertilization by ICSI and flow-cytometric and lipidomic analyzes as described below.

Opening and resuspension of the FD vials and VDE minicapsules

The VDE minicapsules were opened using an *ad hoc* shellOpener (Figures 1C,D,F). The minicapsules were placed in the special housing of the shellOpener, and the shellOpener lid was screwed, ensuring that the tip on the inside of the lid punched a hole in the minicapsules. For the FD vials, the rubber stopper was removed. All samples were rehydrated with bi-distilled water: 100 µL for FD samples and 50 µL for VDE samples. All samples were rehydrated within controlled environments, maintaining a temperature range between 20 and 25°C.

Spermatozoa quality evaluation

Live-dead staining

After rehydration, samples were incubated with 0.1 µg/mL Hoechst 33342 (to mark all nuclei) and 5 µg/mL PI (to detect

spermatozoa with damaged membrane) in PBS for 5 min at RT. Subsequently, a drop (15 µL) was placed on the slide, covered with a coverslip, and observed on an epifluorescence microscope (Eclipse E-600, Nikon, Tokyo, Japan).

Flow cytometry analysis

Measurements were recorded using a Guava EasyCyte 5HT microcapillary flow cytometer (Merck KGaA, Darmstadt, Germany) with fluorescence probes excited by a 20-mW argon ion laser (488 nm). Forward-scatter vs. side-scatter plots were used to separate the spermatozoa from debris by excluding non-sperm events from further analysis. Fluorescence detection was made with three photomultiplier tube detectors: FL-1 (green: 525/30 nm), FL-2 (yellow/orange: 586/26 nm), and FL-3 (red: 690/50 nm). Calibration was made using standard beads (Guava Easy Check Kit, Merck Millipore). We analyzed 5,000 sperm events per sample at a flow rate of 200 cells/s. Compensation for spectra overlap between fluorochromes was set according to the procedures outlined by (17). Data were acquired and analyzed using CytoSoft and EasyCompDNA software (Merck KGaA), respectively.

Acrosomal membrane integrity

Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) and Propidium Iodide (PI) at appropriate concentrations and 2.0×10^5/mL rehydrated spermatozoa were added to Easy Buffer (IMV Technologies, l'Aigle, France). FITC-PNA labeled damaged acrosomes green, whereas PI-stained spermatozoa with damaged membrane red. Samples were incubated for 45 min at 37°C in the dark following the manufacturer's protocol. Three replicates per sample were performed. After gating out non-DNA-containing particles, two populations were detected on the FL1/FL-3 dot plot, spermatozoa with damaged membranes with intact or damaged acrosomes.

Sperm DNA fragmentation

Sperm Chromatin Structure Assay (SCSA) (18) was used as previously described (10). Samples were stained with acridine orange, a fluorochrome that turns from red to green depending on the degree of chromatin compaction, to distinguish between denatured, single-strand, and double-strand DNA regions. The rehydrated FD and VDE sperm concentration was corrected to 20×10^6 spermatozoa/mL. Twenty-five µL of the rehydrated sperm suspension was diluted in 195.75 µL TNE buffer [0.01 M Tris–HCl, 0.15 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4] and then added into 1,200 µL of an acidic solution (0.1% Triton X-100, 0.15 M NaCl, 0.08 M HCl; pH 1.2). After 30 s, the cells were stained with 1.2 mL acridine orange solution (6 µg/mL in 0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl; pH 6.0). After 2.5 min, two replicates per sample were read on the flow cytometer.

Data were acquired and analyzed using cytoSoft and EasyCompDNA software, respectively (Merck KGaA). The DNA damage types were quantified with the following parameters: %DFI, DNA fragmentation index, the extent of DNA denaturation, i.e., the percentage of spermatozoa outside the main sperm population (those with fragmented DNA); %HG, percentage of spermatozoa with high green fluorescence, i.e., the immature cells part of DFI/cells with "high fragmented DNA."

Glycerophospholipid extraction and analysis

Fresh refrigerated and dried spermatozoa of the four experimental groups (FD_4C, FD_RT, VDE_4C, VDE_RT) were analyzed in triplicate by gas chromatography (GC) with a flame ionization detection (FID) system using Agilent 7890B (Agilent Technologies, Milan, Italy) chromatographic system equipped with a Split/Splitless injector, FID system, and a 60 m × 0.25 mm × 0.25 µm DB-23 column.

Fresh chilled (250-µL) and rehydrated (100-µL) samples were centrifuged at 14,000 rpm at 4°C for 15 min. The pellets were transferred to an LNG-R1 robotic unit. The steps performed by the robotic unit were: (a) lipid extraction with 2:1 organic chloroform phase to methanol, collection, anhydrification with anhydrous sodium sulfate in vials, and vacuum-drying a Genevac evaporator; (b) treatment of the samples with 2 mL 0.5 M methanolic potash, stirring for 15 min at RT, adding 0.5 mL H_2O, and extracting the methyl ester fatty acids with *n*-hexane. The extract was carefully mixed with 15 µL hexane, transferred into amber vials, positioned in the GC autosampler (Agilent 7693A), and processed through the analysis sequence. A wash sample (containing only n-hexane) was injected after running one or two samples and at the end of the sequence. The relative quantitative percentages of the fatty acids were calculated automatically by the software (Agilent OpenLab CDS ChemStation Edition 5.03; driver version 1.03). The software allows to express the results also as absolute concentrations in mg/mL.

Oocyte recovery and *in vitro* maturation

Sheep ovaries were obtained from a local slaughterhouse and transferred to the laboratory at 37°C within 2 h from slaughter. Cumulus-oocyte complexes (COCs) were aspirated using 21G needles in the presence of 4-(2- hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered TCM-199 medium (Gibco, Life Technologies, Milan, Italy) with 0.005% (w/v) heparin. Only COCs with at least two layers of compact cumulus cells were selected for *In Vitro* Maturation (IVM). In 4-well dishes, 500 µL of IVM medium [bicarbonate-buffered TCM-199 (Gibco) containing 2 mM glutamine, 0.3 mM sodium pyruvate, 100 µM cysteamine, 10% (v/v) fetal bovine serum (FBS; Gibco), 5 µg/mL follicle-stimulating hormone (Ovagen, ICP, Auckland, New Zealand), 5 µg/mL luteinizing hormone, and 1 µg/mL 17 β-estradiol] was added to each well. Maturation was performed in a humidified atmosphere at 38.5°C and 5% CO_2 in air for 24 h, as previously described (19). After IVM, MII (Metaphase II) oocytes with expanded cumulus and normal morphology were selected for ICSI. The cumulus cells were removed by fast pipetting of the COCs in 500 µL hepes-buffered TCM-199 with 0.4% bovine serum albumin (BSA; w:v; H199) and 300 U/mL hyaluronidase. The oocytes were washed three times in H199 and incubated in a Petri dish, pending injection.

Intracytoplasmic sperm injection

Only oocytes with a visible first polar body were processed by Intracytoplasmic Sperm Injection (ICSI). Rehydrated spermatozoa (5-µL aliquots) were suspended in 100 µL of H199 with 0.4% BSA (w:v) and then diluted 1:1 with 12% (w:v) polyvinylpyrrolidone in PBS. Three 10–µL drops were placed on the lid of a Petri dish on a warm microscope stage (38.5°C) and covered with warm mineral oil (38.5°C). Fertilization was performed on an inverted microscope (Eclipse Ti2-U, Nikon) connected to a micromanipulation system (NT-88NEN, Narishige, Tokyo, Japan) and a piezo-driven

micropipette system (PiezoXpert, Eppendorf, Milan, Italy). The oocytes were injected 24 h after the start of the IVM. The polyvinylpyrrolidone/sperm-containing drops were renewed every 10 oocyte injections. After injection, the oocytes were chemically activated by incubation in 5 µM ionomycin in H199 + 0.4% BSA for 5 min, washed once in H199 + 0.4% BSA for 5 min, and then placed in IVC-Medium (BO-IVC, cat. 71,005; ivf Bioscience, Falmouth, UK) + 10 µg/mL cycloheximide for 3.5 h in humidified atmosphere at 38.5°C and 5% CO_2. Subsequently, they were moved to embryo culture, as described below.

In vitro embryo culture

The embryo culture was performed following our standard lab procedure (16) with slight changes. Briefly, all presumptive zygotes were cultured five per 20-µL IVC-Medium (BO-IVC, cat. 71,005; ivf Bioscience) drop, covered with mineral oil (Mineral Oil, cat. 51,002; ivf Bioscience), and placed in humidified atmosphere at 38.5°C with 5% CO_2 and 7% O_2 for 7–8 days. The *in vitro* development was evaluated 24 h after activation for cleavage (only the 2-cell embryos were considered to have cleaved) and on days 7–8 for expanded blastocyst formation. Embryo observation and image acquisition were made on an inverted microscope (Eclipse Ti2-U; Nikon) using Octax EyeWare Imaging Software (version 2.3.0.372; Vitrolife, Västra Frölunda, Sweden).

Pronuclear staining

To visualize pronuclei (2PN) in embryos fertilized by freeze-dried spermatozoa, a total of 28 presumptive zygotes were fixed in 4% paraformaldehyde (PFA) for 20 min, at 14–16 h after spermatozoa injection/chemical activation. Subsequently, the presumptive zygotes were permeabilized with 0.1% Triton X-100, stained with 5 µg/mL of propidium iodide (PI) for 5 min at room temperature, washed twice in 0.4% polyvinylpyrrolidone (PVP) in PBS, and then mounted on slides. Images were captured using a confocal microscope (Nikon Eclipse Ti-E).

Statistical analysis

Data obtained from flow cytometry (DNA and acrosome integrity), *in vitro* embryo development (cleaved embryos and expanded blastocysts), and glycerophospholipids (spermatozoa fatty acids) were analyzed using SAS, v 9.4 (SAS Institute Inc., Cary, NC, USA). The general linear model evaluated the effect of the four experimental treatments on *in vitro* quality traits (sperm quality and embryo development) and the fatty acids profile. Results are presented as adjusted least squares means ± standard errors of the means.

The global lipidomic and lipidomic data were also assessed in GraphPad Prism for Windows (Version 6.01, GraphPad Software, San Diego, CA, USA) using a one-way [Kruskal–Wallis *H* test and two-way ANOVA nonparametric tests (Friedmann's test)], respectively.

Multi-dimensional preference (MDPREF) analysis explored the *in vitro* quality traits (sperm quality and embryo development) and the fatty acid profile that correlated with the two sperm drying protocols (FD and VDE) and the two storage temperatures (RT and 4°C). It is a principal component analysis that detects linear and non-linear variable transformations using the alternating least squares method, which optimizes the transformed variables' correlation properties or the covariance matrix. MDPREF analysis identifies the most salient variability to the preference patterns of the drying protocols and storage temperatures toward the *in vitro* quality traits (sperm quality and embryo development) and the fatty acids profile, and extracts it as the first principal component.

Statistical significance was set at $p < 0.05$.

Results

Effects of storage conditions and desiccation and packaging protocols on sperm quality

Samples produced by freeze-drying (FD) and vacuum-drying and encapsulation (VDE) were stored for 2 years at 4°C (4C) and room temperature (RT; Figure 1). Both drying and storage techniques preserved a high degree of DNA integrity, with DNA damage levels below the threshold for subfertility. The DNA fragmentation index (%DFI) did not undergo significant variations between the experimental groups. In the other side, the percentage of high green fluorescence (%HG) in FD_RT was lower than FD_4C (Table 1 and Figure 2).

The cellular membrane of all spermatozoa in both desiccation methods and storage types was damaged after rehydration, as confirmed in the total positivity to PI (Figures 3A,B).

Post-rehydration structural assessment of intact acrosome (DI%) were statistically insignificant, anyway the FD_RT group showed the highest proportion of acrosome integrity (Figures 3E,F and Table 1).

Vacuum-dry encapsulation better preserve sperm fertilizing ability after ICSI in RT storage

As an initial assessment to rule out the development of haploid parthenogenic embryos arising from the chemical activation of oocytes fertilized via ICSI (Figure 3C), was examined the rate of 2-pronuclei (2PN) embryo formation. As illustrated in Figure 3D, oocytes that were fertilized using freeze-dried spermatozoa and subsequently subjected to chemical activation involving ionomycin and cycloheximide exhibited a 2PN formation rate of 78.57% (22/28). Rehydrated spermatozoa injected into sheep *in vitro*-matured oocytes (Figure 3C) led to a higher embryo cleavage when stored at RT than 4°C in the FD [54.3% ($n = 62$) vs. 25.4% ($n = 83$); $p < 0.001$] and VDE [46.5% ($n = 53$) vs. 32.0% ($n = 70$); $p < 0.001$; Figure 3H] groups. Additionally, when utilizing frozen/thawed semen (FZ), there was a reduced formation of 2-cell embryos (FZ, 22.22%, $n = 36$) in comparison to FD_RT ($p = 0.0098$) and VDE_RT ($p = 0.0248$). RT also had a higher day-8 expanded blastocyst formation rate than 4°C in the FD (8.7% vs. 3.2%; $p < 0.001$) and, even more apparent, VDE (12.8% vs. 5.8%; $p < 0.001$; Figures 3G,H) groups. Furthermore, in the comparison with the FZ group, there was a greater formation of

TABLE 1 Acrosome integrity and DNA fragmentation evaluated by flow cytometry in dried-rehydrated spermatozoa.

Variable	Experimental group				
	FD_4C	FD_RT	VDE_4C	VDE_RT	EG effect
DI (%)	17.95 ± 5.35	32.73 ± 6.55	24.68 ± 5.35	28.67 ± 5.35	ns
DR (%)	82.05 ± 5.34	67.27 ± 6.54	75.33 ± 5.34	71.33 ± 5.34	ns
% DFI	1.103 ± 0.099	0.860 ± 0.121	1.033 ± 0.099	1.108 ± 0.099	ns
% HG	0.748 ± 0.135^{ab}	0.520 ± 0.166^{a}	1.060 ± 0.135^{b}	0.768 ± 0.135^{ab}	$P < 0.05$

Values are presented as least square means and standard error of the means. Different superscript letters in the same row correspond to a significant ($P < 0.05$) difference between experimental groups, "ns" means not significant. EG, experimental group; FD, freeze-drying; VDE, vacuum-dried and encapsulated; 4C, storage at 4°C; RT, storage at room temperature; DI, dead spermatozoa with intact acrosome; DR, dead spermatozoa with damaged acrosome; %DFI, DNA fragmentation index; %HG, sperm with high green fluorescence, describes poor chromatin condensation.

expanded blastocysts (FZ, 22.22%, $n = 36$) compared to FD_4C ($p = 0.001$) and VDE_4C ($p = 0.0201$).

Dynamics of glycerophospholipid composition in FD and VDE ram spermatozoa

Table 2 provides a selection of the global lipidomic analysis. The entire list of 29 fatty acid can be found as Supplementary Table S1. Docosapentaenoic acid (DPA; C 22:5 ω3) in the FD_RT group was higher than in the FD_4C group (0.70 ± 0.04 vs. 0.55 ± 0.04) and lower in the VDE_RT group than in the VDE_4C group (0.54 ± 0.04 vs. 0.67 ± 0.04). Docosahexaenoic acid (DHA; C 22:6 ω3) in both VDE storage groups was significantly higher than in the respective FD samples. Moreover, all dry semen samples (FD_4C, FD_RT, VDE_4C, and VDE_RT) exhibited a significant elevation in DHA levels when compared to fresh semen. Spermatozoa in the VDE_RT group presented a higher quantity of monounsaturated fatty acids (MUFA; 10.91 ± 0.47) and omega-6 polyunsaturated fatty acids (PUFA ω-6; 11.26 ± 0.23) than all other groups. The VDE_4C group had significantly higher omega-3 PUFA (PUFA ω-3) than the VDE_RT group (59.14 ± 1.72 vs. 53.06 ± 1.72, $p < 0.05$). The total PUFA quantity (PUFA TOT) in the VDE_RT samples was higher than in the FD_RT samples.

Freeze-drying and vacuum-drying with encapsulation shape the polyunsaturated fatty acid distribution

The changes in membrane fatty acid composition of frozen/thawed ram spermatozoa were well documented (20); likewise, this work reports on similar changes in the FD and VDE spermatozoa.

The most dramatic changes were recorded for C20:3 cis ω-3 (Eicosatrienoic acid). It significantly ($p < 0.001$; Figure 4A) decreased from 40.74 ± 5.32 in the fresh samples to 2.95 ± 3.19 in FD_4C, 2.12 ± 0.67 in FD_4C, 1.16 ± 0.27 in VDE_4C, and 1.21 ± 0.22 in VDE_RT. A similar but less distinct trend was noted for C18:2 ω-6 (linoleic acid), decreasing from 3.46 ± 0.71 to the following respective values: 2.70 ± 0.18, 2.55 ± 0.35, 2.40 ± 0.03, and 1.93 ± 0.11; $p < 0.05$; Figure 4B). The C20:3 ω-6 (dihomo-γ-linolenic acid) in FD_RT (1.33 ± 0.31) was higher than in FD_4C (1.25 ± 0.03) and fresh (0.51 ± 0.13). The C20:4 ω-6 (arachidonic acid) level in VDE_RT (8.28 ± 0.40) was higher than in fresh (0.89 ± 0.120, FD_4C (5.13 ± 0.75), FD_RT (4.93 ± 0.59), and VDE_4C (5.73 ± 0.07; $p < 0.001$). Furthermore, the arachidonic acid level in the fresh samples was lower than in all FD and VDE groups ($p < 0.001$), and that in FR_RT lower than in VDE_4C ($p = 0.010$).

The ratio of PUFA to saturated fatty acids (SFA) revealed that the VDE_4C (2.46 ± 0.02) and VDE_RT (2.60 ± 0.01) groups exhibited higher values compared to the remaining groups (Fresh: 1.80 ± 0.18; FD_4C: 1.73 ± 0.19; FD_RT: 1.71 ± 0.13; $p < 0.05$; Figures 4C, D).

Possible association between *in vitro* quality features, fatty acids, desiccation method, and storage conditions

Multi-dimensional preference (MDPREF; Figure 4E) analysis showed that most variability was due to the first two components (~87%). The analysis produced four dimensions based on the four experimental groups (FD_4C, FD_RT, VDE_4C, and VDE_RT), with their relative sperm quality variables' associations.

Among the experimental groups, a positive relationship between the DNA quality variables (%DFI and %HG) and VDE_4C was noted, while FD-RT showed the opposite trend. The embryo development traits (cleavage and expanded blastocyst) and good-quality sperm membrane (intact acrosome) were positively associated with VDE_RT and negatively associated with FD_4C, in which the sperm membrane quality was poor (reacted acrosome) and contained a lower DPA level. A positive association was observed between MUFA and PUFA (ω-3 and DHA, ω-6, and PUFA TOT) and the VDE samples. A good association was noted between SFA and TRANS TOT fatty acid and the FD_RT samples.

Discussion

The first successful fertility preservation of dried spermatozoa has been achieved using lyophilization (21, 22); therefore, this method became the default water extraction strategy in almost all the subsequent studies. This study describes and analyzes a previously unexplored drying technique, vacuum drying, which has been found to be less invasive for the structural integrity of spermatozoa glycerophospholipids, acrosome, and DNA levels. Moreover, this method has demonstrated improved fertilization outcomes when evaluated in pre-implantation stage embryos.

The benefits of this new sperm drying method were tracked even after 2 years of storage at 4°C and RT; remarkedly and unexpectedly, the latter storing approach proved the most appropriate (Table 1). As

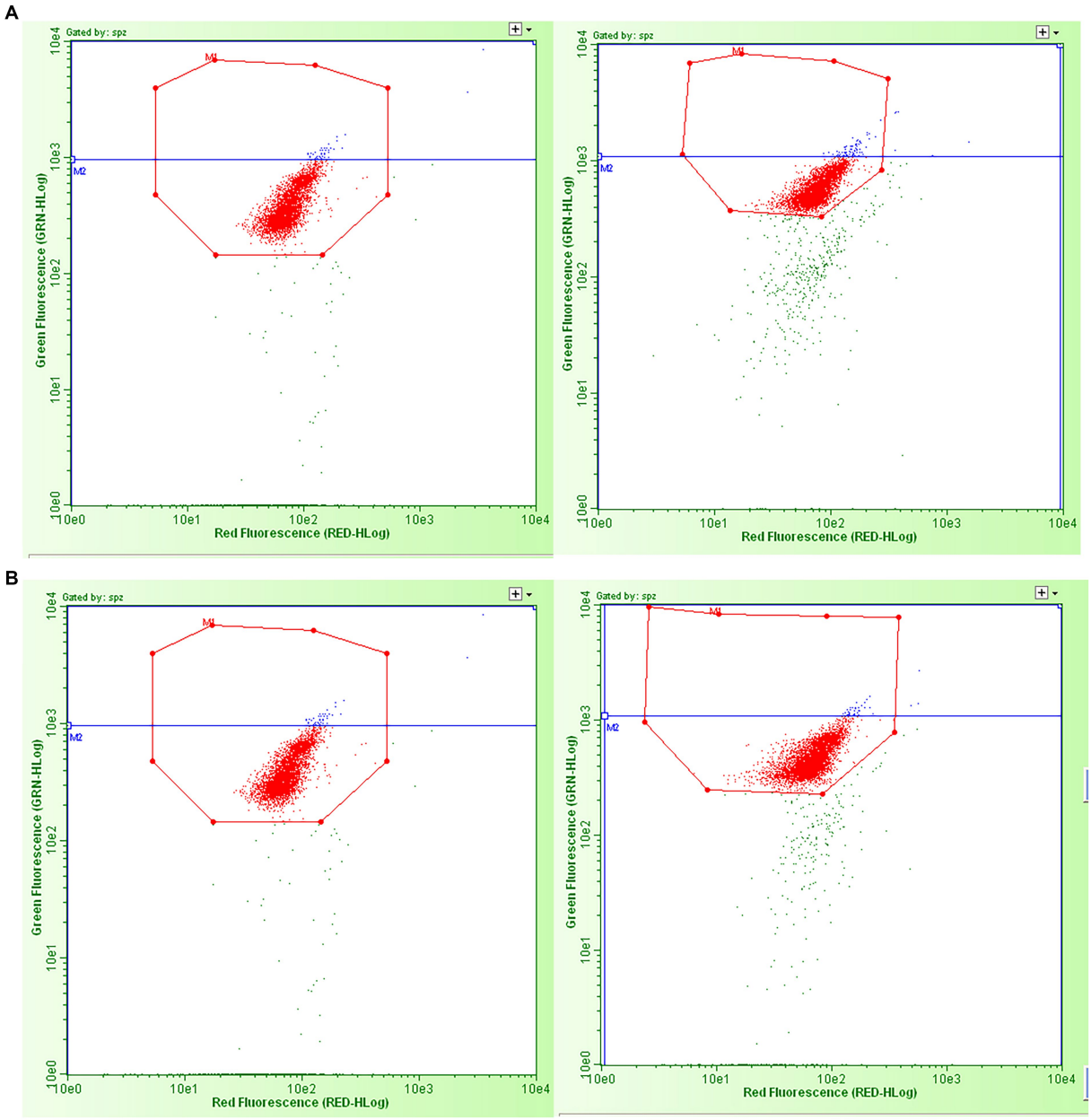

FIGURE 2
Examples of green vs. red fluorescence intensity scatter plots obtained by flow cytometric sperm chromatin structure assay analysis of the dried spermatozoa from the four experimental groups. **(A)** Left: freeze-dried and stored at room temperature; right: vacuum-dried, encapsulated, and stored at room temperature. **(B)** Left: freeze-dried and stored at 4°C; right: vacuum-dried, encapsulated, and stored at 4°C. The red dots in the octagons represent two populations of sperm cells: sperm with the double strands (with no DNA fragmentation) and sperm with single-strand DNA breaks (with "fragmented DNA" - %DFI and "high fragmented DNA - %HG). The blue dots in the octagons represent the percentage of spermatozoa with high green fluorescence, i.e., the immature cells part of DFI/cells with "high fragmented DNA." The green dots outside the octagons are non-sperm events, considered as debris.

previously noted, the data presents an entirely unexpected outcome. However, it is conceivable that an explanation for this phenomenon can be found in the nature of the membrane lipids of ram spermatozoa. These lipids may create more favorable conditions for preservation at RT in anhydrous state, as elaborated upon in the following discussion.

Sperm lipid changes during cryopreservation have been extensively investigated (23). Collectively, the published data indicate susceptibility to cold shock and differences among species in lipid phase transition and sperm survival that seem to be linked to their membranes' PUFA (polyunsaturated fatty acids) to SFA (saturated fatty acids) ratio (24) and cholesterol content (25). However, little is known about lipid changes during sperm drying. To fill this knowledge gap, we analyzed lipid composition and its variations following various drying and storage conditions in ram spermatozoa. Ram spermatozoa, like those of bull (26) and boar (27), are characterized by a high PUFA to SFA ratio (28). Our fresh ram spermatozoa data presented a PUFA to SFA (TOT) ratio of roughly 60:35 (1.71), in agreement with these previous reports. The

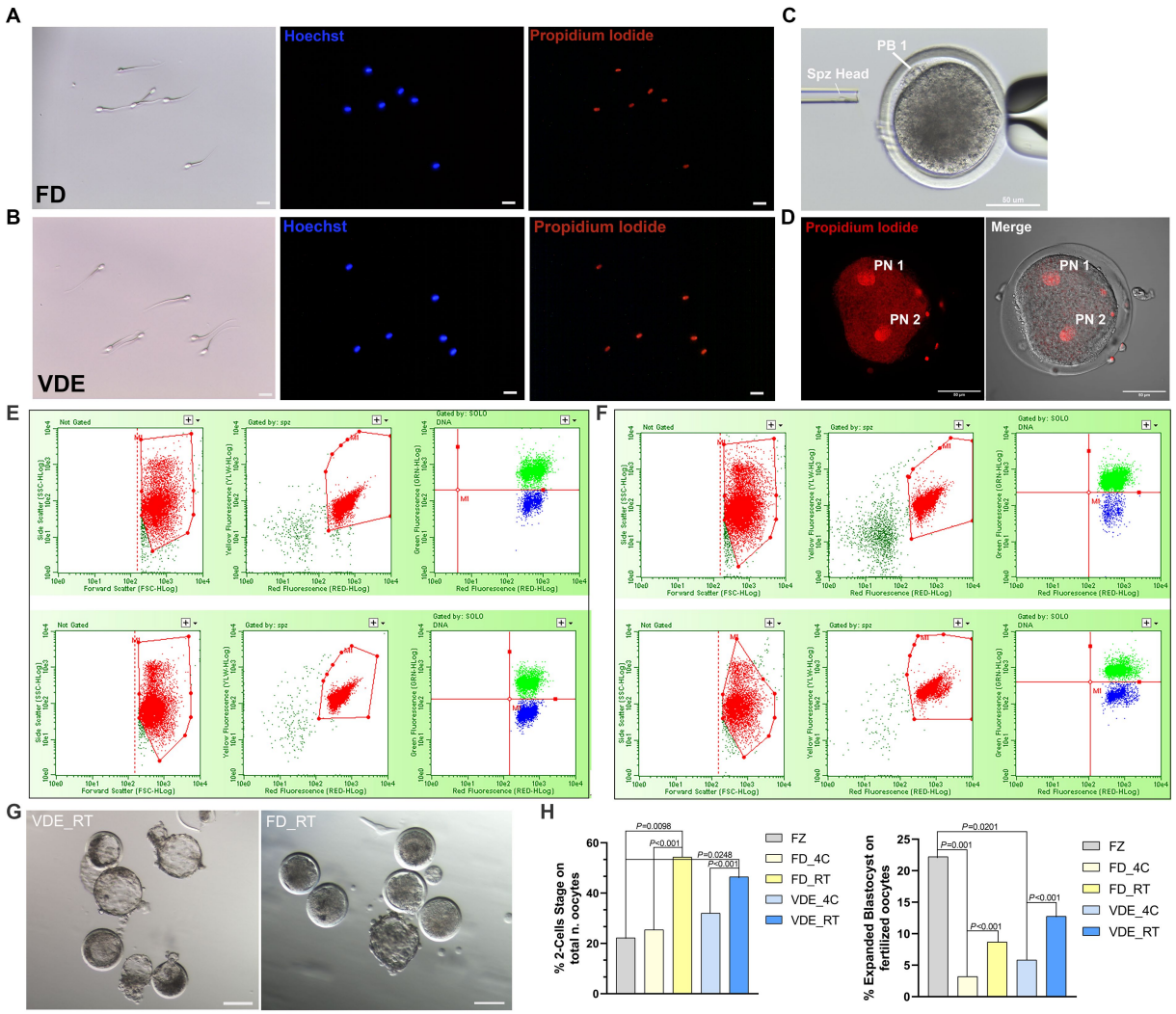

FIGURE 3
Post-rehydration sperm morphology and embryonic development. **(A)** Freeze-dried (FD) and **(B)** vacuum-dried and encapsulated (VDE) spermatozoa stained with Hoechst 33342 (blue signal) and propidium iodide (red signal) after rehydration. All cells were positive for propidium iodide. Scale bar = 20 µm. **(C)** Intracytoplasmic sperm injection of a dried-rehydrated spermatozoon into an *in vitro* matured sheep oocyte. Spz Head, sperm head; PB1, first polar body. Scale bar = 50 µm. **(D)** Activated oocyte fertilized by freeze-dried spermatozoa via ICSI with two distinguishable pronuclei: Pronuclear 1 (PN 1) and Pronuclear 2 (PN 2), Scale bar = 50 µm. Flow cytometric analysis of acrosome integrity in FD **(E)** and VDE **(F)** spermatozoa. The red population represent: in the first two panels, the total events acquired; in the second two panels, the events of the first panel plotted in the second panel considering only sperm cells, excluding debris. The green and blue populations represent dead spermatozoa with damaged and intact acrosomes, respectively. The top row in **(E,F)** shows an example of spermatozoa analyzed after storage for 2 years at 4°C; the bottom row shows the respective results of spermatozoa stored for 2 years at room temperature. **(G)** Blastocysts produced via ICSI of VDE (on the left) and FD (on the right) spermatozoa stored for 2 years at room temperature (RT) at day 7th of *in vitro* embryo culture. Scale bar = 100 µm. **(H)** *In vitro* embryo development outcomes, 2-Cells Stage embryos (on the left) and expanded blastocyst (on the right). FZ, frozen/thawed spermatozoa.

different proportions of MUFA, PUFA, and SFA have a significant effect on the physical and chemical properties of the membrane, including its fluidity at room temperature (29) and lipid phase transition temperature (30). The lipid conformation of ejaculated ram spermatozoa makes them highly susceptible to cold shock (27). This high susceptibility could partially explain the membrane damage and acrosomal loss in our semen samples as they were stored at 4°C during transport to France or frozen at a slow cooling rate (1°C per minute) before water sublimation in the FD groups. It is possible that storage of the dried samples at 4°C for 2 years slowly but progressively enhanced the damage to the membranes. In any case, it's worth noting that post-rehydration, freeze-dried sperm display complete immobility (a condition significantly distinct from that of frozen semen). This observation underscores the impact of lyophilization, likely connected to the inherent nature of the technique, on the integrity of the sperm's outer membrane.

Functional tests using ICSI fertilization indicated a yield of semen stored at 4°C comparable to that stored at RT.

Long-term sperm storage at RT was reported in mice (31) and rabbits (32). However, the length of RT storage in those studies was shorter, a maximum of 1 year (33), than the duration reported in our work. Our storage conditions were intentionally kept straightforward, involving the placement of capsules/vials in a drawer. Nevertheless, it's worth noting that the office environment is carefully controlled, with a temperature ranging between 20°C and 25°C and a humidity level maintained between 40 and 60% (Figure 1E).

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TABLE 2 Dried-rehydrates sperm fatty acid analysis in the four experimental groups.

Sperm fatty acids	Experimental group (Values are presented as a % of the total fatty acids)				
	FRESH	FD_4C	FD_RT	VDE_4C	VDE_RT
Docosapentaenoic acid (DPA) C22:5n3	0.67 ± 0.17	0.55 ± 0.04	0.70 ± 0.04	0.67 ± 0.04	0.54 ± 0.04
Docosahexaenoic Acid (DHA) C22:6n3	5.53 ± 0.78	46.77 ± 2.83$^{\#\dagger}$	47.47 ± 2.83$^{\#\dagger}$	57.13 ± 2.83$^{\#\dagger}$	51.08 ± 2.83$^{\#\dagger}$
Saturated fatty acids (SFA)[a]	30.66 ± 1.65	35.26 ± 1.38	35.39 ± 1.38	27.86 ± 1.38	24.72 ± 1.38
Monounsaturated Fatty acids (MUFA)[b]	13.25 ± 1.30	4.32 ± 0.47	4.48 ± 0.47$^{\#}$	3.65 ± 0.47*†	10.91 ± 0.47$^{*\#}$
Omega-6 fatty Acids (PUFAω6)[c]	5.77 ± 0.39	9.4 ± 0.23†	9.07 ± 0.23$^{\#\dagger}$	9.31 ± 0.23*†	11.26 ± 0.23$^{*\#\dagger}$
Omega-3 fatty Acids (PUFAω3)[d]	48.98 ± 3.12	50.85 ± 1.72	50.99 ± 1.72$^{\#}$	59.14 ± 1.72*	53.06 ± 1.72$^{*\#}$
All polyunsaturated fatty acids (PUFATOT)[e]	54.73 ± 2.78	60.24 ± 1.83	60.05 ± 1.83$^{\#}$	68.45 ± 1.83	64.31 ± 1.83$^{\#}$
All trans fatty acids (TRANSTOT)[f]	1.36 ± 0.10	0.19 ± 0.08	0.10 ± 0.08†	0.06 ± 0.08†	0.08 ± 0.08†

FD, freeze-drying; VDE, vacuum-dried and encapsulated; 4C, storage at 4°C; RT, storage at room temperature. [a]SFA: C14:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C24:0. [b]MUFA: C16:1 9cis + C18:1 9 cis + C18:1 11cis + C22:1 cis + C24:1 cis. [c]PUFAω6: C18:2 ω6 + C18:3 ω6 + C20:2 ω6 + C20:3 ω6 + C20:4 ω6. [d]PUFAω3: C18:3 ω3 + C20:3 cisω3 + C20:5 ω3 + C22:5 ω3 + C22:6 ω3. [e]PUFATOT: PUFAω6+ PUFAω3. [f]TRANSTOT: C18:1 trans 9. *In the same row correspond to a significant difference between storage temperatures within the same drying technique; [#]in the same row correspond to a significant difference between drying techniques within the same storage temperature; [†]in the same row correspond to a significant difference between FRESH group and all remaining groups. Statistical significance was set at $p < 0.05$.

It is well-recognized that the use of chelating agents such as EGTA and EDTA has several protective functions against DNA damage in lyophilized spermatozoa (34). It is well corroborated that the physical processes during lyophilization (freezing and vacuum-drying) can induce DNA breaks (7). Membrane breaks lead to the release of divalent cations that activate endonucleases such as DNase and promote DNA breaks; adding chelating agents to the lyophilization medium could suppress their activity (35). The alkaline pH and EGTA in our lyophilization medium could suppress such DNAse activity (36). We have previously shown how freeze-dried ram spermatozoa can maintain DNA integrity, including EGTA in the lyophilization media (10). This could explain why DNA integrity was maintained in this experimental design, even though the spermatozoa were dried by lyophilization and VDE and stored at RT. Nevertheless, the significant disparity in %HG (high green fluorescence cells), indicative of reduced chromatin condensation, could potentially signify an epigenetic influence on embryos and, perhaps even more notably, on the offspring of large mammals. This aspect, which has not been assessed in this study, demands heightened attention. Especially concerning the welfare of these animals, it is imperative to give substantial consideration to the possibility of ensuring the birth of entirely healthy offspring from freeze-dried spermatozoa, a prudent course of action when dealing with large mammals.

Spermatozoa processed in VDE maintained better fertilizing capacity than the FD ones stored under vacuum, even when kept at RT (Figure 3H). Presumably, the argon (90%) and helium (10%) inside the stainless steel capsules (15, 37, 38) prevented the structural decay in the VDE samples caused by oxidative damage. Our DNA integrity, fertilization capacity, and lipidomic findings jointly support this assertion.

This study was the first to present a lipidomic map of ram spermatozoa that tracked the changes following various dehydration techniques and storage conditions and associated these with membrane integrity, DNA damage, and embryo development. Perhaps the most relevant information gained through this lipid analysis was the high proportion of DHA (40%) in all dry samples in comparison to the control (fresh semen: 5.5%). DHA is highly sensitive to oxidation, particularly at low temperatures (39), explaining why dried spermatozoa packaged in a saturated inert gas atmosphere were preserved better than under incomplete vacuum, as reported in our work. The PUFA TOT value in spermatozoa dried by the VDE technique and stored at RT was higher than in the respective FD ones. Our analysis showed a strong association between PUFA ω-3 and storage at 4°C and between PUFA ω-6 and storage at RT in the VDE samples.

The changes detected by the lipid analysis in the FD and VDE spermatozoa can be put in context with the structural and functional parameters and storage conditions assessed in this study by applying the MDPREF analysis biplot. The most significative association gleaned from the MDPREF analysis plot is the positive association between high MUFA and PUFA ω-6 percentages, structural and DNA integrity, and embryonic development in the VDE samples stored at RT (Figure 4E). Conversely, long-term storage at 4°C appeared positively associated with DNA fragmentation, acrosome loss, and lipid peroxidation. A trait highlighted by MDPREF analysis in all samples was the strong correlation between PUFA TOT and DNA damage, corroborating with Lewis (40), who revealed that spermatozoa were susceptible to lipid peroxidation-mediated DNA damage in the presence of a high proportion of PUFA. Our study also found a positive relationship between DPA and disrupted acrosome rate. The same condition was revealed in mice, where a decrease in DPA was associated with impaired acrosome reaction, resulting in negative consequences for fertilization (41).

High PUFA levels (~50%) were reportedly positively correlated with intact membranes in post-thaw mouse spermatozoa (42). Our findings suggest that the higher levels of DHA, MUFA, PUFA

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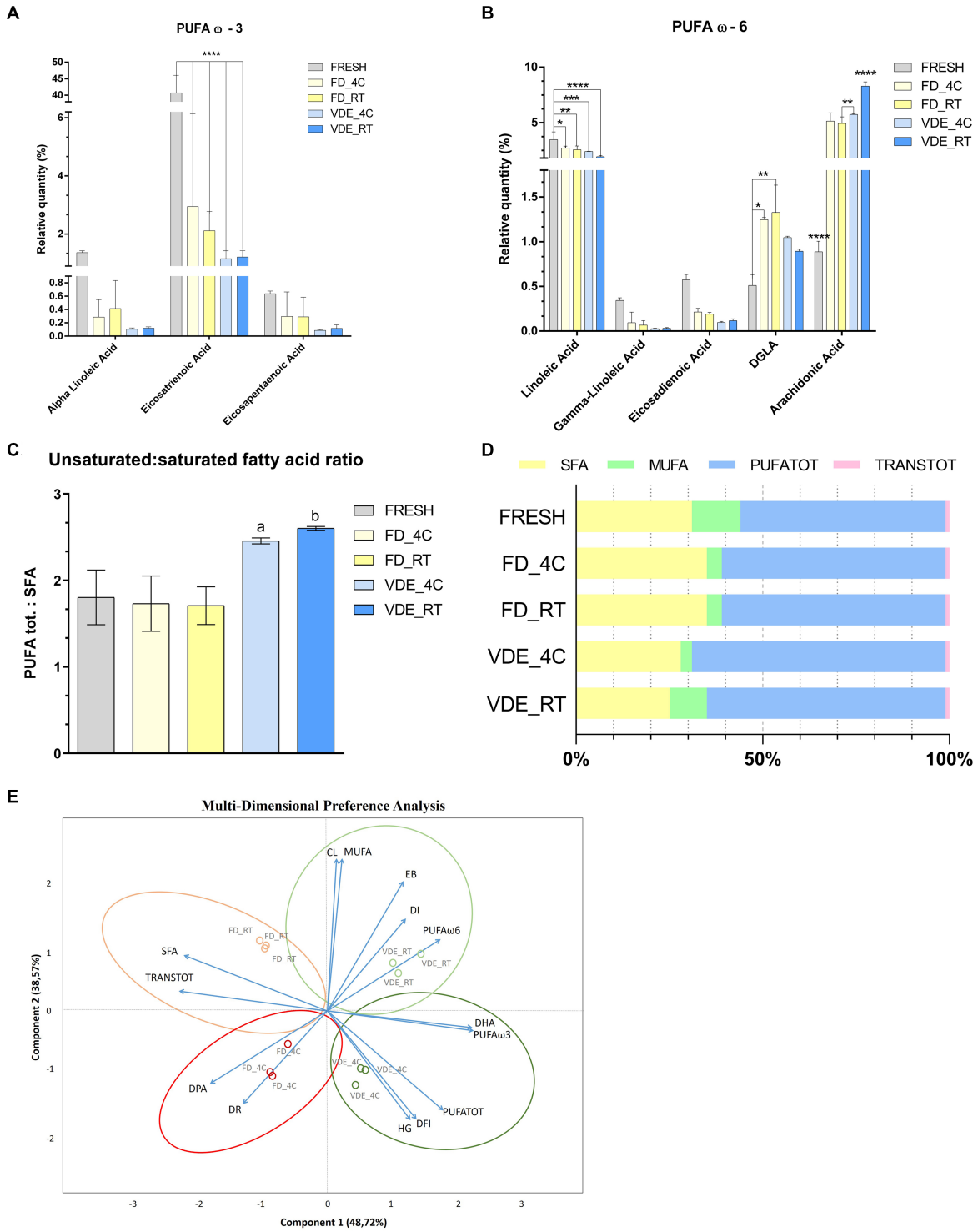

FIGURE 4
Polyunsaturated fatty acid (PUFA) profile and multi-dimensional preference analysis biplot. **(A)** PUFA ω3; **** indicates $p < 0.001$. **(B)** PUFA ω-6; **** indicates $p < 0.001$; *, **, and *** indicate $p < 0.05$. **(C)** PUFA tot. to saturated fatty acid (SFA) ratio. "a" indicates VDE_4C vs. FRESH ($p = 0.0254$), VDE_4C vs. FD_4C ($p = 0.0161$), VDE_4C vs. FD_RT ($p = 0.0152$). "b" indicates VDE_RT vs. FRESH ($p = 0.0114$), VDE_RT vs. FD_4C ($p = 0.070$), VDE_RT vs. FD_RT ($p = 0.0064$). **(D)** Proportion as a percentage of the total fat\ty acids of SFA, MUFA, PUFATOT and TRANSTOT. **(E)** Multi-dimensional preference analysis biplot based on a combination of *in vitro* quality parameters, including sperm quality [acrosome integrity (dead-intact, DI, or dead-reacted, DR) and chromatin stability (DNA fragmentation index, %DFI, and high green fluorescence, %HG)], embryo development [cleaved (CL) and expanded blastocyst (EB)], fatty acid profile (DPA, DHA, SFA, MUFA, PUFA ω-6, PUFA ω-3, PUFATOT, TRANSTOT), and the four experimental groups (FD_4C, FD_RT, VDE_4C, VDE_RT). The dots indicate the exact positions of samples in the four experimental groups between the two components. The ellipses and circle represent clustering based on the *in vitro* quality parameters (sperm quality, embryo development, and fatty acid profile). SFA, all saturated fatty acids combined; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; MUFA, mono-unsaturated fatty acids; PUFATOT (or PUFA TOT), all polyunsaturated fatty acids combined; TRANSTOT, all trans fatty acids combined; FRESH, refrigerated spermatozoa; FD_4C, freeze-dried and stored at 4°C; FD_RT, freeze-dried and stored at room temperature; VDE_4C, vacuum-died, encapsulated, and stored at 4°C; VDE_RT, vacuum-dried, encapsulated, and stored at room temperature.

beneventofrosinoneω-3, and PUFA ω-6 detected in the VDE stored at RT than FD samples were presumably involved in their better structural and functional performances.

While ICSI remains the sole applicable method for freeze-dried semen due to the current lack of motility in post-rehydrated spermatozoa, it still cannot be deemed entirely eco-friendly. This is because it necessitates the use of microscopes, CO_2, disposable plastics, and various other equipment and resources. This stands in stark contrast to the practices of *in vivo* fertilization in animal husbandry, which relies on refrigerated or frozen semen.

Nevertheless, it is worth noting that freeze-drying, in and of itself, has the potential to significantly reduce the environmental impact associated with the use of cryogenic fluids like liquid nitrogen. This inherently offers substantial energy savings and eliminates pollution to a great extent. So far, this alternative preservation method is particularly well-suited for use with wild animals, where the limitations and challenges of using liquid nitrogen are considerable.

In conclusion, this work demonstrated that an alternative drying solution for lyophilization, vacuum-drying with encapsulation, resulted in enhanced preservation of the structural and fertilization potential of ram spermatozoa stored for 2 years both at room temperature and 4°C. Furthermore, from the embryonic development data it can be seen that the storage yield of samples stored at RT is comparable with those stored at 4°C, especially in the VDE method. The explanation could arise from the VDE packaging, an inert atmosphere and a perfectly sealed capsule could allow a potential totally green application of the freeze-dried spermatozoa. Furthermore, the lipidomics data show a strong change in the composition of fatty acids in relation to the freeze-drying method used. Different species have different sperm lipid mapping, so it would be advisable to choose a more compliant freeze-drying method depending on the species of interest. Nevertheless, it is essential to acknowledge that we can only determine the optimal freeze-drying and preservation conditions for ram spermatozoa once we have successfully achieved the birth of the first offspring using freeze-dried sperm. This milestone will serve as a crucial reference point for guiding future research in this field.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by Italian Ministry of Health (No. 200/2017-PR, Prot. 944F0.1 del 04/11/2016). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LP: Conceptualization, Writing – original draft, Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. FT: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft. DA: Methodology, Writing – review & editing. JS: Writing – review & editing. JB: Writing – review & editing. MCo: Writing – review & editing, Methodology. ST: Writing – review & editing, Methodology. FP: Writing – review & editing. AL: Writing – review & editing. KM: Writing – review & editing, Methodology. MCz: Writing – review & editing, Data curation, Funding acquisition. PL: Writing – review & editing, Data curation, Funding acquisition, Conceptualization, Project administration, Resources, Supervision, Writing – original draft.

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Conflict of interest

JB, MC, and ST were employed by the company Imagene.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1270266/full#supplementary-material

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Supplementing semen extenders with a combination of phosphorus and vitamin B12 Improves post-thawed cryopreserved rooster semen quality

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Semen cryopreservation is an important technique for preserving the genetic material of numerous species. However, frozen semen is highly susceptible to sperm DNA damage and reduced motility, resulting in decreased fertility. The standard method for cryopreservation and several approaches have not been elucidated. This study aimed to determine the effects of supplementing rooster semen extender with a combination of phosphorus and vitamin B12 on cryopreserved semen quality. Semen was collected weekly via dorso-abdominal massage from 57 Burmese×Vietnam-crossbred Thai native roosters aged 1–3years. In total, 139 semen samples were collected, pooled, and diluted to 200 million sperm per dose. The pooled sample was divided into six experimental groups: a control group (0.00%) diluted with modified Beltville Poultry Semen Extender (BPSE) and five treatment groups diluted with modified BPSE supplemented with phosphorus and vitamin B12 at concentrations 0.02, 0.04, 0.06, 0.08, and 0.10%, respectively. The semen samples were frozen and evaluated at 0, 15, and 30min after thawing. Sperm kinematic parameters were determined using a computer-assisted sperm analysis system. Sperm quality was evaluated by measuring sperm viability, mitochondrial activity, acrosome integrity, and plasma membrane integrity. Statistical analyses were performed using a general linear mixed model (MIXED) in SAS. Factors in the statistical model were experimental groups, time after thawing, and interaction between experimental groups and time after thawing. Total and progressive motilities were greater in semen supplemented with 0.04% phosphorus and vitamin B12 compared with those in the control ($p<0.05$). At 15min post-thawing, VCL, VAP, and HPA in the 0.04% phosphorus and vitamin B12 supplementation group was greater than that in the control ($p<0.05$). Phosphorus and vitamin B12 supplementation did not affect sperm kinematics at 0 and 30min after thawing ($p>0.05$). All the sperm parameters that were tested for the 0.04% phosphorus and vitamin B12 supplementation group in modified BPSE were the highest at all the timepoints after thawing. Thus, supplementing frozen semen extender with 0.04% phosphorus and vitamin B12 increased sperm motility, sperm kinematic parameters, and sperm quality.

KEYWORDS

extender, frozen semen, phosphorus, Thai native rooster, vitamin B12

1 Introduction

Artificial insemination has been widely applied in poultry for overcoming low fertility, preventing infection transmission, and improving genetics (1, 2). Semen cryopreservation is an important technique for preserving the genetic material of numerous species (3–6). Cryopreserved semen can be used to considerably enhance genetic diversity, especially in animal populations with ongoing or that are at risk for inbreeding (5, 6). However, freeze-thaw cycles during the recovery of cryopreserved rooster semen can reduce sperm viability by compromising sperm cell membrane permeability and damaging the sperm mitochondria, midpiece, and acrosome (7). Particularly, avian spermatozoa have few cytoplasmic antioxidants and abundant polyunsaturated fatty acids in the membrane, rendering avian sperm extremely sensitive to oxidative stress during cryopreservation (8–11). Thus, frozen semen is highly susceptible to sperm DNA damage and reduced motility, resulting in decreased fertility (11, 12). However, to the best of our knowledge, there is no standard method for cryopreservation and several approaches have been reported in previous studies (13–19). However, this technique requires further refinement to increase success in its applications.

Phosphorus is a main energy source for sperm, with cyclic adenosine monophosphate (cAMP) serving as an important factor for sperm motility (20). In hamster sperm, cAMP-dependent phosphorylation of 36- and 65 kDa proteins plays a role in regulating the speed of microtubule motion (21). Furthermore, cyanocobalamin or vitamin B12 is a water-soluble vitamin that acts as a cofactor in numerous important biochemical pathways, such as methionine synthesis and branched-chain amino acid metabolism (22). Previous studies reported that vitamin B12 deficiency increases incomplete sperm formation and impairs sperm motility and velocity in rats (23). Supplementing chilled boar semen with phosphorus and vitamin B12 improved the total motility, progressive motility, sperm viability, and plasma membrane integrity of the sperm (24). In cattle and rams, supplementing semen extender with vitamin B12 improves the quality of thawed spermatozoa (25).

To the best of our knowledge, it is currently unknown whether phosphorus and vitamin B12 supplementation during cryopreservation can improve rooster sperm quality. Therefore, this study aimed to determine the effect of combined phosphorus and vitamin B12 supplementation in semen extender on the quality of frozen-thawed semen in Thai native rooster.

2 Methodology

The study protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Science, Chulalongkorn University (Approval number 2131018) and complies with provisions of "The Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes" (edited by the National Research Council of Thailand).

2.1 Animals

This study employed a case-control design which included 57 roosters in a local Thai native chicken farm in Thailand. In total, 139 ejaculates of semen were collected from Burmese × Vietnam-crossbred Thai native roosters aged between 1 and 3 years. Thai native roosters were kept in individual pens and fed *ad libitum* with paddy rice mixed with water.

2.2 Semen collection and experimental design

Semen was collected using the dorso-abdominal massage method weekly. The macroscopic and microscopic examinations of the rooster semen were performed. Semen samples with total motility of <65% were discarded. Following collection, semen was pooled and diluted to 200 million sperm per dose. The components of modified BPSE (26) were sodium glutamate (8.67 g/L), sodium acetate (0.43 g/L), magnesium chloride (0.34 g/L), potassium citrate (0.64 g/L), dipotassium phosphate (12.7 g/L), monopotassium phosphate (0.65 g/L), TES [*n*-tris (hydroxymethyl) methyl 1–2 amino ethane sulfonic acid] (1.95 g/L), Trehalose (1.9 g/L), Fructose (5 g/L) with pH of 7.5 and osmolarity of 366 mOsm/kg. The pooled semen was divided into six groups. The control group was diluted with modified Beltville Poultry Semen Extender (BPSE) plus 0.5% dimethyl sulfoxide (DMSO). The five treatment groups were diluted with modified BPSE plus 0.5% dimethyl sulfoxide (DMSO) and supplemented with various concentrations of phosphorus and vitamin B12 (Octafos® Octa Memorial Co., Ltd., Bangkok, Thailand). Each pooled ejaculate (12 repetitions) was split into six equal aliquots. Then, put the completed rooster semen extenders [0.00% (Control), 0.02, 0.04, 0.06, 0.08, 0.10%] in randomly. The semen samples in microtubes were wrapped with tissue paper for slowly cooled and kept at 4°C during transportation (27).

2.3 Chemical

The supplementation solution was prepared by adding 100 mg butaphosphan, 0.05 cyanocobalamin mg, and 1 mg methyl paraben to 1 mL solution and diluted to achieve final concentrations of 0.02, 0.04, 0.06, 0.08, and 0.10%.

2.4 Cryopreservation and thawing

Chemical reagents were purchased from Sigma (St. Louis, MO, United States). The freezing procedure was performed as reported previously by Amini et al. (10) with some modifications. After arriving at the laboratory, the diluted semen samples underwent equilibration at 4°C for 2 h, followed by the addition of 3% glycerol and 0.5% dimethyl formamide. The samples were loaded into 0.25 mL. French straw (IMV, L'Aigle, France) incubated for 15 min in a cooled tray. The straws were placed at ~5 cm above the liquid nitrogen vapour for 15 min in a 40 × 20 × 20 cm styrofoam box containing 8,000 cm^3 liquid nitrogen (28). After 1 week, the frozen straws were thawed for 3 min in a water bath at 5°C. Thawed samples were placed at room temperature (20–25°C). At 0, 15, and 30 min, samples were analyzed for sperm evaluation.

2.5 Sperm evaluation

2.5.1 Computer-assisted sperm analysis

The semen samples were evaluated at 0, 15, and 30 min using the computer-assisted sperm analysis (CASA) system (SCA®, Microptic, Barcelona, Spain). The settings were adjusted to detecting avian spermatozoa ($A = 5\,\mu m^3$) Based on general velocity, spermatozoa were classified as static (<10 μm/s), slow-medium (10–50 μm/s), or rapid (>100 μm/s). A minimum of 5 fields and 1,000 sperm tracks in each sample chamber were evaluated at 10× magnification on a phase-contrast microscope (image acquisition rate: 25 frames/s). Thawed semen samples were diluted over the range 1:40 to 1:60 (v/v) with PBS (Phosphate-Buffered Saline) and loaded into chamber at 37°C warm plate. The percentage of total motility and the percentage showing progressive motility were recorded. Sperm kinematic parameters, curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH), beat crossing frequencies (BCF), and hyperactivity (HPA) were also analyzed. Three progression ratios were calculated from the three velocity measurements as follows: linearity (LIN) of forward progression (LIN = VSL/VCL × 100), straightness (STR = VSL/VAP × 100), and wobble (WOB = VAP/VCL × 100), Mean values of VCL, VSL, VAP, ALH, and BCF parameters indicate the vigor of spermatozoa, whereas LIN, STR, and WOB indicate progressiveness (29).

2.5.2 Sperm quality analysis

2.5.2.1 Sperm viability

Sperm viability was measured by determining the percentage of live sperm using SYBR-14/propidium iodide (PI), as described by Chalah et al. (30) and Santiago-Moreno et al. (31). SYBR-14/PI was prepared by adding 4 μL 0.02 mM SYBR-14 and 2 μL 2.4 mM PI to 100 μL HEPES-buffered medium (containing 130 mM NaCl, 4 mM KCl, 14 mM fructose, 10 mM HEPES, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.1% BSA). The semen samples were prepared for the viability test by diluting 10 μL rooster semen with 200 μL phosphate-buffered saline (PBS). A 10 μL aliquot of the diluted semen was mixed with 20 μL SYBR-14/PI in HEPES-buffered medium and incubated at 20–25°C for 15 min. A sample of 200 spermatozoa were observed under a fluorescence microscope at 400× magnification. Live spermatozoa with intact plasma membranes were stained green by SYBR-14. Live spermatozoa with compromised plasma membranes were stained red and green by SYBR-14 and PI. Additionally, dead spermatozoa with damaged plasma membranes were stained red by PI.

2.5.2.2 Mitochondrial activity

Mitochondrial activity was assessed using JC-1 dye (Molecular Probes, Molecular Probes Inc., Eugene, OR). JC-1 was mixed with SYBR-14 and PI in DMSO at concentrations of 0.153, 0.02, and 2.4 mmol, respectively. This mixture was then combined with HEPES-buffered medium with 1.6 μL JC-1, 1 μL SYBR-14, and 1.6 μL PI. Subsequently, 12.5 μL diluted semen and 25 μL prepared stain mixture were combined and incubated at 20–25°C for 30 min. A fluorescence microscope was used to visualize the mitochondria in 200 spermatozoa tails at 400× magnification. Spermatozoa tails with low mitochondrial function were stained green, while those with high mitochondrial function appeared orange (32).

2.5.2.3 Acrosome integrity

To evaluate the percentage of sperm cells with intact acrosomes, Coomassie blue staining (Merck, Germany) was performed using a modification of the protocol reported by Abouelezz et al. (29). The staining solution (100 mL) was prepared by mixing 22.5 mL 0.5% Coomassie blue, 22.5 mL methanol, 54.75 mL distilled water, and 0.25 mL glacial acetic acid. A drop of 10 μL diluted semen sample was applied on a glass slide, smeared as a circle, and allowed to dry. The smear was subsequently fixed with buffered 4% glutaraldehyde in PBS for 30 min at room temperature (25°C) and air-dried. The slide was stained with Coomassie blue staining solution for 5 min, rinsed with distilled water, and air-dried. Finally, 200 spermatozoa with intact acrosomes were counted under a light microscope with oil immersion at a magnification of 1,000× oil. Spermatozoa exhibiting a hooked, swollen, thinned, or absent acrosome were classified as having no acrosome integrity.

2.5.2.4 Plasma membrane integrity

To evaluate plasma membrane integrity, a 100 mOsm/kg hypoosmotic solution was prepared by dissolving 1 g sodium citrate in 100 mL double-distilled water. A solution containing 3 μL diluted semen and 100 μL hypoosmotic solution was incubated at 20–25°C for 30 min. A drop of the incubated solution was spread on a slide and allowed to dry. Coomassie blue mixed with 0.25% acetic acid staining solution was added to the slide for 2 min. A sample of 200 spermatozoa were observed under a light microscope at 1,000× magnification. Spermatozoa with coiled midpieces and tail segments were classified as having positive plasma membrane integrity, while those without coiled tails were classified as negative (33).

2.6 Statistical analysis

Statistical analyses were carried out using SAS (SAS version 9.1cary, NC, United States). The effect of phosphorus and vitamin B12 on sperm motion characteristics and sperm characteristics on time after thawing were analyzed by the general linear mixed model (MIXED). Factors for the statistical model included the experimental groups (control, 0.02, 0.04, 0.06, 0.08, and 0.10% of phosphorus and vitamin B12), time after thawing (0, 15, and 30 min), and interaction between treatment and time after thawing. The following model was applied to analyzed = the data:

$$Y_{ijk} = m + G_i + T_j + R_k + O_{ijk}$$

Where Y_{ijk} is the response variable, m is the overall mean, G_i is the fixed effect of the experimental groups [i.e., 0.00% (control), 0.02, 0.04, 0.06, 0.08, and 0.10% of phosphorus and vitamin B12], T_j is the fixed effect of the time after thawing (i.e., 0, 15, and 30 min), R_k is a random component related rooster and O_{ijk} is the residual error component. The Thai native rooster was included as a random variable. Least square means were obtained from each class of factor and compared using the least significant test (LSD). p-values of <0.05 were considered statistically significant.

3 Results

Sperm motility, VCL and HPA were higher in semen supplemented with 0.04% phosphorus and vitamin B12 than in the control (Table 1). VSL, VAP, STR, and BCF were also higher in the 0.08% phosphorus and vitamin B12 supplementation group than in the control group (Table 1). All supplementation doses increased the sperm quality parameters tested compared with the controls (Table 2). In all the experimental groups, sperm motility, kinematic parameters, and sperm quality decreased over time after thawing (Tables 1, 2).

3.1 Effects of different concentrations of phosphorus and vitamin B12 supplementation and time after thawing on rooster sperm motility

Total motility was the highest in semen supplemented with 0.04% phosphorus and vitamin B12 at all timepoints after thawing (Figure 1A). Immediately after thawing (0 min), the total motility in the 0.04% group (51.1%) was greater than that in the control (46.8%, $p = 0.001$) and 0.02% (48.4%, $p = 0.042$) groups (Figure 1A). At 15 min post-thawing, the total motility in the 0.04% group (48.1%) was greater than that in the control group (43.1%, $p < 0.001$). At 30 min post-thawing, the total motility in the 0.04% group (44.2%) was greater than that in the control (39.7%, $p < 0.001$) and 0.02% (41.5%, $p = 0.042$) groups (Figure 1A). At 0, 15, and 30 min after thawing, progressive motility in the group with 0.04% phosphorus and vitamin B12 supplementation was greater than that in the control ($p < 0.05$) (Figure 1B).

3.2 Effects of different concentrations of phosphorus and vitamin B12 supplementation and time after thawing on rooster sperm kinematics

At 15 min post-thawing, VCL, VAP, and HPA were greater in the 0.04% phosphorus and vitamin B12 supplementation group than in the control ($p < 0.05$) (Table 3). However, 0.04% phosphorus and vitamin B12 supplementation did not affect sperm kinematics immediately and 30 min after thawing when compared with control group ($p > 0.05$) except VCL at 30 min.

3.3 Effects of different concentrations of phosphorus and vitamin B12 supplementation and time after thawing on rooster sperm quality

The effects of phosphorus and vitamin B12 supplementation on sperm quality are presented in Figure 2. At all timepoints after thawing, 0.04% supplementation showed the highest sperm viability (Figure 2A). At 0 min post-thawing, the sperm viability in 0.04% (51.8%) was greater than that in the control (46.2%, $p < 0.001$) and 0.02% (49.6%, $p = 0.026$) (Figure 2A) groups. At 15 min post-thawing, sperm viability in the 0.04% group (48.6%) was greater than that in the control (44.0%, $p < 0.001$), 0.02% (46.6%, $p = 0.038$), and 0.10% (45.3%, $p < 0.001$) groups. At 30 min post-thawing, sperm viability in the 0.04% group (45.9%) was greater than that in the control (40.4%, $p = 0.002$) and 0.02% (43.5%, $p = 0.014$) groups (Figure 2A).

At 0 min post-thawing, the mitochondrial activity in the 0.04% phosphorus and vitamin B12 supplementation group (47.2%) was greater than that in the control (40.8%, $p < 0.001$), 0.02% (43.6%, $p = 0.003$), and 0.10% (44.2%, $p = 0.012$) groups (Figure 2B). At 15 min post-thawing, the mitochondrial activity with 0.04% supplementation (43.5%) was greater than that in the control (36.7%, $p < 0.001$), 0.02% (39.9%, $p = 0.003$), and 0.10% (41.0%, $p = 0.031$) groups. At 30 min post-thawing, mitochondrial activity in the 0.04% group (41.1%) was greater than that in the control (33.7%, $p < 0.001$), 0.02% (38.2%, $p = 0.017$), 0.06% (38.2%, $p = 0.017$), and 0.10% (37.0%, $p < 0.001$) groups (Figure 2B).

Acrosome integrity and membrane integrity were the highest in semen supplemented with 0.04% phosphorus and vitamin B12 at all timepoints after thawing (Figures 2C,D). Acrosome integrity was greater in the 0.04% phosphorus and vitamin B12 supplementation

TABLE 1 The effects of phosphorus and vitamin B12 supplementation in semen extender, irrespective of the time elapsed after thawing, as well as the impact of time after thawing, regardless of the concentrations of phosphorus and vitamin B12, on sperm motility and sperm kinematic parameters analyzed via CASA in frozen-thawed rooster semen.

Parameters	Concentrations of phosphorus and vitamin B12,%						SEM*	Min			SEM*
	0.00	0.02	0.04	0.06	0.08	0.10		0	15	30	
Total motility, %	43.2^{c}	45.4^{b}	47.8^{a}	45.8^{b}	46.5^{ab}	45.8^{b}	1.2	49.5^{a}	45.6^{b}	42.2^{c}	1.1
PR**, %	6.9^{b}	7.8^{a}	8.3^{a}	7.9^{a}	8.0^{a}	8.0^{a}	0.4	9.1^{a}	7.7^{b}	6.8^{c}	0.4
VCL, μm/s	48.3^{b}	49.4^{a}	49.8^{a}	49.8^{a}	49.5^{a}	49.8^{a}	0.6	50.8^{a}	49.4^{b}	48.2^{c}	0.6
VSL, μm/s	14.5^{b}	14.5^{b}	14.6^{ab}	14.6^{ab}	14.9^{a}	14.8^{ab}	0.3	15.0^{a}	14.6^{b}	14.3^{c}	0.3
VAP, μm/s	23.7^{b}	23.8^{b}	24.2^{ab}	24.2^{ab}	24.4^{a}	24.3^{a}	0.4	24.8^{a}	24.1^{b}	23.5^{c}	0.3
LIN, %	33.3^{ab}	32.7^{bc}	32.3^{c}	32.9^{ac}	33.8^{a}	33.3^{ab}	0.7	32.6^{b}	33.2^{ab}	33.4^{a}	0.7
STR, %	56.2^{b}	56.2^{b}	56.2^{b}	56.3^{b}	57.2^{a}	56.6^{ab}	0.6	56.3	56.6	56.4	0.6
WOB, %	50.5^{ab}	49.7^{bc}	50.7^{ab}	51.1^{ab}	51.9^{a}	51.2^{a}	0.7	50.0^{a}	51.2^{b}	51.3^{b}	0.6
ALH, μm	3.41	3.36	3.45	3.42	3.49	3.52	0.08	3.46	3.48	3.38	0.06
BCF, beats/s	4.05^{b}	4.07^{ab}	4.15^{ab}	4.10^{ab}	4.21^{a}	4.06^{b}	0.08	4.30^{a}	4.08^{b}	3.94^{c}	0.07
HPA, %	0.90^{b}	1.09^{a}	1.06^{a}	1.08^{a}	1.01^{ab}	1.08^{a}	0.10	1.14^{a}	1.08^{a}	0.89^{b}	0.09

$^{a, b, c}$ Different superscript letters within rows indicate statistically significant difference ($p < 0.05$). *SEM: maximum standard error of the mean and **Progressive motility.

TABLE 2 The effects of phosphorus and vitamin B12 supplementation in semen extender, irrespective of the time elapsed after thawing, as well as the impact of time after thawing, regardless of the concentrations of phosphorus and vitamin B12, on sperm quality in frozen-thawed rooster semen.

Parameters	Concentrations of phosphorus and vitamin B12,%						SEM*	Min			SEM*
	0.00	0.02	0.04	0.06	0.08	0.10		0	15	30	
Viability, %	43.5^{e}	46.5^{b}	48.8^{a}	47.3^{bc}	48.2^{ac}	45.4^{d}	1.0	49.7^{a}	46.7^{b}	43.5^{c}	0.9
Mitochondria, %	37.1^{d}	40.6^{c}	43.9^{a}	42.2^{b}	44.4^{a}	40.7^{c}	1.1	45.0^{a}	41.2^{b}	38.2^{c}	1.1
Acrosome, %	81.7^{d}	88.5^{b}	91.6^{a}	89.0^{b}	88.7^{b}	84.3^{c}	1.2	93.8^{a}	87.7^{b}	80.4^{c}	1.1
Membrane, %	26.2^{e}	31.7^{c}	35.6^{a}	33.6^{b}	34.6^{ab}	29.6^{d}	1.0	35.6^{a}	31.8^{b}	28.2^{c}	1.0

$^{a, b, c, d, e}$ Different superscript letters within rows indicate statistically significant difference ($p < 0.05$). *SEM: maximum standard error of the mean.

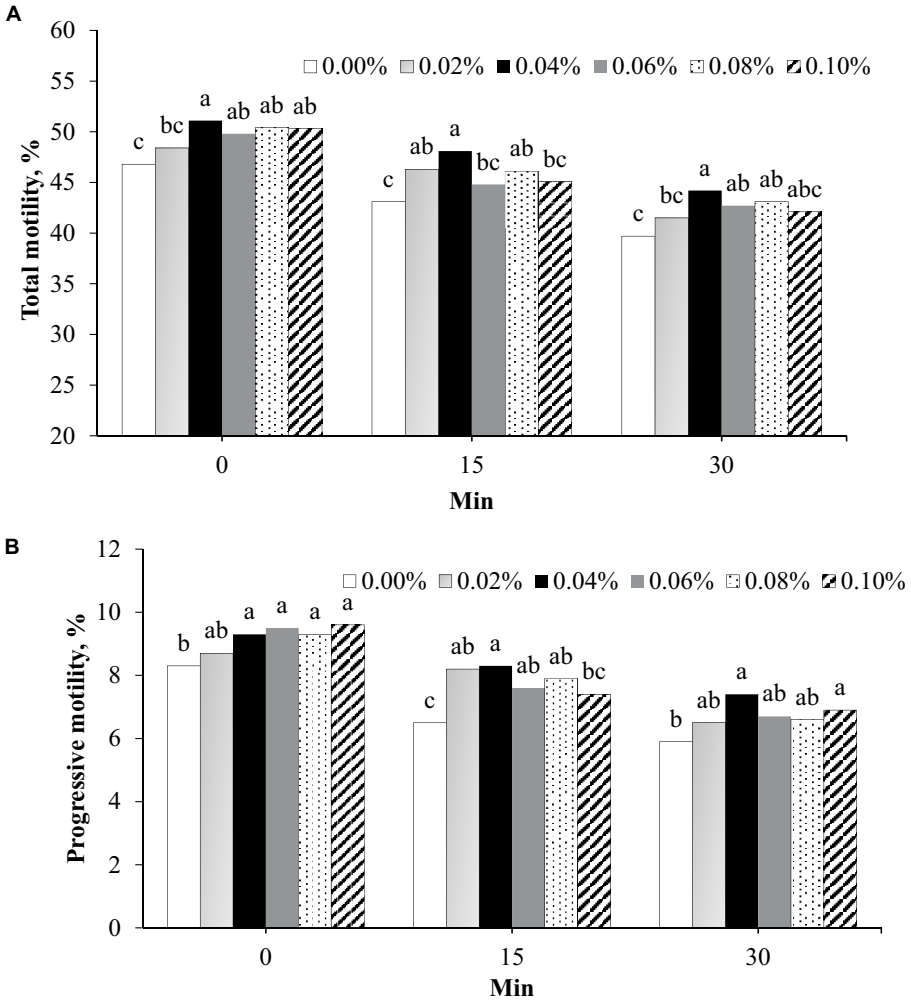

FIGURE 1
Effect of different concentrations of phosphorus and vitamin B12 supplementation (0.00, 0.02, 0.40, 0.06, 0.08, and 0.10%) on frozen rooster semen at different timepoints after thawing. **(A)** Total motility and **(B)** Progressive motility. $^{a, b, c}$ Different superscript letters per timepoint indicate statistically significant differences ($p < 0.05$).

group (95.8%) that in the control group (30.9%, $p = 0.017$) immediately after thawing (Figure 2C). At 15 min post-thawing, acrosome integrity in the 0.04% group (88.8%) was greater than that in the control (83.20%, $p < 0.001$) and 0.10% (85.9%, $p = 0.006$) groups. At 30 min post-thawing, acrosome integrity in the 0.04% group (87.7%) was greater than that in the other concentration groups ($p < 0.05$) (Figure 2C). Membrane integrity in the group 0.04% phosphorus and vitamin B12 supplementation (39.0%) was greater than that in the control (30.9%, $p < 0.001$), 0.02% (35.5%, $p < 0.001$), and 0.10% (33.4%, $p < 0.001$) groups at 0 min after thawing, (Figure 2D). At 15 min after thawing, the membrane integrity in the 0.04% group (35.5%) was greater than that in the control (26.0%), 0.02% (31.6%), and 0.10% (29.6%) ($p < 0.001$) groups. At 30 min after thawing, membrane integrity in the 0.04% group (32.3%) was greater than that in the control (21.9%), 0.02% (32.3%), 0.06% (30.0%), and 0.10% (25.6%) groups ($p < 0.001$) (Figure 2D).

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TABLE 3 The interaction between phosphorus and vitamin B12 supplementation to semen extender and time after thawing in frozen-thawed rooster spermatozoa on the sperm trajectory and velocity.

Parameters	0 Min						SEM*	15 Min						SEM*	30 Min						SEM*
	0.00	0.02	0.04	0.06	0.08	0.10		0.00	0.02	0.04	0.06	0.08	0.10		0.00	0.02	0.04	0.06	0.08	0.10	
VCL, μm/s	50.3	50.7	50.6	51.5	50.6	51.3	0.8	47.3^{b}	50.3^{a}	49.7^{a}	49.8^{a}	49.8^{a}	49.3^{a}	0.8	47.3^{c}	47.3^{bc}	49.1^{a}	48.2^{abc}	48.1^{abc}	48.9^{ab}	0.8
VSL, μm/s	15.1^{ab}	14.4^{b}	15.0^{ab}	15.0^{ab}	15.4^{a}	15.3^{a}	0.4	14.2	14.8	14.8	14.4	14.7	14.8	0.4	14.1	14.2	14.0	14.4	14.7	14.3	0.4
VAP, μm/s	24.7^{ab}	24.1^{b}	24.7^{ab}	25.0^{a}	25.0^{a}	25.1^{a}	0.4	23.3^{b}	24.5^{a}	24.3^{a}	24.0^{ab}	24.2^{a}	24.2^{a}	0.4	23.2^{ab}	22.9^{b}	23.6^{ab}	23.6^{ab}	23.9^{a}	23.7^{ab}	0.4
LIN, %	33.2^{ab}	31.4^{c}	31.7^{bc}	32.2^{abc}	33.6^{a}	33.2^{ab}	0.9	33.2	33.2	33.0	32.9	33.4	33.8	0.9	33.5^{ab}	33.5^{ab}	32.1^{b}	33.7^{ab}	34.4^{a}	33.0^{ab}	0.9
STR, %	56.5^{ab}	55.3^{b}	56.2^{ab}	55.7^{b}	57.4^{a}	56.7^{ab}	0.8	56.1	56.5	56.9	56.3	56.7	57.1	0.8	56.1^{ab}	56.8^{ab}	55.5^{b}	56.8^{ab}	57.4^{a}	56.0^{ab}	0.8
WOB, %	48.9^{bc}	47.0^{c}	50.8^{ab}	50.7^{ab}	51.6^{a}	51.2^{ab}	1.0	51.2	51.2	50.9	51.0	51.5	51.5	1.0	51.4	50.8	50.3	51.5	52.5	51.0	1.0
ALH, μm	3.44	3.41	3.47	3.50	3.44	3.51	0.11	3.28^{b}	3.42^{ab}	3.44^{ab}	3.44^{ab}	3.69^{a}	3.63^{a}	0.11	3.52	3.26	3.43	3.32	3.34	3.41	0.11
BCF, beats/s	4.24	4.23	4.34	4.34	4.42	4.24	0.11	4.03	4.13	4.09	4.05	4.08	4.09	0.11	3.89^{ab}	3.84^{b}	4.01^{ab}	3.91^{ab}	4.11^{a}	3.84^{b}	0.11
HPA, %	1.14	1.14	1.14	1.09	1.11	1.20	0.13	0.82^{b}	1.20^{a}	1.12^{a}	1.20^{a}	1.02^{ab}	1.13^{a}	0.13	0.73	0.92	0.91	0.94	0.90	0.90	0.13

^{a,b,c} Different superscript letters within rows indicate significant difference ($p < 0.05$). *Maximum standard error of the mean (SEM).

4 Discussion

To the best of our knowledge, this is the first study to report data on the effects of phosphorus and vitamin B12 supplementation on frozen-thawed rooster semen. Our results demonstrated that supplementation with 0.04% phosphorus and vitamin B12 increased sperm motility, sperm kinematic parameters, and sperm quality. These findings provide a basis for adding phosphorus and vitamin B12 in semen extenders to improve sperm quality in cryopreserved Thai native rooster semen.

Cryopreservation can decrease sperm viability and survival after thawing. This could be attributed to increased lipid peroxidation and sperm acrosome, plasma membrane, DNA, and mitochondria disruption during freezing (12). Previous studies reported that freezing causes the greatest structural damage to the mitochondria, midpiece, and perforatorium (34). Furthermore, rooster spermatozoa exhibit unique characteristics that render them potentially more vulnerable to freezing-induced damage, such as a small cytoplasm, fewer mitochondria, lower cytoplasmic antioxidants, and abundant plasma membrane polyunsaturated fatty acids (9). Additionally, cryopreservation induces the production of reactive oxygen species (ROS) and cellular defense systems, resulting in oxidative stress (35). ROS and free radicals, such as hydrogen peroxide, abolish sperm motility, while hydroxyl radicals can reduce all movement characteristics except straightness and linearity (36).

Thus, some investigators have attempted to improve semen quality by adding antioxidants, inhibitors of lipid peroxidation, and cryoprotectants. Antioxidants, such as resveratrol, lycopene, quercetin, melatonin, vitamin C, E, amino acid, glutathione, and selenium, have been reported to improve frozen rooster semen (37). The amino acid serine decreases lipid peroxidation and improves semen quality and fertilizing ability in frozen-thawed Thai native rooster semen (17). Cryoprotectants act by inducing cell dehydration and reducing intracellular ice crystals formation (38). Sugar is a cryoprotectant that improves semen quality and fertility (18).

Phosphorus enhances sperm motility by acting as substrate for ATP, AMP, and phosphocreatinine production and regulating gluconeogenesis and glycogenesis, which are related to energy metabolism (39, 40). Furthermore, vitamin B12 is a cofactor in the conversion of methylmalonyl coenzyme A (CoA) to succinyl CoA during gluconeogenesis (41). The result of the present study demonstrated that all concentrations of phosphorus and vitamin B12 improved sperm total motility, progressive motility, and sperm kinematics, consistent with the results of a previous study that reported improved motility following phosphorus and vitamin B12 supplementation in chilled boar semen (24). Thus, the supplementation of a combination of phosphorus and vitamin B12 may improve energy efficiency in cryopreserved semen.

The antioxidant activity of vitamin B12 prevents stress-induced membrane lipid peroxidation in sperm, such as in the freezing-thawing process. Many studies also suggest that vitamin B12 plays an important role in spermatogenesis and increases glutathione peroxidase activity (42). Glutathione is a major intracellular antioxidant that protects the cell against oxidative stress. A reduction in spermatozoa glutathione levels after freezing has been reported in bulls, boar, and human semen (43). Our

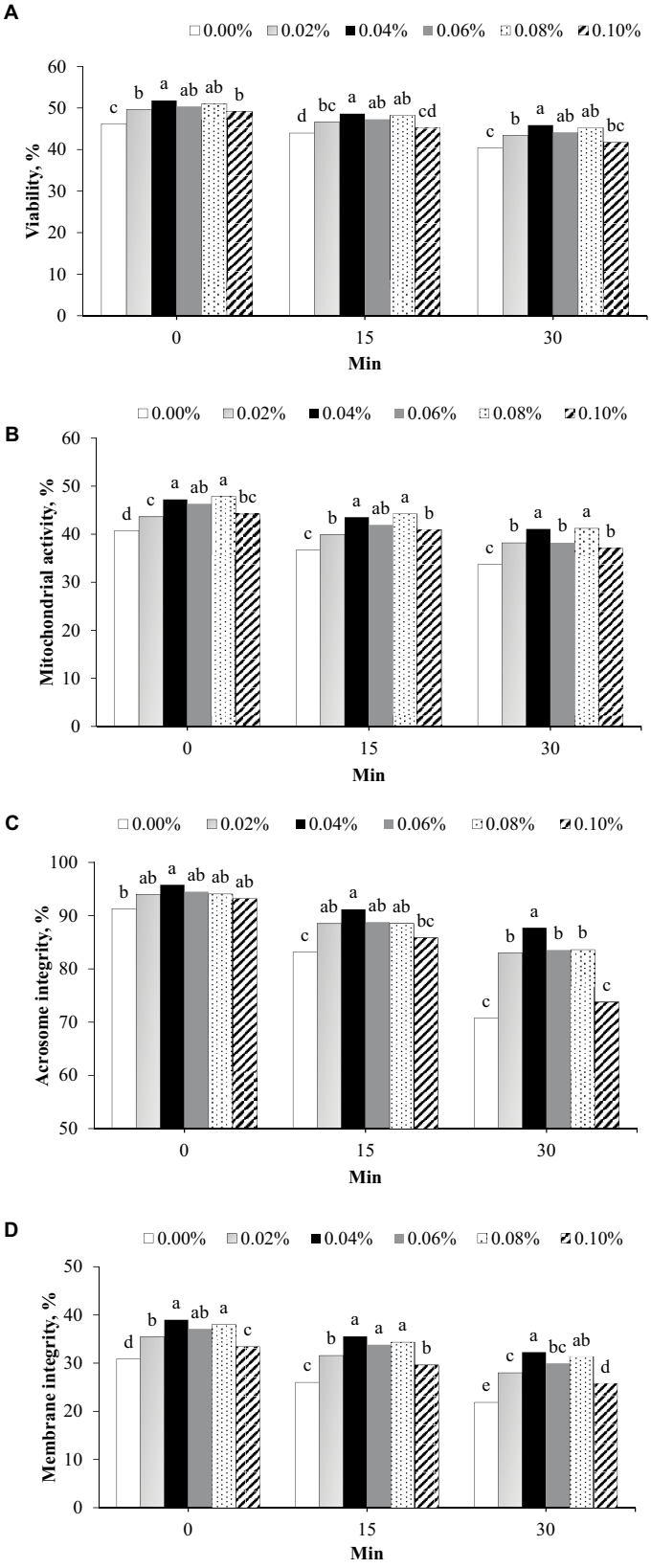

FIGURE 2
Effect of different concentrations of phosphorus and vitamin B12 supplementation (0.00, 0.02, 0.40, 0.06, 0.08, and 0.10%) on frozen rooster semen at different timepoints after thawing. **(A)** Sperm viability, **(B)** Mitochondrial activity, **(C)** Acrosome integrity, and **(D)** Plasma membrane integrity. [a, b, c, d, e] Different superscript letters per timepoint indicate statistically significant differences ($p < 0.05$).

results revealed that 0.04% phosphorus and vitamin B12 supplementation increased sperm viability by 5.3%, mitochondrial activity by 6.8%, sperm plasma membrane integrity by 9.4%, and acrosome integrity by 9.9%. Supplementation with 0.04% phosphorus and vitamin B12 improved VCL and all sperm quality parameters 30 min after thawing. As reported previously, VAP and VCL are good predictors of the ability of spermatozoa to migrate in cervical mucus (44) and are significantly correlated with fertility in bulls (45). Furthermore, the addition of 0.08% phosphorus and vitamin B12 enhanced VAP, LIN, STR, and BCF as well as sperm viability, mitochondrial activity, and membrane integrity 30 min after thawing. These suggest that higher doses of phosphorus and vitamin B12 allow the sperm cell to harness energy to sperm kinematics and quality.

Conversely, the concentration of 0.1% decreased acrosome integrity and membrane integrity, which may be because of potential toxicity at high doses. Thus, optimal concentration of phosphorus and vitamin B12 supplementation could be in the range of 0.4–0.8%.

These findings corroborate those of previous studies, reporting the beneficial effect of vitamin B12 supplementation on semen quality in numerous species (46–48). Moreover, phosphorus and vitamin B12 supplementation obviously improved the quality of chilled semen from Thai Native Chicken (49). Therefore, the addition of an optimized amount of vitamin B12 into the freezing extender could prevent the generation of oxygen radicals, resulting in decreased peroxidation and membrane damage and ultimately improving sperm motility and viability (47). Moreover, the addition of vitamin B12 to bovine semen *in vitro* increased sperm motility, sperm velocity, and proportion of intact sperm by increasing catalase and glutathione reductase activities (50). Therefore, the supplementation of phosphorus and vitamin B12 can improve rooster sperm by protecting the plasma membrane from damage and preventing oxidative stress during semen cryopreservation.

5 Conclusion

The supplementation of frozen semen extender with 0.04% phosphorus and vitamin B12 increased sperm motility, kinematics, and quality. Thus, the potential of phosphorus and vitamin B12 supplementation in semen extender in improving frozen rooster sperm quality should be applied in rooster semen cryopreservation.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal studies were approved by The study protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Science, Chulalongkorn University (Approval number 2131018) and complies with provisions of "The Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes" (edited by the National Research Council of Thailand). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

JS: Conceptualization, Methodology, Software, Writing – original draft, Writing – review & editing. PK: Investigation, Methodology, Writing – original draft. GN: Investigation, Methodology, Writing – original draft. CK: Methodology, Writing – original draft. BO: Investigation, Methodology, Writing – original draft. PS: Investigation, Methodology, Writing – original draft. MN: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Alpha-lipoic acid improves the quality of ram spermatozoa stored at 4°C by reducing oxidative stress and increasing mitochondrial potential

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Introduction: Ram spermatozoa inevitably produce a large number of reactive oxygen species (ROS) during liquid storage, leading to oxidative stress and a decline of spermatozoa quality. Therefore, it is particularly important to add exogenous antioxidants during the process of semen liquid preservation. The purpose of this study is to investigate whether adding alpha-lipoic acid (ALA) to ram semen can reduce oxidative stress and enhance spermatozoa quality during the liquid storage at 4°C.

Methods: Different concentrations of ALA (0, 0.025, 0.05, 0.1, 0.5, 1 mM) were added to semen and stored at 4°C. During storage at 4°C, spermatozoa motility, kinetic parameters, membrane integrity, acrosome integrity, energy metabolism parameters (mitochondrial membrane potential (ΔΨM) and adenosine triphosphate (ATP)) and oxidative stress parameters [ROS, malondialdehyde (MDA), total antioxidant capacity (TAC), superoxide dismutase (SOD)] were assessed.

Results and discussion: The results indicated that 0.1 mM ALA significantly ($p<0.05$) improved spermatozoa total motility (TM) and progressive motility (PM), plasma membrane integrity, acrosome integrity, ΔΨM, ATP, TAC, and SOD, while significantly ($p<0.05$) reducing spermatozoa ROS and MDA content compared to the control group. In conclusion, ALA can reduce damage caused by oxidative stress in spermatozoa and effectively improve the quality of semen preserved at 4°C. And the optimal concentration is 0.1 mM.

KEYWORDS

alpha-lipoic acid, sheep, spermatozoa, oxidative stress, low temperature

1 Introduction

Hu sheep is a world-famous breed known for its high productivity (1). Compared with specialized mutton sheep breeds, the excellent characteristics of Hu sheep, such as high fertility and multiple births, stand out in large-scale house feeding (2). Artificial insemination (AI) plays an important role in the reproductive process, and the collection, processing, and preservation of semen are important factors that affect the efficiency of AI (3). In addition, low-temperature preservation involves diluting semen, slowly cooling it, and then storing it at 4°C. It induces dormancy in spermatozoa and reduces metabolic activity, thereby prolonging the lifespan of spermatozoa (4).

Oxidative stress is an important factor that impacts semen quality during the process of *in vitro* semen preservation. Semen possesses an antioxidant system that is safeguarded by various antioxidant enzymes, which help prevent oxidative stress resulting from an excess of reactive oxygen species (ROS) in the spermatozoa (5). And spermatozoa can produce a small amount of ROS, which plays an important role in spermatozoa capacitation and acrosome reaction (6, 7). However, as the duration of semen storage increases, abnormal and dead spermatozoa can generate significant amounts of ROS, leading to an imbalance in ROS level and subsequent oxidative stress (8–10). On the one hand, excessive ROS will also attack polyunsaturated fatty acids (PUFAs) in the spermatozoa plasma membrane, leading to the integrity of the spermatozoa membrane structure and causing lipid peroxidation reaction (LPO) in the spermatozoa plasma membrane (11). On the other hand, ROS can damage spermatozoa DNA by breaking DNA double strands and inducing apoptosis (12, 13). Therefore, to reduce the negative impact of oxidative stress on semen quality, antioxidants are usually added to the diluent (14). The primary mechanisms by which antioxidants exert their effects include directly removing the ROS produced, inhibiting the production of spermatozoa ROS, and removing or repairing damage caused by ROS (15).

Alpha-lipoic acid (ALA) is a vitamin-like biological non-enzymatic antioxidant with high efficiency (16, 17). ALA is a mitochondrial coenzyme synthesized from mitochondrial octanoic acid through a mitochondrial enzymatic reaction and it plays a role in the tricarboxylic acid cycle (18). Moreover, it catalyzes the generation and transfer of acyl groups in the oxidative decarboxylation process of the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complex, and plays an antioxidant and metabolic role at the cellular level (19). It is reported that ALA can play an active role in the treatment of diseases related to the human reproductive system (20, 21). Furthermore, oral administration of ALA has been shown to enhance sperm quality in both rats (22) and humans (23). However, the preservation effect of ALA at 4°C on Hu ram spermatozoa has not been demonstrated in any research. The aim of this study is to investigate whether ALA can enhance the quality of Hu ram semen preservation at 4°C by reducing oxidative stress.

2 Materials and method

2.1 Animals and semen collection

The ejaculates of five proven fertile Hu rams were collected using an artificial vagina according to procedures approved by the Animal Ethics Committee of Yangzhou University (SYXK[Su]2017–0044) and Hu sheep are known to be in estrus all year round. The study selected healthy rams aged 2 to 4 years, free of parasites. The rams were free to drink water and ingest licking bricks rich in minerals. The ram was fed 0.4 kg concentrate and 0.2 kg alfalfa every day. A total of 90 ejaculates (three times a week for each ram) were used for the experiments and the experiments were repeated five times. Ejaculates were collected from October to December 2022. The computer-assisted sperm analyzer (CASA) was utilized to evaluate the collected spermatozoa. Semen with spermatozoa motility greater than 80%, abnormal rate less than 15%, semen volume between 0.6 ~ 1.3 mL and the spermatozoa concentration between $2 \times 10^9 \sim 2.5 \times 10^9$ was selected. The qualified semen was evenly mixed to eliminate individual differences.

2.2 Semen extender and evaluation

The 500 mL basic extender was composed of 2.5 g fructose, 12 g sodium citrate, 0.5 g Soy Lecithin, 250,000 IU penicillin sodium and streptomycin sulfate (24). ALA was dissolved in water and should be prepared in the dark. ALA (Purity>99%, Beyotime, Shanghai, China) was added to the basic extender at concentrations of 0.025, 0.05, 0.1, 0.5, and 1.0 mM, while the control was the basic extender without ALA (The concentration range of ALA was determined by a pre-experiment.). All the samples were diluted to 2×10^8 sperm/ml using an extender containing different concentrations of ALA. The semen samples were stored at 4°C in a refrigerator. It is recorded as 0 d when the sample is placed at 4°C and it is recorded as 1 d after 24 h.

Spermatozoa motility parameters, functional integrity parameters (including plasma membrane and acrosome integrity), oxidative stress parameters [ROS, malondialdehyde (MDA), superoxide dismutase (SOD), total antioxidant capacity (TAC)], and energy metabolism-related parameters [mitochondrial membrane potential ($\Delta\Psi$M), adenosine triphosphate (ATP)] were assessed on the first, third, and fifth day of semen preservation at 4°C.

2.3 Spermatozoa motility parameters

Parameters such as Total motility (TM, %), Progressive motility (PM, %), Straight line velocity (VSL, μm/s), Curvilinear velocity (VCL, μm/s), Average path velocity (VAP, μm/s), and Amplitude of lateral head displacement (ALH, μm) were assessed using CASA (Instrument number: ML-608JZ II, Mailang, Nanning, China). The CASA software recorded data at 30 frames per second. Samples diluted with the basic extender were incubated at 37°C for 4 min. A total of 1.4 μL semen sample was placed on a MACRO sperm counting chamber (YA-1, Yucheng, Nanjing, China) and examined at 37°C using a phase-contrast microscope (ML-800, Mailang, Nanning, China) at a magnification of 100×, equipped with a CCD-camera (MD06200C, Mailang, Nanning, China).

2.4 Spermatozoa functional integrity parameters

The integrity of the spermatozoa plasma membrane was assessed using the hypotonic swelling test (HOST). Semen samples preserved at 4°C were mixed with a hypotonic solution (consisting of 0.245 g sodium citrate and 0.45 g fructose dissolved in 50 mL distilled water) in a 1: 10 ratio. The osmotic pressure of the hypotonic solution was 108 m Osm/L. The evenly mixed samples were incubated at 37°C for 30 min. After incubation, 1.4 μL of the mixture was dispensed onto a specialized plate for spermatozoa counting. The coiling rate of the spermatozoa was examined using a phase-contrast microscope (CX31, Olympus Corporation, Tokyo, Japan) at a magnification of 400×, until 200 spermatozoa had been counted.

Giemsa staining was utilized to assess the integrity of the spermatozoa acrosome. Smears were taken from 10 μL of semen samples stored at 4°C. After 5 ~ 10 min, allow it to air dry naturally, and then fix it with 4% paraformaldehyde. After that, it was soaked in Giemsa stain for 3 h. After air-drying, the spermatozoa were examined under a phase-contrast microscope (CX31, Olympus Corporation, Tokyo, Japan) at a magnification of 400×, until 200 spermatozoa had been counted.

The result of HOST incubation was shown in Figure 1A. There were two types of sperm tail: A and B, in which the tail curl type A represented intact membrane sperm, and the tail non-curl type B represented sperm with damaged membrane. The result of Giemsa staining was shown in Figure 1B. There were two types of sperm head: C and D. If the sperm head was unstained, then the acrosome was not intact (D). If the sperm head was evenly purplish red, then the acrosome was intact (C).

2.5 Spermatozoa oxidative stress parameters

ROS generation was measured using an ROS Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China) following the manufacturer's instructions. Briefly, the ROS content was measured by incubating spermatozoa with 300 μL DCFH-DC solution for 30 min at 37°C in dark conditions, followed by three rounds of centrifugation and washing in PBS. The samples were analyzed using a multifunctional microplate reader (488 nm excitation and 525 nm emission for DCF) and the ROS content was determined based on the fluorescence intensity.

Measurement of MDA generation was conducted using an MDA Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China) following the manufacturer's instructions. Briefly, the MDA content was measured by incubating spermatozoa with 200 μL MDA working solution for 15 min at 100°C, followed by a single centrifugation at room temperature. The samples were evaluated using a multifunctional microplate reader (absorbance at 532 nm) and the MDA content was determined based on the standard curve.

SOD activity was measured using a Total Superoxide Dismutase Assay Kit with WST-8 (Beyotime Institute of Biotechnology, Shanghai, China) following the manufacturer's instructions. Briefly, the semen samples were lysed using the lysis buffer in the kit, followed by measuring the protein concentration using a Detergent Compatible Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China). The SOD activity was measured by incubating the spermatozoa with a series of reagents at 37°C for 30 min. The samples were evaluated using a multifunctional microplate reader (absorbance at 450 nm) and the SOD activity was calculated using the instruction formula.

TAC was measured using a TAC Assay Kit (Nanjing Jiancheng Bioengineering Institute, China) by measuring the absorbance of 2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate)$^+$ ($ABTS^+$) following the manufacturer's instructions. Briefly, the samples were centrifuged, and the supernatant was collected. A series of reagents was added to a 96-well plate to quantify TAC. The samples were evaluated using a multifunctional microplate reader ($ABTS^+$ absorbance at 405 nm) and the TAC was determined based on the standard curve.

2.6 Spermatozoa energy metabolism parameters

Mitochondrial membrane potential (ΔΨM) was measured using a ΔΨM Assay Kit with JC-1 (Beyotime Institute of Biotechnology, Shanghai, China) following the manufacturer's instructions. The formation of JC-1 aggregates can produce red fluorescence, indicating high ΔΨM. JC-1 is a monomer that can produce green fluorescence, indicating a lower ΔΨM. Briefly, the samples were centrifuged, and the supernatant was removed. Added 500 μL JC-1 working solution to the samples and incubated them for 20 min in the dark. The samples were evaluated using a multifunctional microplate reader with an excitation wavelength of 488 nm and an emission wavelength of 525 nm for JC-1 monomer and an excitation wavelength of 525 nm and an emission wavelength of 590 nm for JC-1 aggregates. The formula provided in the instructions was used to calculate the ΔΨM.

ATP content was measured using an ATP Assay Kit (Solarbio, Beijing, China) following the manufacturer's instructions. Briefly, the samples were centrifuged at 1000 × g for 10 min to obtain the supernatant. Add 1 mL extractive solution to 100 μL supernatant, mix

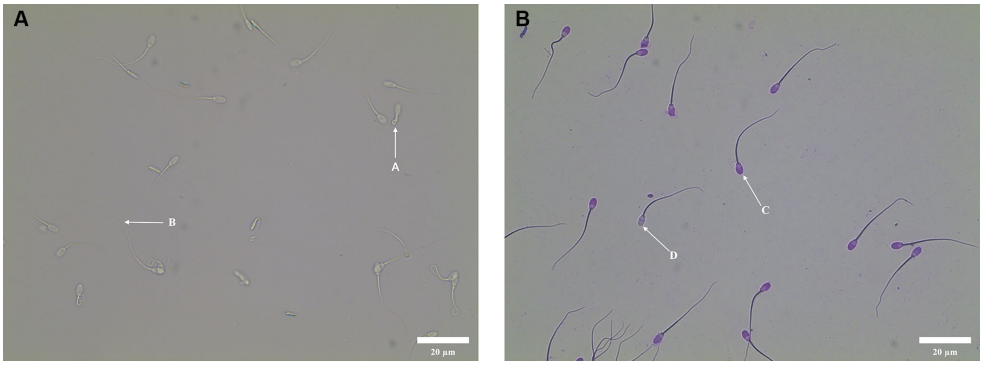

FIGURE 1
Sperm functional integrity test. **(A)** Morphology of curly tail of sperm in HOST. A: Sperm with intact membrane; B: Sperm with damaged membrane. **(B)** Acrosome morphology of sperm stained with Giemsa. D: Sperm with damaged acrosome; C: Sperm with intact acrosome.

well, and centrifuge at 10000 × g for 10 min to obtain the supernatant. Add 500 μL chloroform to the supernatant, mix well, and centrifuge at 10000 × g for 3 min to obtain the supernatant. Add a series of reagents to the supernatant and use a multifunctional microplate reader to measure the absorbance at 340 nm at 10 s and 3 min 10 s. The ATP content can be obtained using the formula.

2.7 Statistical analysis

The Shapiro–Wilk test was used to analyze the normality of the data (SPSS 25.0 software). After testing, the data showed a normal distribution. The parameters were evaluated and analyzed using a two-way repeated measures ANOVA. $p < 0.05$ means a significant difference. All results were expressed as "Mean ± SEM."

3 Result

3.1 Effects of ALA supplementation on spermatozoa TM and PM

As shown in Table 1, the TM and PM of all ALA groups were significantly higher ($p < 0.05$) than those of the control group from day 1 to 5. The TM of the 0.1 mM group was significantly higher ($p < 0.05$) than that of the 0 mM, 0.025 mM and 0.5 mM groups on the third day. On the fifth day, the TM of the 0.1 mM group was significantly higher ($p < 0.05$) than that of the 0 mM, 0.025 mM, 0.5 mM and 1 mM groups. On the third day, the PM of 0.05 mM, 0.1 mM and 0.5 mM groups were significantly higher ($p < 0.05$) than in the other groups. The PM of the 0.1 mM group was significantly higher ($p < 0.05$) than in the 0 mM, 0.025 mM and 1 mM groups on the fifth day.

3.2 Effects of ALA supplementation on spermatozoa kinetic parameters

As shown in Table 2, the VSL of the 0.1 mM group was higher ($p < 0.05$) than that of the 0 mM, 0.025 mM, 0.5 mM and 1 mM groups from day 1 to 5. On the first day, the VSL of all ALA groups was higher ($p < 0.05$) than that of the control group. The 0.1 mM group was higher ($p < 0.05$) compared to the 0 mM, 0.025 mM, 0.5 mM and 1 mM groups on the first day, but it did not show a significant difference ($p > 0.05$) compared to the 0.05 mM group. On the third day, the VSL of the 0.1 mM group was significantly higher ($p < 0.05$) than that of the control, 0.025 mM, 0.5 mM and 1 mM groups, but it was not significantly higher ($p > 0.05$) than the 0.05 mM group. On the fifth day, the VSL of the 0.1 mM group was higher ($p < 0.05$) than that of the other groups.

As indicated in Table 2, the VCL of the ALA groups was significantly higher ($p < 0.05$) than that of the control group from day 1 to 5. Specifically, on the first day, the VCL of the 0.1 mM, 0.5 mM and 1 mM groups was significantly higher ($p < 0.05$) than that of the other groups. On the third day, the VCL of the 0.1 mM group was significantly higher ($p < 0.05$) compared to the other groups. On the fifth day, the VCL of the 0.05 mM, 0.1 mM and 0.5 mM groups were significantly higher ($p < 0.05$) than those of the other groups.

As indicated in Table 2, the VAP of the 0.1 mM, 0.5 mM and 1 mM groups was significantly higher ($p < 0.05$) than that of the other groups from day 1 to 3. On the fifth day, the VAP of the 0.1 mM and 0.5 mM groups was significantly higher ($p < 0.05$) than that of the other groups.

Results showed that the ALH of the 0.1 mM, 0.5 mM and 1 mM groups was significantly higher ($p < 0.05$) than that of the other groups on the first day. Additionally, there were no significant differences ($p > 0.05$) in ALH between these three groups. On the third and fifth days, the ALH of the 0.1 mM group was significantly higher ($p < 0.05$) than that of the 0 mM, 0.025 mM, 0.05 mM and 1 mM groups, but it was not significantly higher ($p > 0.05$) than the 0.5 mM group.

3.3 Effects of ALA supplementation on spermatozoa plasma and acrosome membrane integrity

The plasma membrane integrity of the 0.05 mM and 0.1 mM groups was higher ($p < 0.05$) than that of the other groups on the first day. Additionally, these two groups were not significantly ($p > 0.05$) different from each other on the first day as shown in Table 3. The integrity of the plasma membrane in the 0.1 mM group was significantly higher ($p < 0.05$) than in the other groups from day 3 to 5.

On the first day of preservation, the integrity of the acrosome membrane in the 0.05 mM and 0.1 mM groups was significantly higher ($p < 0.05$) compared to the other groups on the first day as indicated in Table 3. The integrity of the acrosome membrane in the 0.1 mM group was significantly higher ($p < 0.05$) than in the other groups from day 3 to 5 and the acrosome membrane integrity of the control group was significantly lower ($p < 0.05$) than that of the ALA groups on the fifth day.

TABLE 1 Effect of different concentrations of ALA on spermatozoa TM and PM.

Parameter	Time	0 mM	0.025 mM	0.05 mM	0.1 mM	0.5 mM	1 mM
TM (%)	1 d	82.18 ± 0.29^{Ac}	87.43 ± 0.67^{Aab}	88.31 ± 0.39^{Aa}	88.54 ± 0.37^{Aa}	87.75 ± 0.58^{Aab}	86.51 ± 0.65^{Ab}
	3 d	76.87 ± 1.48^{Bc}	80.41 ± 0.40^{Bb}	85.93 ± 0.38^{Aa}	85.89 ± 0.47^{Ba}	82.11 ± 0.50^{Bb}	85.28 ± 0.12^{Aa}
	5 d	55.26 ± 0.28^{Cd}	66.16 ± 2.16^{Cc}	76.31 ± 1.19^{Ba}	78.31 ± 0.21^{Ca}	72.49 ± 0.61^{Cb}	71.44 ± 0.79^{Bb}
PM (%)	1 d	75.29 ± 0.60^{Ab}	81.28 ± 1.78^{Aa}	81.28 ± 0.72^{Aa}	81.42 ± 0.69^{Aa}	81.92 ± 0.96^{Aa}	81.54 ± 0.34^{Aa}
	3 d	68.97 ± 0.58^{Bd}	71.60 ± 0.26^{Bc}	78.30 ± 0.44^{Aa}	79.14 ± 0.47^{Ba}	78.48 ± 0.73^{Aa}	75.08 ± 1.63^{Bb}
	5 d	46.60 ± 0.79^{Cd}	57.44 ± 1.50^{Cc}	65.59 ± 1.69^{Bab}	68.20 ± 0.72^{Ca}	65.12 ± 2.04^{Bab}	61.57 ± 0.96^{Cbc}

Different superscripts (lowercase) in the same row show significant differences ($p < 0.05$). Different superscripts (uppercase) in the same column show significant differences ($p < 0.05$).

TABLE 2 Effect of different concentrations of ALA on spermatozoa kinetic parameters.

Parameter	Time	0 mM	0.025 mM	0.05 mM	0.1 mM	0.5 mM	1 mM
VSL (μm/s)	1 d	44.42 ± 0.14^{Ad}	46.48 ± 0.20^{Abc}	48.02 ± 0.46^{Aa}	48.60 ± 0.26^{Aa}	46.98 ± 0.31^{Ab}	45.67 ± 0.37^{Ac}
	3 d	43.65 ± 0.12^{ABc}	44.69 ± 0.53^{Bbc}	45.40 ± 0.46^{Bab}	46.37 ± 0.18^{Ba}	44.58 ± 0.09^{Bbc}	44.78 ± 0.39^{Ab}
	5 d	42.47 ± 0.67^{Bb}	42.85 ± 0.32^{Cb}	42.56 ± 0.22^{Cb}	44.98 ± 0.26^{Ca}	43.13 ± 0.44^{Cb}	42.59 ± 0.06^{Bb}
VCL (μm/s)	1 d	79.91 ± 0.49^{Ac}	80.98 ± 0.37^{Ab}	81.33 ± 0.10^{Ab}	83.55 ± 0.15^{Aa}	83.54 ± 0.34^{Aa}	84.08 ± 0.44^{Aa}
	3 d	76.03 ± 0.17^{Bc}	79.19 ± 0.08^{Bb}	79.64 ± 0.07^{Bb}	82.44 ± 0.42^{Ba}	79.49 ± 0.62^{Bb}	79.65 ± 0.56^{Bb}
	5 d	69.34 ± 0.06^{Cd}	76.24 ± 0.30^{Cb}	77.37 ± 0.13^{Ca}	78.01 ± 0.21^{Ca}	78.19 ± 0.37^{Ba}	73.78 ± 0.61^{Cc}
VAP (μm/s)	1 d	56.74 ± 0.36^{Ab}	57.94 ± 0.52^{Ab}	58.21 ± 0.31^{Ab}	62.57 ± 0.33^{Aa}	62.29 ± 0.28^{Aa}	62.54 ± 0.94^{Aa}
	3 d	54.77 ± 0.09^{Bd}	56.15 ± 0.14^{Bc}	57.08 ± 0.38^{Ab}	59.34 ± 0.24^{Ba}	59.65 ± 0.26^{Ba}	58.85 ± 0.44^{Ba}
	5 d	52.25 ± 0.16^{Cd}	53.41 ± 0.12^{Cc}	55.04 ± 0.42^{Bb}	57.73 ± 0.15^{Ca}	57.81 ± 0.52^{Ca}	54.35 ± 0.15^{Cb}
ALH (μm)	1 d	23.6 ± 0.16^{Ac}	24.76 ± 0.08^{Ab}	24.34 ± 0.09^{Ab}	26.41 ± 0.37^{Aa}	25.8 ± 0.12^{Aa}	25.81 ± 0.26^{Aa}
	3 d	22.5 ± 0.17^{Bd}	23.19 ± 0.1^{Bcd}	23.64 ± 0.16^{Bbc}	24.88 ± 0.26^{Ba}	24.33 ± 0.17^{Bab}	23.92 ± 0.47^{Bbc}
	5 d	20.73 ± 0.41^{Cc}	22.08 ± 0.01^{Cb}	22.37 ± 0.21^{Cb}	23.65 ± 0.22^{Ca}	23.57 ± 0.24^{Ca}	22.51 ± 0.06^{Cb}

Different superscripts (lowercase) in the same row show significant differences ($p < 0.05$). Different superscripts (uppercase) in the same column show significant differences ($p < 0.05$).

TABLE 3 Effect of different concentrations of ALA on spermatozoa plasma and acrosome membrane integrity.

Parameter	Time	0 mM	0.025 mM	0.05 mM	0.1 mM	0.5 mM	1 mM
Plasma membrane (%)	1d	76.53 ± 0.63^{Ad}	77.35 ± 0.15^{Acd}	81.27 ± 0.36^{Aa}	82.64 ± 0.81^{Aa}	78.65 ± 0.23^{Abc}	79.51 ± 0.20^{Ab}
	3d	68.69 ± 0.51^{Bd}	70.65 ± 0.28^{Bc}	72.17 ± 0.43^{Bb}	73.79 ± 0.32^{Ba}	72.25 ± 0.49^{Bb}	70.39 ± 0.39^{Bc}
	5d	57.45 ± 1.06^{Cc}	59.20 ± 0.24^{Cc}	61.01 ± 0.43^{Cb}	64.15 ± 0.53^{Ca}	57.96 ± 0.24^{Cc}	57.98 ± 0.28^{Cc}
Acrosome integrity (%)	1d	77.17 ± 0.28^{Ab}	78.06 ± 0.08^{Ab}	79.93 ± 0.09^{Aa}	80.12 ± 0.12^{Aa}	78.09 ± 0.47^{Ab}	78.08 ± 0.88^{Ab}
	3d	71.58 ± 0.33^{Bc}	71.98 ± 0.24^{Bc}	73.77 ± 0.24^{Bb}	75.53 ± 0.83^{Ba}	71.52 ± 0.08^{Bc}	71.24 ± 0.42^{Bc}
	5d	66.24 ± 0.11^{Ce}	69.53 ± 0.18^{Cbc}	69.78 ± 0.13^{Cb}	72.56 ± 0.25^{Ca}	69.23 ± 0.12^{Cc}	68.58 ± 0.06^{Cd}

Different superscripts (lowercase) in the same row show significant differences ($p < 0.05$). Different superscripts (uppercase) in the same column show significant differences ($p < 0.05$).

3.4 Effects of ALA supplementation on spermatozoa ROS content

As shown in Figure 2, the ROS level in the control group was significantly higher ($p < 0.05$) than that in the group with ALA on the first and third days. The addition of ALA can reduce the ROS content in spermatozoa, and the ROS content in the 0.1 mM ALA group was the lowest from day 1 to 5. On the fifth day, the ROS level decreased initially with the increase of ALA concentration in each ALA addition group, and then increased. The ROS level in the 0.1 mM group was significantly lower ($p < 0.05$) than that of the other groups, and the inhibitory effect of the 0.1 mM group on the ROS level was more pronounced.

3.5 Effects of ALA supplementation on spermatozoa MDA content

On the first, third and fifth days, the spermatozoa MDA content in the ALA group was significantly lower ($p < 0.05$) than that of the control group as shown in Figure 3. MDA production gradually decreased with increasing concentration on the third and fifth days. However, when the concentration of ALA exceeded 0.1 mM, MDA production began to increase once more. On the fifth day of preservation, the spermatozoa MDA content of the 0.1 mM group was significantly lower ($p < 0.05$) than that of the other groups.

3.6 Effects of ALA supplementation on spermatozoa SOD activity

As shown in Figure 4, on the first day of preservation, the spermatozoa SOD activity in the 0.1 mM group was the highest and significantly higher ($p < 0.05$) than in the 0, 0.025 mM and 0.05 mM groups. On the third and fifth days, the spermatozoa SOD activity in the ALA addition group was significantly higher ($p < 0.05$) than that in the control group. Compared to other groups of ALA supplementation, the spermatozoa SOD activity was higher in the 0.1 mM group.

3.7 Effects of ALA supplementation on semen TAC

Compared to other groups, the decline in semen TAC in the 0.1 mM group was the slowest as shown in Figure 5. On the first, third, and fifth days, the TAC of the group with added ALA was significantly higher ($p < 0.05$) than that of the control group. On the first day, the semen TAC in the 0.1 mM, 0.5 mM and 1 mM groups was higher than that in the other three groups ($p < 0.05$). On the third day, the TAC of the 0.1 mM and 0.5 mM groups was significantly higher ($p < 0.05$) than that in the other four groups. On the fifth day, the TAC of the 0.1 mM group was significantly higher ($p < 0.05$) than that of other groups.

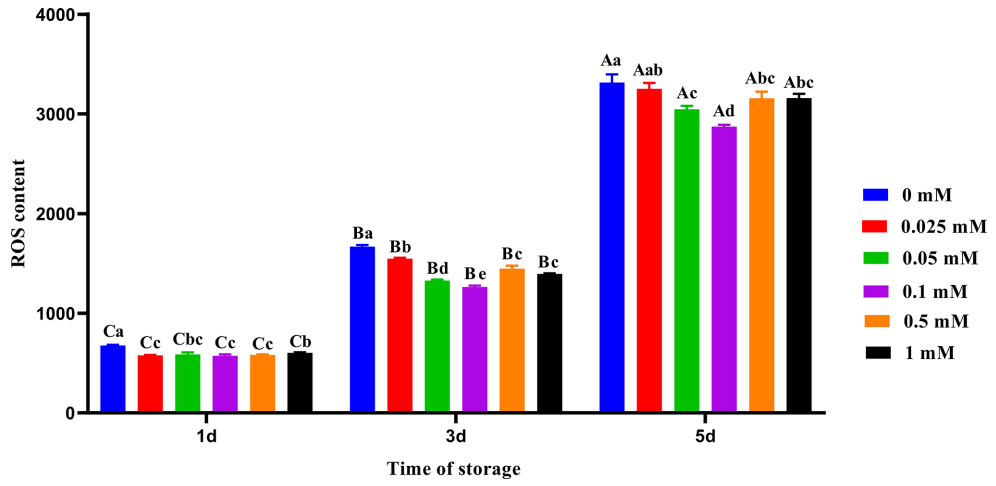

FIGURE 2
Effect of different concentrations of ALA on the ROS level of chilled spermatozoa from day 1 to 5. Values with different lowercase letters indicate difference ($p < 0.05$) among groups at each time point. Values with different uppercase letters indicate difference ($p < 0.05$) over time within the groups.

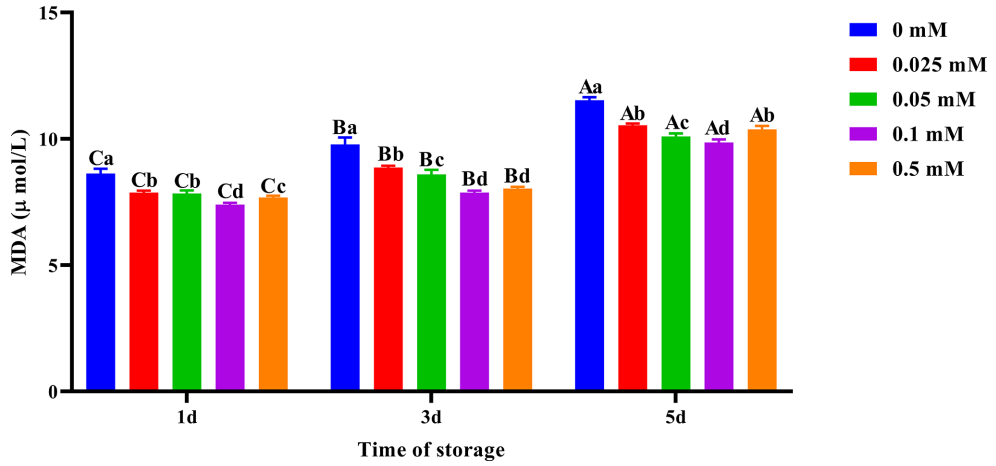

FIGURE 3
Effect of different concentrations of ALA on MDA content of chilled spermatozoa from day 1 to 5. Values with different lowercase letters indicate difference ($p < 0.05$) among groups at each time point. Values with different uppercase letters indicate difference ($p < 0.05$) over time within the groups.

3.8 Effects of ALA supplementation on spermatozoa mitochondrial membrane potential

As shown in Figure 6, the decrease in spermatozoa ΔΨM in the ALA addition group was slower compared to the control group, and the spermatozoa ΔΨM in the 0.1 mM group was higher. On the first day of preservation, the spermatozoa ΔΨM in the 0.1 mM group was significantly higher ($p < 0.05$) than that of the other groups. Meanwhile, the spermatozoa ΔΨM in the control group was significantly lower ($p < 0.05$) than that of the groups with added ALA. On the third day, the spermatozoa ΔΨM in the 0.1 mM group was significantly higher ($p < 0.05$) than that of the other groups. On the fifth day of preservation, the spermatozoa ΔΨM in the 0.05 mM and 0.1 mM groups was significantly higher ($p < 0.05$) than that of the other groups.

3.9 Effects of ALA supplementation on spermatozoa ATP content

As shown in Figure 7, the spermatozoa ATP content in the ALA addition group slowly decreased compared to the control group. On the first day, the spermatozoa ATP content in the 0.1 mM group was the highest. On the third day, the spermatozoa ATP content in the 0.05 mM and 0.1 mM groups was significantly higher ($p < 0.05$) than that of other groups. On the fifth day, the spermatozoa ATP content in the 0.1 mM group was significantly higher ($p < 0.05$) than that of the other groups.

4 Discussion

Spermatozoa are prone to the accumulation of ROS during liquid storage at 4°C, which can lead to the destruction of the spermatozoa

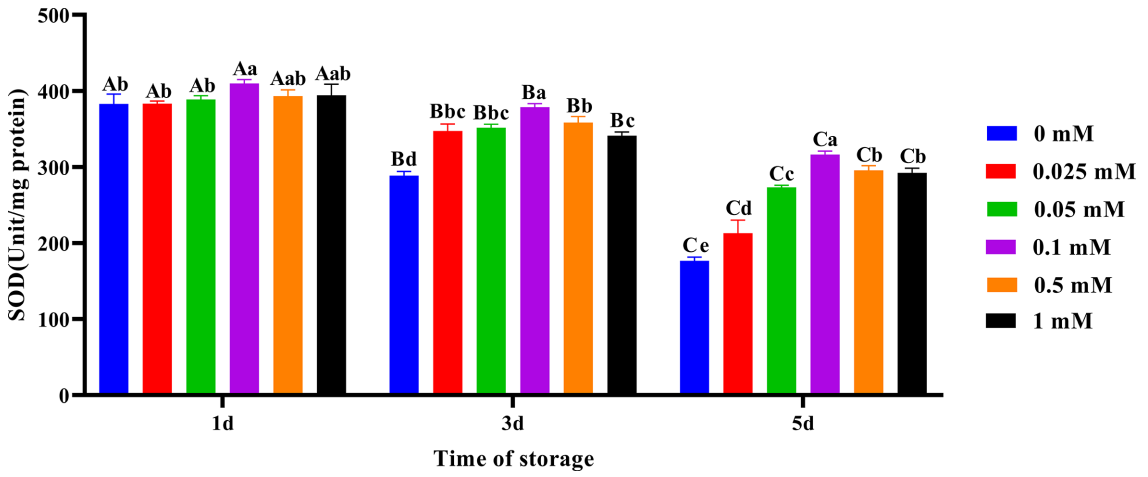

FIGURE 4
Effect of different concentrations of ALA on SOD activity of chilled spermatozoa from day 1 to 5. Values with different lowercase letters indicate difference ($p < 0.05$) among groups at each time point. Values with different uppercase letters indicate difference ($p < 0.05$) over time within the groups.

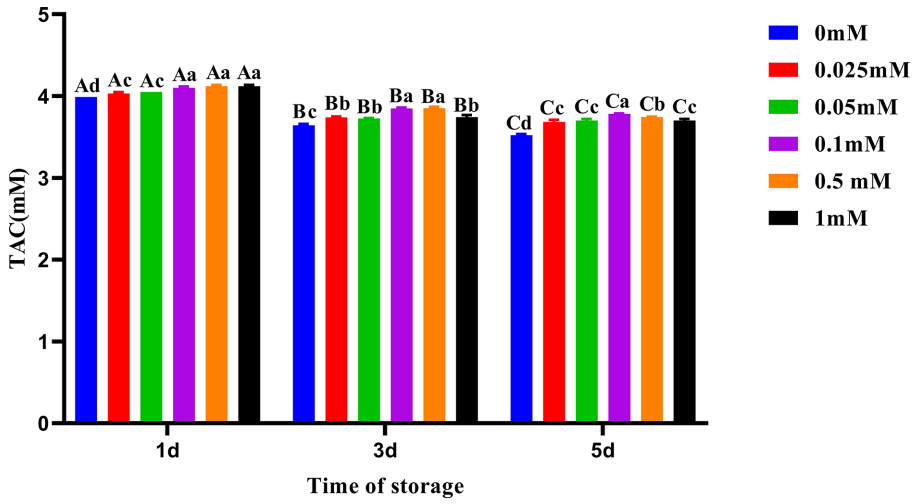

FIGURE 5
Effect of different concentrations of ALA on TAC of chilled spermatozoa from day 1 to 5. Values with different lowercase letters indicate difference ($p < 0.05$) among groups at each time point. Values with different uppercase letters indicate difference ($p < 0.05$) over time within the groups.

structural integrity (25, 26). The quality of semen will deteriorate further with prolonged storage time, ultimately leading to a loss of spermatozoa function and fertilization ability. There are two ways for spermatozoa to produce ROS at 4°C. First, it is produced by the electron transport chain of spermatozoa mitochondria (27). Second, it is produced by the NADPH-dependent oxidase system on the spermatozoa plasma membrane (28). Therefore, controlling the production of ROS and LPO is crucial for reducing oxidative damage to spermatozoa. Moreover, ALA is a nucleophilic reagent containing a mercaptan group, which can react with endogenous electrophilic reagents (29). Therefore, it can effectively remove ROS during semen preservation and has a good scavenging effect on free radicals, such as hydroxyl free radicals, nitric oxide free radicals, and peroxynitrite (30).

TM and PM are the most direct and important parameters of sperm quality. VSL, VCL, VAP, and ALH are important parameters for evaluating sperm kinematic parameters, and fertilization ability is closely related to these kinematic parameters. In this study, the addition of exogenous ALA significantly improved spermatozoa TM, PM and kinetic parameters compared to the control group. This may be due to the high concentration of ROS in semen affecting the parameters of spermatozoa motility. This study demonstrated that the semen diluent containing 0.1 mM ALA resulted in lower ROS content on the third and fifth days. It has been demonstrated that 0.1 mM ALA exhibits a strong ability to scavenge ROS. Furthermore, it can effectively reduce the oxidative damage to spermatozoa caused by ROS and maintain optimal spermatozoa motility parameters. Kasimanickam et al. (31) observed that the spermatozoa SOD activity had a positive effect on the spermatozoa kinetic parameters during liquid storage at 4°C. The spermatozoa SOD activity in the ALA group was significantly higher than that of the control group from day 3 to 5. Additionally, the spermatozoa VCL, VAP, and ALH were also significantly higher than those of the control group on the fifth day in

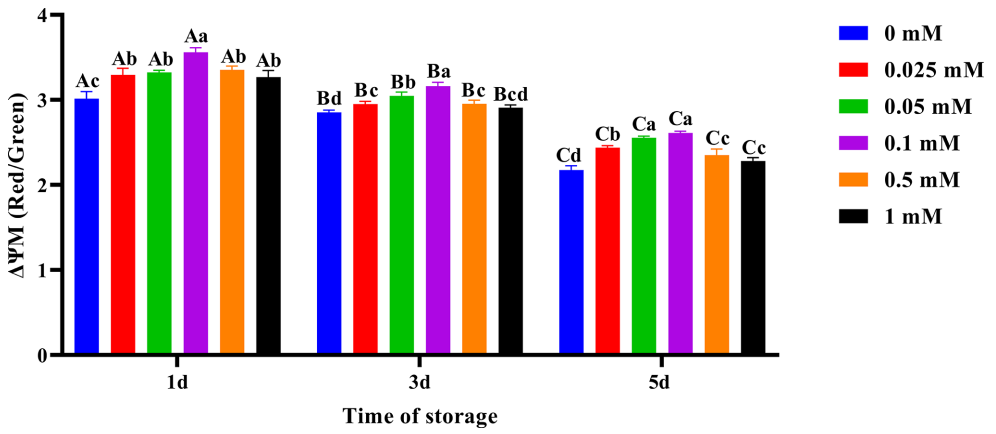

FIGURE 6
Effect of different concentrations of ALA on the mitochondrial membrane potential of chilled spermatozoa from day 1 to 5. Values with different lowercase letters indicate difference ($p < 0.05$) among groups at each time point. Values with different uppercase letters indicate difference ($p < 0.05$) over time within the groups.

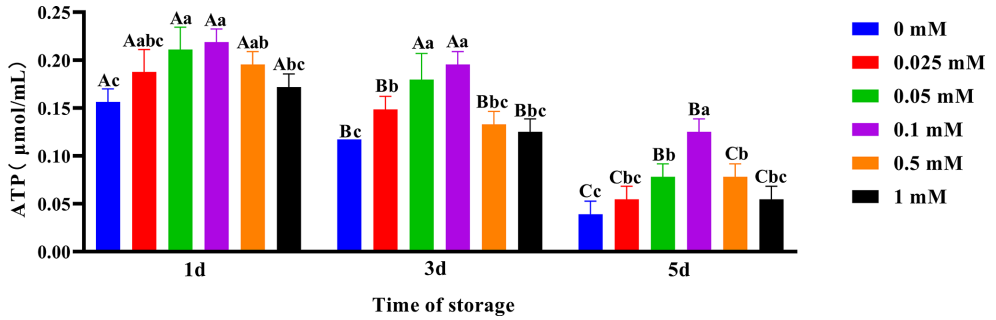

FIGURE 7
Effect of different concentrations of ALA on ATP content of chilled spermatozoa from day 1 to 5. Values with different lowercase letters indicate difference ($p < 0.05$) among groups at each time point. Values with different uppercase letters indicate difference ($p < 0.05$) over time within the groups.

this study. Ibrahim et al. (32) reported that a concentration of 0.02 mmol/mL ALA significantly improved the motility of Boer bucks' spermatozoa after thawing. In a study on cryopreserved boar spermatozoa, Shen et al. (33) reported that a concentration of 6 mg/mL ALA was found to be the most effective in significantly increasing spermatozoa motility after thawing. These results are consistent with the findings of this study, indicating that ALA can enhance spermatozoa quality. However, it is possible that the concentration of ALA varies among different species and under different preservation conditions.

Spermatozoa plasma membrane contains a lot of unsaturated fatty acids, which are prone to oxidation and can result in sperm damage (25, 26). At the same time, ROS produced by spermatozoa metabolism can also lead to peroxidation damage to the spermatozoa structure of Hu ram. Because ALA is both fat-soluble and water-soluble. Therefore, it will combine with a phospholipid bilayer and liquid components that envelop the spermatozoa when it is absorbed by the spermatozoa membrane (34). Finally, a barrier is formed on the spermatozoa membrane to enhance the spermatozoa tolerance to free radical attack and ultimately ensure the spermatozoa structural integrity (35). In this study, the integrity rate of the plasma membrane and spermatozoa acrosome in the ALA group was significantly higher than that in the control group, indicating that ALA had a protective effect on the structural integrity of the spermatozoa membrane. Onder et al. (36) reported that ALA could enhance the plasma membrane integrity of ram spermatozoa after thawing. Avdatek and Gündoğan (37) found that the addition of 1 mM ALA had a postive protective effect on the structural integrity of cryopreserved goat spermatozoa. These studies were consistent with the findings of our study. MDA is the product of LPO, which can disrupt the membrane structure and ultimately impact spermatozoa function. Excessive ROS can lead to damage to the integrity of the spermatozoa plasma membrane, its fluidity, and enzyme receptor function (38). This study found that the exogenous addition of ALA provided a layer of protection to the spermatozoa membrane, inhibited LPO, and significantly reduced the content of MDA in semen (39). These findings indicate that ALA had a protective effect on the spermatozoa plasma membrane and acrosome. This may be the cause of the decrease in MDA.

Spermatozoa motility depends on the supply of energy. Oxidative decarboxylation with ALA will impact the concentration of cytochrome c and alter the mitochondrial membrane potential (40). Fayyaz et al. (41) research demonstrated that an optimal concentration of ALA can regulate mitochondrial coenzyme metabolism and utilization efficiency, maintain a high mitochondrial

membrane potential, and ultimately protect mitochondrial function. This study found that compared to the control group, the addition of suitable ALA could maintain a higher level of mitochondrial membrane potential in spermatozoa, which was beneficial for spermatozoa motility. However, the protective effect of a high concentration of ALA is diminished, and the mitochondrial membrane potential was reduced. This may be due to a higher concentration of antioxidants causing some damage to spermatozoa mitochondria (42). In all ALA treatment groups, the addition of 0.05 mM and 0.1 mM ALA can result in higher mitochondrial membrane potential in spermatozoa on the fifth day of preservation. Therefore, an optimal concentration of ALA may reduce oxidative damage by ultimately Dihydrolipoic Acid (DHLA), maintaining high mitochondrial membrane potential to protecte mitochondrial function, and ultimately enhancing semen quality. This guarantees the implementation of AI in production. Considering the results of the present study, future research should assess how ALA exerts beneficial effects on the 4°C preservation of Hu ram spermatozoa and conduct IVF experiments.

5 Conclusion

The results of the work revealed the protective effects of ALA on Hu ram spermatozoa membrane functionality and motility during storage at 4°C. In addition, supplementation with ALA to semen decreased the contents of MDA and ROS, increased the SOD activity, the ATP content and spermatozoa ΔΨM and TAC, and ultimately improved the motility of Hu ram spermatozoa preserved at 4°C. The optimal concentration of ALA was determined to be 0.1 mM.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal studies were approved by Animal Ethics Committee of Yangzhou University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

XS: Writing – review & editing. LZ: Writing – original draft, Software. YK: Methodology, Investigation, Writing – original draft. XW: Data curation, Writing – review & editing. CJ: Software, Writing – review & editing. JW: Resources, Writing – review & editing. TS: Visualization, Writing – review & editing. YL: Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Effect of selenium nanoparticles on the quality and fertility of short-term preserved boar semen

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This study was carried out to investigate the effect of different concentrations of selenium nanoparticles (Se-NPs) in the Beltsville Thawing Solution (BTS) extender on the semen quality and fertility of Hampshire crossbred pigs. For the study, semen was collected from four boars (10 ejaculates/boar) by the gloved hand method. Each ejaculate was extended @ 1:2 with the BTS extender and split into four aliquots. The control (C) samples were without the supplementation of Se-NPs, whereas the other three were supplemented with 0.5 (T1), 1 (T2), and 2 μg ml^{-1} of Se-NPs (T3) and stored at 15°C in a BOD incubator. Extended semen was evaluated at 0 (immediately after dilution), 24, 48, 72, and 96 h of storage for sperm motility, live sperm, plasma membrane integrity, acrosome integrity, DNA integrity, and mitochondrial membrane potential (MMP). The mean percentage of sperm motility, live sperm, and sperm with intact plasma membrane and acrosome, and MMPs were significantly ($p < 0.01$) higher in all treated groups in comparison to control at 24, 48, 72, and 96 h of storage. Sperm with intact DNA in all treated groups increased significantly at 48 ($p < 0.05$), and 72 and 96 ($p < 0.01$) h of storage in comparison to the control group. The concentration of 1 μg ml^{-1} of Se-NPs was found to be the best among other concentrations. In each group, 10 sows were artificially inseminated with the liquid semen preserved for 72 h at 15°C. Supplementation of 1 μg ml^{-1} of Se-NPs yielded the highest conception rate in comparison to other groups. In conclusion, supplementation of 1 μg ml^{-1} of Se-NPs in the BTS extender resulted in the best semen quality and conception rate during the short-time liquid preservation of boar semen.

KEYWORDS

selenium nanoparticles, boar semen, BTS extender, liquid preservation, antioxidants

1 Introduction

Artificial insemination is frequently carried out in swine farms all over the world with extended liquid storage of semen to maintain the sperm fertilizing ability (1). Semen extenders allow the extraction of numerous doses from a single ejaculate (2); additionally, they allow the maintenance of sperm viability at 16–18°C for days (3). Commercial extenders are categorized as short, medium, and long-term depending on their capacity to keep sperm for 1 to 2, 3 to 4, or 7 to 10 days after collection (4). However, due to the fact that cell metabolism does not slow down or cease on cooling (5°C) or freezing (−196°C), which creates favorable conditions for

microbes to damage the quality of the sample, their storage capacity is restricted (5, 6). Modification of the sperm collection protocol can reduce contamination in raw sperm by 49.85% in bacteria and by 9.67% in fungi (7). The bacteria and filamentous fungi in boar semen could be controlled by sanitary procedures and by using commercial extenders (8). They also reported that the fertility of AI in sows with lower microspermia generated better results compared to the high one. Boar semen, kept in liquid condition that day or held at 15–20°C for 1–5 days, is used in almost 99% of artificial inseminations that are performed globally (1). The sperm quality deteriorates gradually on storage for prolonged periods due to oxidative stress (9, 10). The majority of the cells neutralize the toxic effects of various reactive oxygen species (ROS); however, the antioxidant system in the sperm cell is much lower as compared to other cells and more susceptible to oxidative stress (11). Mammals, fish, and birds have an abundance of unsaturated fatty acids and phospholipids in their sperm plasma membrane. Increased levels of unsaturated fatty acids with multiple bonds make the sperm cell sensitive to lipid peroxidation, which has a positive correlation with male infertility (12). ROS are generated when fatty acids are oxidized. Under normal circumstances, these radicals are required for several actions and physiological processes in the sperm; however, an excess amount of ROS generation may cause a decrease in membrane fluidity, DNA bridging, damaged proteins, and finally lowered sperm motility and fertility (13). Selenium is a potent natural antioxidant that has the capacity to stop spermatozoa from oxidizing. Recently, nanoparticles emerged as promising alternatives that suppressed toxicity but maintained the positive effects of selenium on an organism (14). Urbankova et al. studied the effects of sub-lethal doses of Se-NPs on the health status of rats and suggested that short-term Se-NP supplementation can be safe and beneficial in cases of Se deficiency or specific treatment (15). Several studies reported the beneficial effect of supplementing semen extenders with antioxidants on semen quality during processing and cryopreservation (16). Researchers also reported a strong favorable relationship between sperm quality and selenium levels in the seminal plasma.

The enzyme GSH-PX uses selenium as one of its constituents and protects against peroxidative injury to cell membranes and other organelles that constitute lipids (17). Over the past two decades, a growing corpus of research has examined the effects of NPs on semen characteristics. NPs with a diameter of less than 100 nm can be applied to different reproductive biology techniques due to their physiochemical characteristics (18). The antioxidant properties of certain NPs are the most intriguing aspect of their usage in maintaining sperm cell activity during preservation (19). Horkey et al. reported that the oral supplementation of selenium nanoparticles has not shown an improving effect on sperm quality. However, this could be considered a safe alternative to inorganic selenium, as well as having the potential to enhance the antioxidant properties of the semen of boars (20).

The bioactive abilities of selenium in nanoforms obtained by the application of nanotechnology can be exploited in reproduction, growth, cell freezing, digesting, and antibacterial activities. Additionally, a number of clinical and experimental studies have looked at the impact of Se-NP administration on the quality of the semen in goat bucks, rats, and roosters grown *in vitro*. However, there is no study to optimize the level of supplementation of Se-NPs in the extender for improving boar semen quality. As selenium has antioxidant capabilities, it was hypothesized that this would protect the sperm population from oxidative damage and improve semen quality and fertility.

2 Materials and methods

The study was conducted at the Division of Animal and Fisheries Sciences, ICAR Research Complex for the North Eastern Hill Region, Umiam, Meghalaya, India. Four mature, healthy Hampshire crossbred boars, ranging in age from 2.5 to 3 years with normal reproductive characteristics, were used for the experiment. The selenium nanoparticles (Se-NPs) were commercially purchased from Sai-Biotech, India (catalog number 282492). The size of the selenium nanoparticles was 80 nm. The research study was duly approved by the Institution Animal Ethics Committee of the College of Veterinary Science and Animal Husbandry, Central Agricultural University, Aizawl, India.

2.1 Semen collection and processing

Forty semen ejaculates were collected from trained crossbred Hampshire boars (n = 4, 10 ejaculates from each boar) by the gloved hand method using a dummy by the same technician following a twice-weekly schedule. The boars were aged between 14 and 18 months and managed under similar conditions. Following ejaculation, the gel fraction was separated by filtering through gauze using a Buchner funnel. Fresh semen samples were evaluated for volume by using a measuring cylinder, sperm motility by direct observation under a microscope, and sperm concentration by a Neubauer counting chamber. Samples exhibiting mass activity ≥3+, sperm motility ≥70%, viability ≥75%, and sperm concentration ≥ 100 million/ml were processed further for the experiment.

Each ejaculate was extended with a BTS extender @ 1:2 (v/v) ratio to achieve a final concentration of at least 50 million spermatozoa per ml of semen and divided into four aliquots. Each aliquot was supplemented with 0 (control), 0.5 (T1), 1 (T2), and 2 (T3) μg ml^{-1} Se-NPs and stored at 15°C in a BOD incubator. At 0, 24, 48, 72, and 96 h of preservation, the stored semen samples were evaluated for sperm motility by the conventional method, live sperm count by eosin-nigrosin staining, plasma membrane integrity by the hypo-osmotic swelling test (HOST), acrosomal integrity by Giemsa staining, DNA integrity by acridine orange staining, and MMP using JC-1 dye.

2.2 Total sperm motility

A drop of semen that had been pre-warmed to 37°C was put on a clean glass slide, covered with a cover slip, and examined under 40x magnification under a microscope. The proportion of motile spermatozoa was recorded.

2.3 Live sperm

The live sperm percentage was determined by the eosin–nigrosin staining technique (21). The straining solution (10 μL) was mixed with

10 µL of extended semen and allowed to stand for 60 s. A smear was prepared on a clean glass slide and examined under a 100X magnification microscope. Spermatozoa that were fully or partially stained were recorded as dead and those that were unstained were recorded as live. A total of 200 spermatozoa were counted, and the percentage was calculated.

2.4 Plasma membrane integrity

Plasma membrane integrity was evaluated using HOST. Briefly, 0.1 mL of semen was mixed with 1 mL of 150 mOsmol HOST solution in a glass tube and incubated for 60 min at 37°C (22). A phase contrast microscope was used to observe a drop of the incubated suspension at 40x magnification. A total of 200 sperm were counted. Spermatozoa with different forms of tail swelling were counted as sperm with an intact plasma membrane.

2.5 Acrosomal integrity

The percent intact acrosome was evaluated using the Giemsa staining method (23). On a glass slide, a smear was prepared using 20 µL of extended semen. It was air-dried and stained with Giemsa stain. A total of 200 numbers of spermatozoa were examined under 100X magnification of a microscope, and different forms of acrosomal damage were recorded. The percentage of spermatozoa with intact acrosomes was calculated and recorded.

2.6 Sperm DNA integrity

The DNA integrity of the sperm cells was evaluated by the acridine orange (AO) staining technique (24). Thin smears were prepared on glass slides, air-dried, and fixed in Carnoy's solution (one part glacial acetic acid to three parts methanol) for 2 h. Following fixation, smears were air-dried and stained for 5 min in the dark with freshly prepared AO stain (concentration: 0.19 mg/mL; TC262 HiMedia Labs), rinsed with distilled water, and then immediately examined with a fluorescent microscope (Nikon Eclipse T2i) using an excitation wavelength of 450–490 nm and a 530 nm barrier filter. Sperm cells with intact DNA fluoresced green and those with damaged DNA fluoresced from yellow-green to red (Figure 1).

2.7 Mitochondrial membrane potential

The MMPs were assessed using a cationic carbocyanine dye called JC-1(5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolyl carbocyanine iodide) (25). Briefly, 100 µL of semen sample was mixed with 1 µL of JC-1, counterstained with 4 µL of PI stock solutions, and incubated at 37°C in the dark for 30 min. Following incubation, sperm cells were spread on a grease-free glass slide and observed under a fluorescent microscope (Nikon Eclipse T2i) to count at least 200 cells. Those cells having mid-piece JC-1 aggregate-induced yellowish to orange fluorescence were considered to have high MMP levels, while those with green fluorescence were considered to have low MMP (Figure 1).

2.8 Artificial insemination

Semen was preserved in a BTS extender in 90-ml disposable plastic tubes for storage at 15°C. Each insemination dose (90 mL) contains at least 50 million spermatozoa per ml after warming to 37°C. The cervical insemination method was applied, and all the inseminations were carried out by the same technician. Sows in the second or third parity exhibiting natural estrus after weaning were maintained under standard housing, and management conditions were inseminated on the second and third days of oestrus. The sows were randomly distributed into four groups (control, T1, T2, T3, and T4). The control group received semen without any supplementation, while the T1, T2, and T3 groups received semen supplemented with 0.5, 1, and 2 µg ml^{-1} of Se-NPs, respectively. A total of 10 sows were inseminated from each of the groups. The boars and sows were randomly selected for insemination. For the insemination, semen preserved for up to 72 h was used.

2.9 Conception rate and litter size

The pregnancy was diagnosed using a veterinary ultrasound scanner (ExaGo Veterinary Scanner, IMV Technologies) after 25 days of gestation in sows. After farrowing, the fetuses born live, dead, mummified piglets, and total litter size were recorded.

2.10 Statistical analysis

The SPSS 20.0 statistical software was used to analyze the data. Data obtained in the present experiment were analyzed statistically for the main effect of experimental groups or hours of preservation using univariate analysis as per (26). Duncan's new multiple range test (27) was used to test the significance of mean differences. The significance of different selenium nanoparticle-supplemented semen on conception rate was tested using a non-parametric chi-square (χ^2) test. p-values < 0.05 were considered significant.

3 Results

The volume (ml), concentration (x 10^6/ml), mass activity (0–5 grade), initial motility (%), live sperm (%), intact plasma membrane (%), and intact acrosome (%) recorded in the fresh semen were 238.50 ± 7.93, 173.25 ± 6.39, 4.15 ± 0.04, 90.72 ± 0.33, 87.27 ± 0.38, 65.12 ± 0.46, and 93.30 ± 0.29, respectively. The sperm quality attributes in different experimental groups at different hours of preservation are presented in Table 1.

3.1 Sperm motility

The mean percentage of total motile sperm in the T1 and T2 groups was significantly ($p < 0.01$) higher as compared to control (C) at 24, 48, 72, and 96 h of preservation. At 96 h of preservation, significantly ($p < 0.01$) higher sperm progressive motility was observed in all treatment groups as compared to the control group. The ejaculates supplemented with 1 µg ml^{-1} of Se-NPs (T2) showed significantly ($p < 0.01$) higher sperm progressive motility as compared to the rest of the groups.

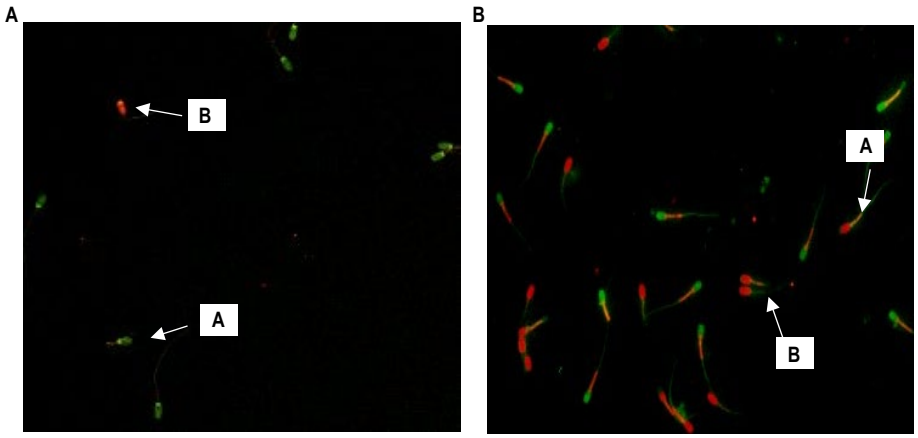

FIGURE 1
(A) Sperm with the arrow mark "A" emitting green fluorescence have intact DNA, whereas those with orange to red fluorescence are sperm with damaged DNA (400X). **(B)** Sperm with the arrow mark "A" emitting red fluorescence in the mid-piece region indicates high MMP, whereas those with green fluorescence are sperm with low MMP (400X).

3.2 Live sperm

The mean percentage of live sperm also showed a similar trend as progressive motility, and it was observed that live spermatozoa were significantly ($p < 0.01$) higher in the T2 group (μg ml^{-1} Se-NPs) in comparison to other treatment and control groups at 24, 48, 72, and 96 h of preservation.

3.3 Plasma membrane integrity

The mean percentage of sperm with an intact plasma membrane was significantly ($p < 0.01$) higher in the T2 group as compared to other groups at 48, 72, and 96 h of preservation. No difference was observed between the T1 and T3 groups at any stage of preservation.

3.4 Acrosomal integrity

The mean percentage of sperm with intact acrosomes was significantly ($p < 0.01$) higher in the T1 and T2 groups in comparison to the control group at 24, 48, 72, and 96 h of preservation. No difference was observed the between T1 and T3 groups at any stage of preservation.

3.5 Sperm DNA integrity

The mean percentage of sperm with intact DNA was significantly ($p < 0.05$) higher in the T1 and T2 groups at 48, 72, and 96 h of preservation as compared to the control group, but there was no significant difference at 0 and 24 h of preservation.

3.6 Mitochondrial membrane potential of sperm cell

The mean percentage of sperm MMP was significantly ($p < 0.05$) higher at 0 h and 24, 48, 72, and 96 h ($p < 0.01$) of preservation in 1 μg ml^{-1} of Se-NPs supplemented group (T2) among all the groups.

3.7 Conception rate and litter size

The conception rate and litter size in the control and three treatment groups are presented in Table 2. The conception rate was significantly ($p < 0.01$) higher in T2 as compared to other treatment and control groups. The average litter size was observed to be higher in all treatment groups as compared to the control group, but the difference was non-significant. The average litter size at birth in control (C), 0.5 μg ml^{-1} (T1), 1 μg ml^{-1} (T2), and 2 μg ml^{-1} (T3) selenium nanoparticles supplemented groups was found to be 7.20 ± 0.35, 7.50 ± 0.42, 8.30 ± 0.55, and 7.30 ± 0.57, respectively.

4 Discussion

The sperm membrane is put under a great deal of stress during preservation because of the generation of free radicals. It is generally known that raising ROS production slows down cell metabolism and makes sperm undergo an acrosome reaction. Although semen contains a variety of enzymatic and non-enzymatic antioxidants, their natural protection may not be enough to minimize the harmful effects of ROS. In such a situation, the antioxidant capabilities of semen can be increased by utilizing extenders enriched with exogenous antioxidants that either regulate, suppress, or block the oxidation process or prevent the generation of ROS in order to maintain sperm quality (17). Several NPs can be used in reproductive biological methods, particularly during semen preservation because of their antioxidant properties (18). Nevertheless, for the success of assisted reproductive outcomes, the potentially harmful effects of certain NPs should be taken into consideration. A study identified NPs as a driving force that triggers apoptosis and cell cycle arrest; therefore, it would be interesting to investigate any potential dose- and time-dependent harmful effects on the testes and male germ cells (28).

Reduced fertility may be due to severe injury to the sperm DNA, motility mechanism, plasma membrane, and acrosomal cap damaged during the processing of sperm. Oxidative stress causes lipid peroxidation in biomembranes leading to sperm abnormalities. The semen may be supplemented with different antioxidants that may act as free radical scavengers to protect the spermatozoa from reactive

TABLE 1 Evaluation of sperm quality attributes in different selenium nanoparticles supplemented and without supplemented groups at different hours of preservation.

Parameters	Hours	Control (without supplementation)	T1 (0.5 μL ml^{-1})	T2 (1.0 μL ml^{-1})	T3 (2.0 μL ml^{-1})	*p*-value
Total sperm motility (%)	0	$87.4^{A} \pm 0.45$	$88.37^{A} \pm 0.47$	$88.47^{A} \pm 0.46$	$87.75^{A} \pm 0.43$	0.340^{NS}
	24	$75.10^{cB} \pm 0.57$	$77.30^{bB} \pm 0.56$	$79.60^{aB} \pm 0.47$	$75.52^{cB} \pm 0.56$	0.000**
	48	$63.00^{cC} \pm 0.64$	$67.25^{bC} \pm 0.56$	$71.25^{aC} \pm 0.55$	$64.00^{cC} \pm 0.69$	0.000**
	72	$51.62^{cD} \pm 0.60$	$56.75^{bD} \pm 0.60$	$61.12^{aD} \pm 0.52$	$53.25^{cD} \pm 0.70$	0.000**
	96	$40.75^{dE} \pm 0.52$	$45.75^{bE} \pm 0.52$	$50.12^{aE} \pm 0.48$	$42.75^{cE} \pm 0.53$	0.000**
	***p*-value**	0.000**	0.000**	0.000**	0.000**	
Live sperm (%)	0	$86.12^{A} \pm 0.41$	$86.72^{A} \pm 0.37$	$87.05^{A} \pm 0.28$	$86.22^{A} \pm 0.43$	0.279^{NS}
	24	$79.25^{dB} \pm 0.40$	$81.40^{bB} \pm 0.31$	$82.47^{aB} \pm 0.28$	$80.22^{cB} \pm 0.34$	0.000**
	48	$69.40^{cC} \pm 0.56$	$72.17^{bC} \pm 0.46$	$74.37^{aC} \pm 0.42$	$70.52^{cC} \pm 0.51$	0.000**
	72	$60.05^{cD} \pm 0.71$	$62.95^{bD} \pm 0.62$	$65.37^{aD} \pm 0.58$	$61.57^{bcD} \pm 0.60$	0.000**
	96	$50.57^{cE} \pm 0.76$	$54.25^{bE} \pm 0.64$	$56.82^{aE} \pm 0.63$	$52.82^{bE} \pm 0.68$	0.000**
	***p*-value**	0.000**	0.000**	0.000**	0.000**	
Plasma membrane integrity (%)	0	$63.60^{A} \pm 0.46$	$63.92^{A} \pm 0.52$	$64.62^{A} \pm 0.47$	$63.70^{A} \pm 0.49$	0.451^{NS}
	24	$52.30^{bB} \pm 0.53$	$53.57^{abB} \pm 0.53$	$54.95^{aB} \pm 0.55$	$52.70^{bB} \pm 0.45$	0.002**
	48	$42.50^{cC} \pm 0.61$	$44.37^{bC} \pm 0.62$	$46.10^{aC} \pm 0.61$	$43.25^{bcC} \pm 0.57$	0.000**
	72	$33.92^{cD} \pm 0.60$	$36.20^{bD} \pm 0.65$	$38.07^{aD} \pm 0.67$	$35.07^{bcD} \pm 0.61$	0.000**
	96	$26.15^{cE} \pm 0.57$	$28.32^{bE} \pm 0.58$	$30.12^{aE} \pm 0.61$	$27.15^{bcE} \pm 0.57$	0.000**
	***p*-value**	0.000**	0.000**	0.000**	0.000**	
Acrosomal integrity (%)	0	$92.82^{A} \pm 0.33$	$93.20^{A} \pm 0.33$	$93.27^{A} \pm 0.38$	$92.92^{A} \pm 0.31$	0.754^{NS}
	24	$82.85^{cB} \pm 0.42$	$84.22^{abB} \pm 0.38$	$85.15^{aB} \pm 0.37$	$83.07^{bcB} \pm 0.39$	0.000**
	48	$72.80^{bC} \pm 0.51$	$74.95^{aC} \pm 0.46$	$76.17^{aC} \pm 0.42$	$73.65^{bC} \pm 0.44$	0.000**
	72	$63.45^{cD} \pm 0.64$	$65.87^{abD} \pm 0.60$	$67.07^{aD} \pm 0.62$	$64.60^{bcD} \pm 0.62$	0.000**
	96	$53.75^{cE} \pm 0.70$	$56.27^{abE} \pm 0.66$	$57.92^{aE} \pm 0.67$	$55.10^{bcE} \pm 0.70$	0.000**
	***p*-value**	0.000**	0.000**	0.000**	0.000**	
Sperm DNA integrity (%)	0	$99.70^{A} \pm 0.07$	$99.77^{A} \pm 0.06$	$99.85^{A} \pm 0.05$	$99.80^{A} \pm 0.06$	0.439^{NS}
	24	$99.60^{AB} \pm 0.07$	$99.67^{AB} \pm 0.07$	$99.75^{A} \pm 0.06$	$99.70^{AB} \pm 0.07$	0.547^{NS}
	48	$99.40^{bB} \pm 0.07$	$99.52^{abB} \pm 0.07$	$99.70^{aA} \pm 0.07$	$99.50^{abB} \pm 0.08$	0.057*
	72	$98.95^{cC} \pm 0.10$	$99.25^{abC} \pm 0.08$	$99.42^{aB} \pm 0.09$	$99.05^{bcC} \pm 0.08$	0.002**
	96	$98.22^{cD} \pm 0.12$	$98.80^{aD} \pm 0.08$	$98.97^{aC} \pm 0.06$	$98.50^{bD} \pm 0.10$	0.000**
	***p*-value**	0.000**	0.000**	0.000**	0.000**	
Mitochondrial membrane potential of sperm cell (%)	0	$85.77^{bA} \pm 0.30$	$86.25^{abA} \pm 0.34$	$86.85^{aA} \pm 0.29$	$85.75^{bA} \pm 0.30$	0.046*
	24	$79.72^{dB} \pm 0.23$	$81.52^{bB} \pm 0.22$	$82.70^{aB} \pm 0.16$	$80.35^{cB} \pm 0.15$	0.000**
	48	$72.62^{dC} \pm 0.29$	$74.77^{bC} \pm 0.31$	$76.17^{aC} \pm 0.21$	$73.65^{cC} \pm 0.29$	0.000**
	72	$65.57^{cD} \pm 0.29$	$67.50^{bD} \pm 0.31$	$69.40^{aD} \pm 0.28$	$66.67^{bD} \pm 0.31$	0.000**
	96	$57.55^{dE} \pm 0.31$	$60.07^{bE} \pm 0.26$	$62.15^{aE} \pm 0.27$	$59.17^{cE} \pm 0.20$	0.000**
	***p*-value**	0.000**	0.000**	0.000**	0.000**	

T1: Supplemented with 0.5 μg ml−1 of Se-NPs; T2: supplemented with 1 μg ml−1 of Se-NPs; T3: supplemented with 2 μg ml−1 of Se-NPs.
**$p < 0.01$; *$p < 0.05$; ^{NS}Non-significant.
Means with different superscripts in a row (a, b, c, d) differed significantly.
Means with different superscripts in a column (A, B, C, D, E) differed significantly.

oxygen species (29). This antioxidant defense potential of semen is reduced during processing and preservation. Supplementation of antioxidants in the freezing diluents had a protective impact against lipid peroxidation, maintaining metabolic activity, and cellular viability (30, 31).

The present findings for sperm progressive motility, live sperm, plasma membrane integrity, and acrosomal integrity were in close agreement with the observations (32) and (33). They reported that nanoselenium supplementation of 1 μg/mL to the ram semen significantly ($p < 0.01$) improved sperm progressive motility, live sperm, plasma

TABLE 2 Conception rate and litter size in the different experimental groups.

Parameters	Control (Without supplementation)	T1 (0.5 μg ml^{-1})	T2 (1 μg ml^{-1})	T3 (2 μg ml^{-1})
Conception rate (%)	80^{a}	90^{a}	100^{b}	90^{a}
Litter size	7.20 ± 0.35	7.50 ± 0.42	8.30 ± 0.55	7.30 ± 0.57

Means bearing different superscripts in a row differed significantly ($p < 0.01$).

membrane integrity, and acrosome integrity compared to 2 μg/mL of nanoselenium and non-treated groups. Dorostkar et al. investigated the effect of *in vitro* supplementation of selenium on fresh and frozen semen of buffalo, and the results showed that the supplementation of 1 and 2 μg/mL of selenium in the extender significantly ($p < 0.01$) improved semen parameters (33). The present findings for sperm DNA integrity were in close agreement with the observations mentioned in Refs. (34) and (32). During the preservation phase, ROS production is more pronounced. The function of selenium in boosting antioxidant defenses, notably glutathione, which in turn controls excessive peroxide levels that might destabilize chromatin material, may explain why Se-NP-treated sperm samples had better DNA integrity. One of the primary techniques previously advised for evaluating sperm quality in human spermatozoa was the detection of MMP alterations (35). MMP, which is linked to intact mitochondria and may affect sperm motility, can therefore be used to detect the energetic condition of mitochondria. In addition, it was suggested that sperm with lower MMP were less likely to react to acrosomes (36). Such studies support the idea that NP supplementation (selenium) would be able to maintain the sperm MMP, giving insight into the sperm's ability to fertilize. The present finding for the mitochondrial membrane potential of sperm cells was in close agreement with the observation (37), (38). The improvement of semen quality can be related to diminishing the effect of ROS by adding antioxidants, i.e., selenium nanoparticles, to the diluent. Kumaresan et al. reported that selenium is an important component of glutathione peroxidase, which, in turn, is important for preserving the structural integrity of the sperm membrane (39). According to the authors mentioned in Ref. (40), the addition of different levels of nano-selenium to the freezing medium was able to reduce free radicals and increase the motility and viability of post-thaw sperm. The present findings for conception rate and average litter size were higher than the observations (41). The sows used in the present study were Hampshire and *Niang Megha* (indigenous breed from India) crosses with 75% Hampshire inheritance. The litter size recorded in the study is typical for this crossbred. The conception rate and litter size were found to be higher in the treated selenium nanoparticle-treated groups. This indicates that the quality of the preserved boar semen improved after the addition of selenium nanoparticles to the extender that was used for insemination.

5 Conclusion

In conclusion, supplementation of Se-NPs in BTS extenders improves the semen quality of Hampshire crossbred boar spermatozoa during short-term preservation at 15°C. The concentration of 1 μg ml^{-1} of Se-NPs resulted in the best semen quality and conception rate in comparison to other concentrations.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by the Institution Animal Ethics Committee of The College of Veterinary Science & Animal Husbandry, Central Agricultural University, Aizawl, India.The study was conducted in accordence with local legislation and institutional requirements.

Author contributions

DP: Investigation, Writing – original draft. DT: Investigation, Writing – review & editing. FA: Writing – original draft, Formal analysis. KL: Writing – original draft, Validation. GKa: Writing – original draft, Validation. TT: Formal analysis, Writing – original draft. HC: Writing – original draft, Investigation. RK: Formal analysis, Writing – original draft. GKh: Writing – original draft, Data curation. SD: Conceptualization, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The characterization of CellROX™ probes could be a crucial factor in ram sperm quality assessment

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Several authors have demonstrated that low levels of reactive oxygen species (ROS) are necessary for the physiological functions of sperm, such as capacitation, hyperactivation, acrosomal reaction and fertilization. However, high levels of ROS are associated with oxidative stress and detrimental effects on fertility. Consequently, deep characterization of ROS presence using different fluorescent probes could be crucial. In this sense, the study of intracellular ROS localization and the relationships between ROS and other conventional parameters could improve the characterization of sperm quality for semen preservation protocols in rams. In this work, a multiparametric study was carried out by analyzing four experimental groups of ram sperm with different initial qualities: fresh semen (from both breeding and nonbreeding seasons), frozen-thawed semen and, a positive control group treated with hydrogen peroxide (300 μM) as a marker of extreme damage. Sperm analyses, including viability, apoptosis, lipid peroxidation, motility and kinetic parameters, were applied to compare several experimental groups with different sperm qualities. After that, the signals from two different ROS probes: CellROX™ Deep Red (CRDR) and Green (CRG), were examined by flow cytometry (percentage of cells that express ROS) and fluorescence microscopy (intracellular ROS location). Comparing conventional parameters, fresh samples from the breeding season showed the highest sperm quality, while the positive control samples showed the worst sperm quality. Concerning the ROS probes, the CRDR levels were higher in fresh samples from the breeding season than in the positive control and cryopreserved samples. Surprisingly, CRG presented its highest level ($P < 0.05$) in the positive control group treated with peroxide by flow cytometry. CRDR and CRG presented opposite labeling patterns that were corroborated by fluorescence microscopy, which determined that the probes localized in different parts of sperm. CRDR was found in the sperm mitochondrial region, while CRG was observed in the cell nucleus, suggesting that ROS localization is an important factor. Finally, our study indicates that CRDR is correlated with proper viability and sperm motility, and could be associated with high mitochondrial activity, while CRG is associated with sperm damage.

KEYWORDS

CellROX probes, lipid peroxidation, ovine, oxidative stress, reactive oxygen species, sperm

1 Introduction

Oxidative stress is one of the leading causes of male infertility that is a consequence of an imbalance in reactive oxygen species (ROS) (1, 2). This effect has been previously demonstrated by different authors in some mammalian species, including rams (3–5), stallions (6), red deer (7) and bulls (8). Sperm cells are very susceptible to oxidative stress due to the high number of unsaturated lipids that compose their cell membranes (9); for this reason, lipid peroxidation is highly correlated with ROS content in sperm cells (5). Sperm preservation methods (cooling and cryopreservation) are the main cause of oxidative stress in domestic animals (10). Sperm cells suffer detrimental effects due to the drastic temperature differences, including the overproduction of ROS, reduced acrosome integrity or impairment of mitochondrial membrane potential (10). An imbalance in the redox system due to an increase in ROS levels triggers a decrease in sperm quality. Specifically, high ROS levels cause lipid peroxidation, DNA fragmentation, apoptosis, and consequently low fertility (11, 12). ROS include superoxide anions (O2), hydrogen peroxide (H_2O_2), proxyl (·ROO), and hydroxyl (·OH) radicals (5). Balanced ROS generation is essential for sperm capacitation, hyperactivation, the acrosomal reaction and fertilization (13–15). Moreover, seminal plasma contains different antioxidants that are very relevant for sperm protection, including enzymatic antioxidants [superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT)] and nonenzymatic antioxidants such as vitamins (A, C, and E) (2, 11). High levels of ROS can be compensated for the seminal plasma antioxidant system (11). Nevertheless, an imbalance caused by excessive ROS production and decreased levels of antioxidant enzymes provokes oxidative stress (13, 14).

Therefore, a key point for improving sperm quality analyses should focus on the redox status of sperm (11, 16). This status can be assessed by direct measurements (ROS production) or indirect measurements (malondialdehyde (MDA), high DNA fragmentation index (hDFI), and SOD and GPX levels) (11, 17). In recent decades, the amount of MDA present in sperm cells has been used as a measure of lipid peroxidation (11, 18) and is an indirect measure of ROS production and oxidative damage (18). The reaction of MDA with thiobarbituric acid (TBA) forms a product that can be identified colorimetrically or fluorometrically, and its signal is proportional to the amount of MDA present. Moreover, several authors have noted an association between a high DNA fragmentation index and oxidative stress (19). Different techniques have been used to evaluate DNA fragmentation, in particular Sperm Chromatin Structure Assay (SCSA®) technique described by Everson (20) which allows the use of flow cytometry. In addition, there are other techniques used for the evaluation of DNA fragmentation including Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL assay), *in situ* nick translation assay (ISNT), Sperm Chromatin Dispersion (SCD) or Single-cell gel electrophoresis assay (Comet) (21).

In recent years, new approaches based on multiparametric analyses by flow cytometry have been developed as innovative technologies to evaluate ROS production in ram sperm (22). Flow cytometry is a useful tool for the evaluation of sperm quality. This technique allows the analysis of a large number of cells, capturing many features of each of them in a few seconds (23). Its ability to analyze multiple sperm characteristics allows for a better understanding of sperm functionality (24), which will enable the development of sperm quality analysis (25, 26). Multiparametric analysis by flow cytometry has been developed to ensure adequate sperm quality to perform fertility studies in different mammalian species, such as humans (27, 28), bulls (29), and stallions (30). Some oxidative stress measurement techniques have been developed for the evaluation of specific ROS and other oxidative species can be carried out by reagents that accumulate intracellularly and become fluorescent upon oxidation (23). These fluorochromes include MitoSOX Red probe used to identify superoxide anion and hydrogen peroxide in human sperm (31), 2′, 7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) to detect hydrogen peroxide (32, 33) or 4,5-diaminofluorescein diacetate (DAF-FM) used for identify nitric oxide in stallion sperm (34).

Recently, novel CellROX fluorescent probes in different colors have been introduced as ROS markers: CellROX™ Deep Red, CellROX™ Orange and CellROX™ Green. CellROX™ Deep Red and Green can detect hydroxyl radicals and superoxide anions in sperm cells (35–37). On the other hand, CellROX™ Orange detects hydroxyl peroxide, hydroxyl radicals, nitric oxide, peroxide nitrile anions and superoxide anions in sperm cells (37, 38). Additionally, CellROX probes can be used in fluorescence microscopy and spectrophotometry, unlike previous probes (35). Therefore, these innovative probes allow for deep characterization and identification of intracellular ROS localization. In the case of sperm cells, this characterization is more interesting because these cells are composed of a head (where the nucleus is located), middle piece (where the mitochondria are placed), and tail (39). ROS can be localized to the mitochondria but also in the nucleus, where they promote DNA damage (40). For this reason, these novel fluorescent probes should be analyzed.

The objective of this work was to validate the efficiency of the fluorescent probes CellROX™ Deep Red (CRDR) and CellROX™ Green (CRG) in identifying the presence of ROS [the hydroxyl radical (OH) and superoxide anion (O_2)], including their intracellular location and concentrations in ram sperm. In addition, the relationships between the fluorescent probes and different parameters of sperm quality were studied. For this purpose, motility and kinetic parameters, multiparametric flow cytometry analyses (viability and apoptosis), hDFI and, MDA measurements were correlated with the novel fluorescent probes. This evaluation could improve semen preservation protocols in rams.

2 Materials and methods

2.1 Animal care and sperm collection

The study was performed following the Guidelines of the European Union Council (86/609/EU), modified by 2010/63/UE, according to the national laws (RD 2013) for laboratory animals. The experimental instructions were approved by Animal Care and Use Committee of the University of León (Spain) (ÉTICA-ULE-050-2022). Seven healthy adult Assaf rams were used in this experiment during the breeding and nonbreeding seasons. Rams were owned by the National Association of Assaf Sheep Breeders (ASSAF.E) and kept under uniform nutritional conditions

at the Animal Selection and Reproduction Center of Junta de Castilla y León (CENSYRA) located in Villaquilambre (León, Spain). Trained males were used to carry out the experiments (twice weekly semen collection). Fourteen ejaculates (two per male) were collected by an artificial vagina (40°C) in two different seasons: (i) breeding season (BS) and (ii) nonbreeding season (NBS). Immediately after collection, the ejaculates were kept in a water bath at 30°C during the initial evaluation of semen quality. This assessment consisted of volume measurements (graduated tubes) and mass motility (a 5 μL drop on a microscope was prepared with a warmed plate, and a subject score from 0 to 5 was determined).

2.2 Experimental groups and sample treatments

Experimental groups with different sperm qualities were used: fresh samples [breeding season (BS) and nonbreeding season (NBS)], frozen-thawed NBS samples and positive control samples from the BS (oxidative stress induction with hydrogen peroxide). The first and second ejaculates from each male were mixed, and each pool was split into two subsamples to obtain the four experimental groups: BS and positive control (PC) from the breeding season and NBS and cryopreserved from the nonbreeding season (Supplementary Figure 1). Samples from the breeding and nonbreeding seasons were diluted to the same volume (1:1) in a dilution medium design by our group -Itra-ULE- (41, 42). This medium was composed by TES solution (325 mOsm/kg) and TRIS solution (325 mOsm/kg), mix to pH= 7.2, D-Fructose solution (325 mOsm/kg), supplemented with 20% egg yolk, penicillin G (sodium salt) (500,000 IU/L), and dihydrostreptomycin sulfate (625 mg/L) (41, 42). After that, the samples were immediately transported to the laboratory in a water bath at 30°C. Once there, the sperm concentrations were determined by a cell counter (Nucleocounter SP-100, ChemoMetec, Allerod, Denmark). Samples were diluted to 1,600 × 10^6 spermatozoa/mL in TTFM. Immediately, the semen was refrigerated by cooling at a rate of −0.5°C/min from 30°C to 15°C in a programmable bath (CC-K8, Huber, Germany). Positive control samples were submitted to oxidative stress induction by treatment with 3% hydrogen peroxide (Viviar, Valencia, Spain) diluted in PBS for a final hydrogen peroxide concentration of 300 μM for 24 h at 37°C, as described by Soliman et al. (16). Cryopreserved samples were diluted to the same volume (1:1) in TTFM supplemented with 20% clarified egg yolk and 4% glycerol made by our group (41). Samples were cryopreserved following the protocol previously described by our group (41, 43). Semen was diluted to 100 × 10^6 sperm/mL in TTFM. Before that, the samples were refrigerated by cooling at a rate of −0.25°C/min to 5°C using a water bath in the refrigerated chamber. After 2 h of equilibration at 5°C, the diluted samples were packed into 0.25 mL French straws. Then, using a programmable biofreezer (Kryo 10 Series III; Planes PLC, Sunbury-on-Thames, UK), the straws were frozen by cooling at a rate of −20°C/min to −100°C and finally dropped into liquid nitrogen. Samples were kept in liquid nitrogen containers until being thawed. The straws were thawed in a water bath at 65°C for 5 s.

2.3 Sperm motility and kinetic parameters

Sperm motility and kinetic parameters were determined using the CASA system (computer assistant sperm analysis) (Sperm Class Analyzer -SCA- 6.3.0.59; Microptic S.L., Barcelona, Spain). Pooled ejaculates from each male were diluted to 2 × 10^6 in TES-TRIS-fructose medium supplemented with 1% egg yolk and warmed to 37°C on a warming plate for the samples from each experimental group (breeding season, nonbreeding season, positive control and frozen-thawed samples). Five microliters of the diluted sample was dropped into a Makler counting chamber (10 μm depth; Sefi Medical Instruments, Mumbai, India) and analyzed with the CASA system. The SCA system consisted of an optical phase-contrast Nikon Eclipse microscope (Nikon, Tokyo, Japan) equipped with a Basler acA1300-200uc digital camera (Basler Vision Technologies, Ahrensburg, Germany) and a warmed stage (37°C) and observations were made with a 10x objective with negative phase contrast specifically set for ram spermatozoa (1 μm < particular area < 20 μm^2). This program was set to capture at 100 frames/second (particles with an area of 20-70 μm^2). The sperm quality parameters included in our study were the percentage of total motile spermatozoa (TM, %), defined as the percentage of sperm with VCL>15 μm/s; progressive motility (PM, %), defined as the percentage of sperm with VCL>45 μm/s; and certain kinetic parameters: curvilinear velocity (VCL, μm/s) and amplitude of the lateral displacement of the sperm head (ALH, μm). A total of seven sperm samples from each experimental group were analyzed.

2.4 Multiparametric flow cytometry analyses

Sperm samples from different experimental groups were analyzed by flow cytometry. Different fluorochromes were combined to evaluate sperm quality: Zombie Violet™ Fixable Viability Kit (excitation 405 nm, emission 423 nm) (Biolegend, San Diego, California, EEUU), used to determine viability associated with membrane integrity; CellEvent™ Caspase-3/7 Green Detection Reagent (excitation 502 nm, emission 530 nm) (Invitrogen, Eugene, Oregon, EEUU), an apoptosis marker; and CellROX™ Deep Red (excitation 644 nm, emission 665 nm) (Invitrogen, Eugene, Oregon, EEUU) and CellROX™ Green (excitation 485 nm, emission 520 nm) (Invitrogen, Eugene, Oregon, EEUU), markers of ROS content. The Zombie Violet™ Fixable Viability Kit is an amine- reactive fluorescent dye that is non-permeant to live cells but permeant to the cells with compromised membranes. Thus, it can be used to assess the live and dead status of mammalian cells by determining two subpopulations: (1) the subpopulation with intact membranes (viable sperm low stained by Zombie Violet™), and (2) the subpopulation showing compromised membranes (dead sperm high stained by Zombie Violet™). Each sample was diluted in PBS (Merck, Madrid, Spain) to obtain a total of 2 × 10^6 sperm per sample, and the samples were centrifuged (from 0 to 14,100 × g in 12 s and maintained at that speed until 15 s) (MiniSpin Plus, Eppendorf, Hamburg, Germany). The sperm pellet was incubated with 96 μL of Zombie Violet (1:1,000 final dilution), 2 μL of CellEvent

Caspase-3/7 (4 μM final concentration) and 2 μL of CRDR (5 μM final concentration) in the dark at room temperature for 30 min. On the other hand, with respect to CRG because it presents the same emission/excitation spectrum as CellEvent™ Caspase-3/7 Green Detection Reagent, it was included with Zombie Violet™ (1:1,000 final dilution) in a separate tube following the same procedure as the other samples (5 uM final concentration of CRG). Different emission/excitation spectrum from each fluorochrome are shown in Supplementary Figure 2. Samples were then washed to stop cell staining, and the pellet was resuspended in 1 mL of PBS. Additionally, following the procedure described above, another study was performed including in the same tube Zombie Violet (1:1,000 final dilution), CRDR (5 μM final concentration) and CRG (5 μM final concentration), analyzing both CellROX™ tests in the same population of viable sperm. This analysis was performed in the best sperm quality group (fresh samples), and in the worst sperm quality group (positive control treated with peroxide samples). Sperm samples were analyzed using a flow cytometer (MACSQuant Analyzer 10, Miltenyi Biotech, Bergisch Gladbach, Germany) equipped with three lasers emitting at 405, 488, and 635 nm (violet, blue and red, respectively) and 10 photomultiplier tubes. Violet fluorescence was detected in V1 (excitation 405 nm, emission 450/50 nm), green fluorescence was detected in B1 (excitation 488 nm, emission 525/50 nm), and red fluorescence was detected in R1 [excitation 635 nm, emission 655–730 nm (655LP + split 730)]. The system was controlled by MACS Quantify software (Miltenyi Biotech, Bergisch Gladbach, Germany), and a total of 40,000 events were recorded for each sample with a flow rate of 200–300 cells/second. Data analysis was carried out by FlowJo v.10.2 (Ashland, Wilmington, DE, USA). In flow cytometry analysis, the population corresponding to the sperm was selected, eliminating the rest of the events (debris). In addition, singlets were selected, eliminating doublets from the analysis. Also, unstained controls were used to determine positive and negative events. Representative cytograms of the assay and gating strategy are shown in Supplementary Figure 3. A total of seven sperm samples from each experimental group were analyzed.

It is important to note that the CRDR probe cannot be fixed with formaldehyde, in contrast to the manufacturer specifications. This fact makes it necessary to read the samples in the flow cytometer at the time of processing. On the other hand, CellROX™ Green probe is fixable with formaldehyde according to the manufacturer's specifications.

2.5 CellROX™ Deep Red and Green intracellular location

Two samples from the most differentiated quality experimental groups (fresh sample from the breeding season like highest quality control and positive damage sample treated with 300 μM hydrogen peroxide like lowest quality control) were used to verify the intracellular location of the two CellROX probes. Two hundred microliters (25×10^6 sperm/mL) of each semen sample was washed in PBS and centrifuged (from 0 and 14,100 × g in 12 s and maintained at that speed until 15 s). Sperm cells were incubated with 4 μL of CRG (1 mM) and 4 μL of CRDR (1 mM) with 100 μL of PBS in the dark at room temperature for 30 min following the protocol described by Lanconi et al. (44). After that, another washing step was carried out to stop cell staining, and the sperm cells were resuspended in 50 μL of PBS. Afterward, an aliquot of 5 μL of the stained solution was placed between a slide and coverslip and observed by epifluorescence microscopy (Eclipse Ni-E) (Nikon, Tokyo, Japan) equipped with a Photometrics BSI digital camera (Prime BSI, Photometrics, USA) using a 40× objective (total magnification: 400×). The same field was captured in a light field with a FITC filter (CRG detection) and a Cy5 filter (CRDR detection).

2.6 Lipid peroxidation assay

MDA generation was measured with a Lipid Peroxidation Assay kit (Lipid Peroxidation MDA Assay Kit (MAK085), Sigma–Aldrich, Darmstadt, Germany). One million sperm cells were homogenized on ice in 300 μL of MDA lysis buffer containing 3 μL of BHT. Samples were centrifuged at 13,000 × g for 10 min to remove insoluble material. Next, 600 μL of TBA solution was added to form the MDA-TBA adduct in the standard and each sample. Then, the samples were incubated at 95°C for 60 min. After that, the samples were cooled to room temperature in an ice bath for 10 min. Two hundred microliters of each sample reaction mixture were added to a well in duplicate. The fluorescence intensity at 532 nm and 553 nm was measured with a plate reader (Biotek, Gene 5 Microplate Reader, Winooski, VT, USA). A calibration curve was prepared from the MDA standard included on the plate. Finally, the concentration of MDA in the samples was calculated using the equation obtained from linear regression of the standard curve. Seven samples from the same males were analyzed in each of the four experimental groups (twenty-eight samples in total). Analysis was performed in duplicate, and the mean of the two values was taken as the result.

2.7 Sperm chromatin structure assay (SCSA)

The SCSA is a technique that quantifies the metachromatic shift from green fluorescence (double-stained DNA) to red (denatured single-stranded DNA). Acridine orange staining was performed following the procedure described by Everson et al. (45). Previously, samples from each experimental group were diluted to 2 million sperm/mL with TNE buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4). After that, 0.1 mL of sperm sample was mixed with 0.2 mL of detergent solution composed of 0.1% Triton X-100 in 0.08 N HCl, and 0.15 M NaCl was added to induce partial DNA denaturation. Thirty seconds later, the sperm samples were stained with 0.6 mL of acridine orange solution (6 μg/mL electrophoretically purified acridine orange in citrate-phosphate buffer). Three minutes after staining, the sperm samples were analyzed by flow cytometry. The DNA fragmentation index (DFI) was calculated based on the intensity of red fluorescence divided by the total (red plus green) fluorescence, indicating the amount of denatured sperm DNA relative to the total amount of DNA in each sperm. A cut-off value in DFI at 0.75 was performed to obtain the percentage of sperm with high DFI (hDFI) (20, 23).

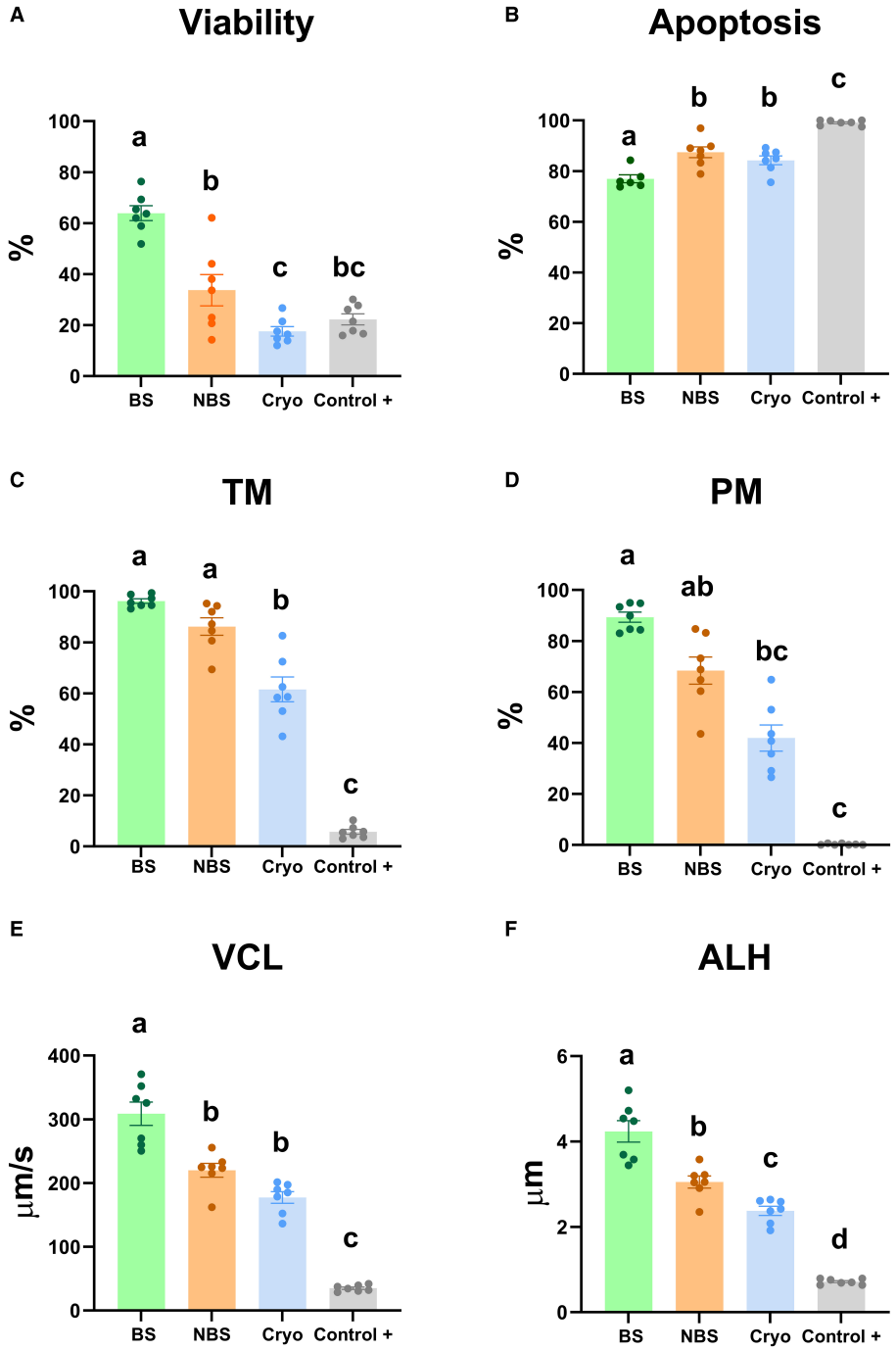

FIGURE 1
Multiparametric flow cytometry analyses and motility and kinetic parameters of ram sperm from the four experimental groups [fresh in breeding season (BS), fresh in nonbreeding season (NBS), positive control treated with hydrogen peroxide (300 µM) (Control +) and cryopreserved (Cryo)]. **(A)** Viability (%), **(B)** apoptosis (%), **(C)** total motility (TM, %), **(D)** progressive motility (PM, %), **(E)** curvilinear velocity (VCL, µm/s), and **(F)** amplitude of the lateral displacement of the sperm head (ALH, µm). Significant differences ($P < 0.05$) among experimental groups are noted with different lowercase letters (a, b). The same seven males were analyzed in each experimental group.

2.8 Statistical analysis

Prism 8 (GraphPad Software, San Diego, CA, USA) was used to perform statistical analysis. Differences were considered significant when p values were < 0.05. Data were analyzed by the Kolmogorov–Smirnov and Levene tests to verify the normality and homogeneity of variances, respectively. Data were evaluated by one-way ANOVA (normally distributed data) or Kruskal–Wallis (nonnormally distributed data). The results are expressed as the mean ± S.E.M. Pearson and Spearman correlation coefficients between seminal parameters were calculated. The reliability of the scoring systems was evaluated by the correlation coefficient

(R squared). The number of asterisks (*) indicates the level of significance: one asterisk (*) indicates $P < 0.05$, two asterisks (**) indicate $P < 0.01$, and three asterisks (***) indicate $P < 0.001$.

3 Results

3.1 Multiparametric sperm quality analyses

The viability of the samples from the nonbreeding season, those treated with hydrogen peroxide (positive control) and those frozen-thawed samples decreased significantly ($P < 0.05$) compared to BS sperm (Figure 1A). However, the viability of the sperm in the NBS and positive control samples were not significantly different (Figure 1A). Similarly, frozen-thawed samples did not show significant differences from the positive control samples (Figure 1A). On the other hand, as expected, apoptosis increased significantly ($P < 0.05$) in NBS, frozen-thawed and positive control samples in comparison with BS (Figure 1B). Moreover, apoptosis decreased significantly ($P < 0.05$) in frozen-thawed samples compared to the positive control (Figure 1B). Nevertheless, apoptosis was not significantly different between the NBS and frozen-thawed samples (Figure 1B).

Concerning motility parameters, TM decreased significantly ($P < 0.05$) in the positive control and frozen-thawed samples in comparison with BS (Figure 1C). In contrast, TM did not show a significant difference between the BS and NBS samples (Figure 1C). Moreover, TM decreased significantly ($P < 0.05$) in the positive control compared to the frozen-thawed samples (Figure 1C). PM decreased significantly ($P < 0.05$) in the frozen-thawed samples and positive control compared to the BS samples (Figure 1D). Moreover, PM did not show a significant difference between the positive control and frozen-thawed samples (Figure 1D). Likewise, there was no significant differences between the breeding and nonbreeding season samples (Figure 1D).

VCL decreased significantly ($P < 0.05$) in the NBS, frozen-thawed and positive control samples in comparison with BS (Figure 1E). However, VCL did not show a significant difference between the NBS and frozen-thawed samples (Figure 1E). However, VCL increased significantly ($P < 0.05$) in frozen-thawed samples compared to positive control samples (Figure 1E). ALH decreased significantly ($P < 0.05$) in the NBS, positive control and frozen-thawed samples in comparison with BS (Figure 1F). Moreover, ALH decreased significantly ($P < 0.05$) in the positive control and frozen-thawed samples in comparison with NBS (Figure 1F). Nevertheless, ALH decreased significantly ($P < 0.05$) in the positive control compared to the frozen-thawed samples (Figure 1F).

3.2 Redox sperm status

The CRDR- and CRG-positive sperm populations were characterized by flow cytometry (Figures 2A, B). The number of CRDR-positive cells increased significantly ($P < 0.05$) in BS compared with NBS, positive control and frozen-thawed samples (Figure 2A). However, the NBS samples did not show significant differences between the positive control and frozen-thawed samples in CRDR-positive cells (Figure 2A). In contrast, the number of CRDR-positive cells decreased significantly ($P < 0.05$) in the positive control samples in comparison with the frozen-thawed samples (Figure 2A). Nevertheless, the number of CRG-positive cells increased significantly ($P < 0.05$) in the positive control compared to the BS and frozen-thawed samples (Figure 2B). In contrast, BS did not show significant differences from the NBS and frozen-thawed samples (Figure 2B). Both MDA generation and the high DNA fragmentation index increased significantly ($P < 0.05$) in the positive control samples in comparison with the fresh samples from breeding and nonbreeding seasons (Figures 2C, D). Likewise, the MDA concentration and high DNA fragmentation index did not show significant differences from the BS and NBS fresh samples (Figures 2C, D). Moreover, the MDA concentration was not significantly different between the frozen-thawed and positive control samples (Figure 2C), while the high DNA fragmentation index increased significantly ($P < 0.05$) in the positive control samples in comparison with the frozen-thawed samples (Figure 2D).

Different correlations among quality parameters were found when we analyzed sperm viability, apoptosis, MDA concentration and motility and certain kinetic parameters when comparing with CRDR and CRG (Figure 3). Some correlations were also found among CRDR- and CRG-positive cells in terms of the other sperm quality parameters. On the one hand, CRDR-positive cells presented significant positive correlations with viability (R^2=0.69), motility and kinetic parameters (TM (R^2=0.71), PM (R^2= 0.75), VCL (R^2= 0.77), ALH (R^2= 0.76)) ($P < 0.001$) (Figure 3). On the other hand, CRDR-positive cells presented significant negative correlations with apoptosis ($R^2 = -0.81$) ($P < 0.001$) and MDA concentration ($R^2 = -0.61$) ($P < 0.01$) (Figure 3). CRG-positive cells presented a significant positive correlation with MDA concentration ($R^2 = 0.40$) ($P < 0.05$) showing an opposite correlation pattern with CRDR probe (Figure 3).

Moreover, the correlations of the two CellROX™ probes with each other have been studied. A significant negative correlation was found between CRDR- and CRG-positive cells ($R^2 = -0.45$) ($P < 0.05$) (Figure 3).

Additionally, with respect to CellROX tests performed on viable sperm population, viable CRDR-positive cells were significantly ($P < 0.001$) increased in fresh compared to the positive control samples (Figure 5A). In contrast, viable CRG-positive sperm were significantly increased in the positive control compared to fresh samples ($P < 0.05$) (Figure 5B).

3.3 CellROX™ localization in sperm samples

The intracellular location of CellROX™ was determined by confocal microscopy. During the breeding season, the middle piece of the sperm cells was stained red by CRDR (Figures 4A, C, 5C, E). However, sperm in the positive control group did not show their middle piece stained red after incubation with CRDR (Figure 5F). Concerning CRG, sperm cells from the positive control sample showed green nuclear staining (Figures 4B, D, 5D, H), while sperm cells from the BS sample did not show nuclear staining (Figure 5G). Furthermore, with respect to the number of stained sperms, the

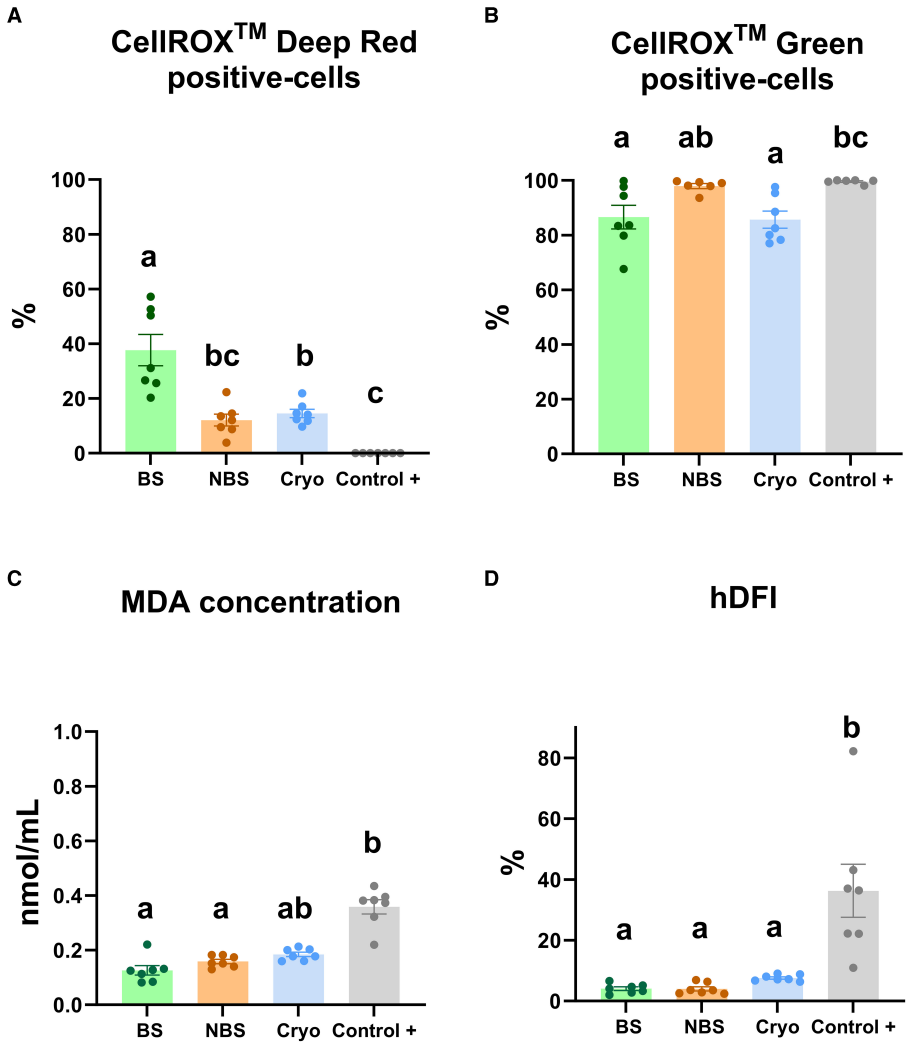

FIGURE 2
Redox status analyses in ram sperm from the four experimental groups using different assays [fresh in breeding season (BS), fresh in nonbreeding season (NBS), positive control treated with hydrogen peroxide (300 μM) (Control +) and frozen-thawed (Cryo)]. **(A)** CellROX™ Deep Red-positive cells (%), **(B)** CellROX™ Green-positive cells (%), **(C)** MDA generation (nmol/mL), and **(D)** high DNA fragmentation index (hDFI, %). Significant differences ($P < 0.05$) among experimental groups are noted with different lowercase letters (a, b). The same seven males were analyzed in each experimental group.

highest number of CRDR-positive cells was registered in the best quality sample (breeding season) (Figures 5C, E), while in the case of CRG, the highest number of positively stained cells was detected in the positive damage control group (Figures 5D, H).

4 Discussion

Redox balance is essential for living systems, including sperm cells (46). The balance between electron loss (oxidation) and electron gain (reduction) is crucial for adequate cellular functionality (11, 12, 47). Therefore, oxidative stress occurs when ROS generation exceeds the protective capacity of the antioxidant mechanisms (11, 12, 46). This can be due to a lack of antioxidant protection or the overproduction of ROS (11, 12, 46). Excessive ROS production may be cytotoxic to sperm, causing a decrease in sperm quality (4, 48). Furthermore, it should be noted that ROS are necessary for the normal physiological functions of sperm and are involved in important signal transduction pathways (40). Moreover, ROS are required for sperm chromatin compaction, providing protection against oxidative DNA damage (49).

Recently, some fluorescent probes, such as CellROX™ Deep Red and CellROX™ Green, have been described as markers of sperm damage, as they can evaluate the presence of specific ROS in different mammalian species (35, 38, 44). CRDR has been considered an ROS marker in ram (35, 50), bull (38, 51) and stallion sperm (44, 52–54). However, contradictory results have been published concerning their correlation with sperm quality in rams (42, 55, 56) and stallions (57, 58). These results could suggest that there may be a species-specific effect, which we have decided to study in depth.

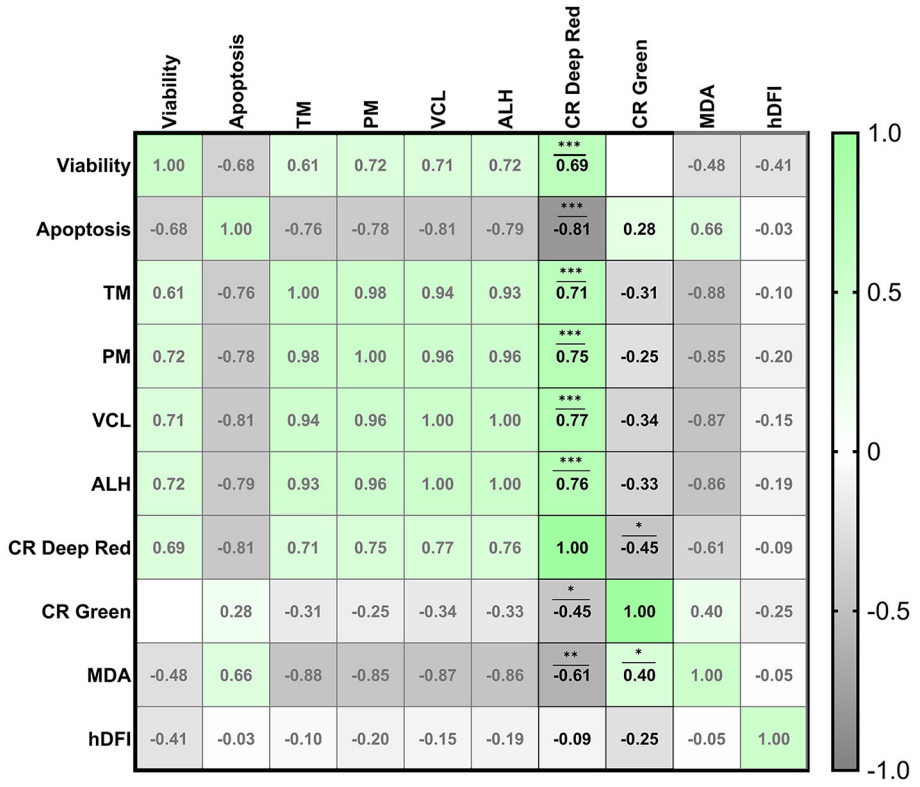

FIGURE 3
Correlation matrix of all sperm quality markers evaluated highlighting the correlations between the CellROX™ Deep Red and CellROX™ Green fluorescent probes. The four experimental groups [fresh samples from breeding and nonbreeding seasons, frozen-thawed and positive control treated with hydrogen peroxide (300 μM)] were included to construct the correlation matrix. Viability (Zombie-negative cells), apoptosis (caspase-3/7-positive cells), TM (total motility), PM (progressive motility), VCL (curvilinear velocity), ALH (amplitude of the lateral displacement of the sperm head), CR Deep Red (CellROX™ Deep Red-positive cells), CR Green (CellROX™ Green-positive cells), MDA (malondialdehyde generation), and hDFI (high DNA fragmentation index). The R squared value between two parameters is noted in each cell. Blue indicates positive correlations, and red indicates negative correlations. The color intensity represents the strength of the correlation between two sperm quality parameters. A total of seven males were analyzed (the same number of samples is required for this analysis). Asterisks show significant correlations among sperm quality parameters. The number of asterisks (*) indicates the level of significance: (*) indicates $P < 0.05$, two asterisks (**) indicate $P < 0.01$, and three asterisks (***) indicate $P < 0.001$.

New markers based on redox status, including deep characterization of ROS probes, could be very useful to analyze sperm quality to optimize ram sperm preservation protocols (cooling and freezing). For this purpose, four experimental groups with different initial sperm qualities were established. These experimental groups included fresh (BS and NBS), frozen-thawed and positive control samples (as a damage control). Afterward, multiparametric analyses of the sperm, including viability, apoptosis, lipid peroxidation, motility and kinetic parameters (VCL and ALH), were applied to corroborate the differences in quality of the previously established experimental groups. As expected, after analyzing the quality of the semen from the different experimental groups, two were of high quality with low levels of oxidative stress (fresh samples in the BS and NBS groups). In addition, two groups with low sperm quality and a high level of oxidative stress (thawed samples provided in the NBS and positive damage control samples) were obtained. However, Mendoza et al. (59) previously described increases in apoptotic markers, such as phosphatidylserine translocation, mitochondrial membrane potential or caspase activation, in samples from the nonbreeding season compared with those from the breeding season. Apoptosis appears to be triggered by oxidative stress, which leads to the activation of ROS generation (60). In addition, we chose the experimental groups with more damage (frozen-thawed and positive control) because they were considered the most suitable to experience oxidative stress and therefore an imbalance in ROS. On the one hand, frozen-thawed samples are subject to oxidative stress due to the drastic temperature changes during the cryopreservation process or the high dilution rates to which they are subjected (61, 62). As previously demonstrated, caspases 3–7 are activated, triggering apoptosis as a consequence of drastic temperature change (cryopreservation and thawing process) as a result of the generated ice crystals, which could result in damage to the sperm (42, 61, 63). On the other hand, the positive control samples were incubated with hydrogen peroxide as described by Soliman et al. (16). Oxidative stress induced by hydrogen peroxide causes a drastic decrease in sperm viability, as several authors have previously demonstrated (16, 64). Furthermore, Pujianto et al. (65) demonstrated that a high concentration of hydrogen peroxide is responsible for oxidative stress as a consequence of excessive ROS production in sperm that causes a decrease in sperm quality, including an increase in cell apoptosis. As expected, our

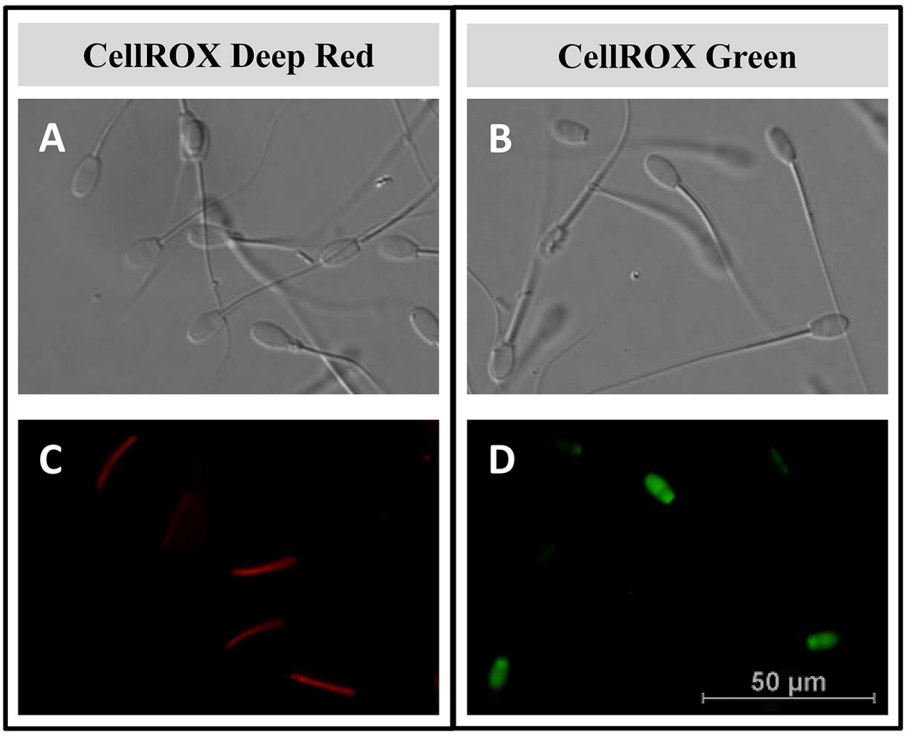

FIGURE 4
Intracellular reactive oxygen species localization by confocal microscopy in ram sperm samples. **(A, B)** Light field image of ram sperm samples. **(C)** Middle piece of the sperm stained red with the CellROX™ Deep Red fluorescent probe. **(D)** Sperm nucleus stained green with the CellROX™ Green fluorescent probe.

results revealed that the fresh BS samples were of the highest quality among the experimental groups. The BS samples showed the highest viability and motility and kinetic parameters (TM, PM, VCL, ALH) and the lowest level of apoptosis (Figures 1A–F) compared to the group in which oxidative stress was induced (positive control samples). Some authors previously described this detrimental effect in oxidative stress-induced samples (16). With respect to the fresh samples inside and outside the reproductive season, while apoptosis significantly increased in the NBS group compared to the BS group, the viability and kinetic parameters increased significantly in the BS compared to the NBS samples (Figures 1A–F). This was reported previously by Kafi et al., who described lower viability in winter than in summer in Karakul rams located in Shiraz (Iran) (66). Moreover, we included cryopreserved sperm samples as a group with intermediate quality that gave values between the fresh and peroxide-treated samples for most of the analyzed parameters. As previously described by several authors and according to our results, cryopreservation induces detrimental effects to sperm quality parameters, including motility and viability (16, 42). To improve sperm quality analyses and confirm the initial differences among the experimental groups in terms of ROS production, the MDA concentration was used as a traditional marker for the assessment of lipid peroxidation and therefore oxidative stress (16). In our results, contrary to previous studies in rams, lipid peroxidation increased significantly in samples assessed after incubation with hydrogen peroxide for 24 h (positive damage control) compared to fresh samples (Figure 2C) (16). This may be because, in contrast to our study, Peris et al. (16) used synthetic oviduct fluid (SOF) as a diluent for both the fresh and peroxide-treated samples. However, we did not observe any differences in lipid peroxidation between the fresh and thawed samples, which is in agreement with the results of Soliman et al. (16). In accordance with previous studies in rams, a similar trend was observed for hDFI, which increased significantly in the positive control samples compared to the fresh samples (16). However, the absence of a difference between the fresh and thawed samples could be explained by the low DNA fragmentation index in rams compared to the other species (67). These results indicate that more accurate ROS tests should be performed to discriminate between fresh samples and cryopreserved samples in rams.

Consequently, after analyzing the differences in sperm quality among the experimental groups, we performed a specific study of two commercial ROS detection probes (CRG and CRDR) for ram sperm quality evaluation.

Our results revealed that CRDR and CRG gave opposite labeling patterns. The number of CRDR-positive cells was higher in fresh BS samples than in frozen-thawed and positive control samples (Figure 2A). Because of this, some significant positive correlations were found between CRDR-positive cells and positive markers of sperm quality, including motility and viability (Figure 3). Furthermore, attending to the viable sperm population, a higher expression of CRDR-positive cells was observed in fresh samples compared to peroxide-treated samples (Figure 5A). In accordance with our results, some authors have previously hypothesized that CRDR is a positive quality

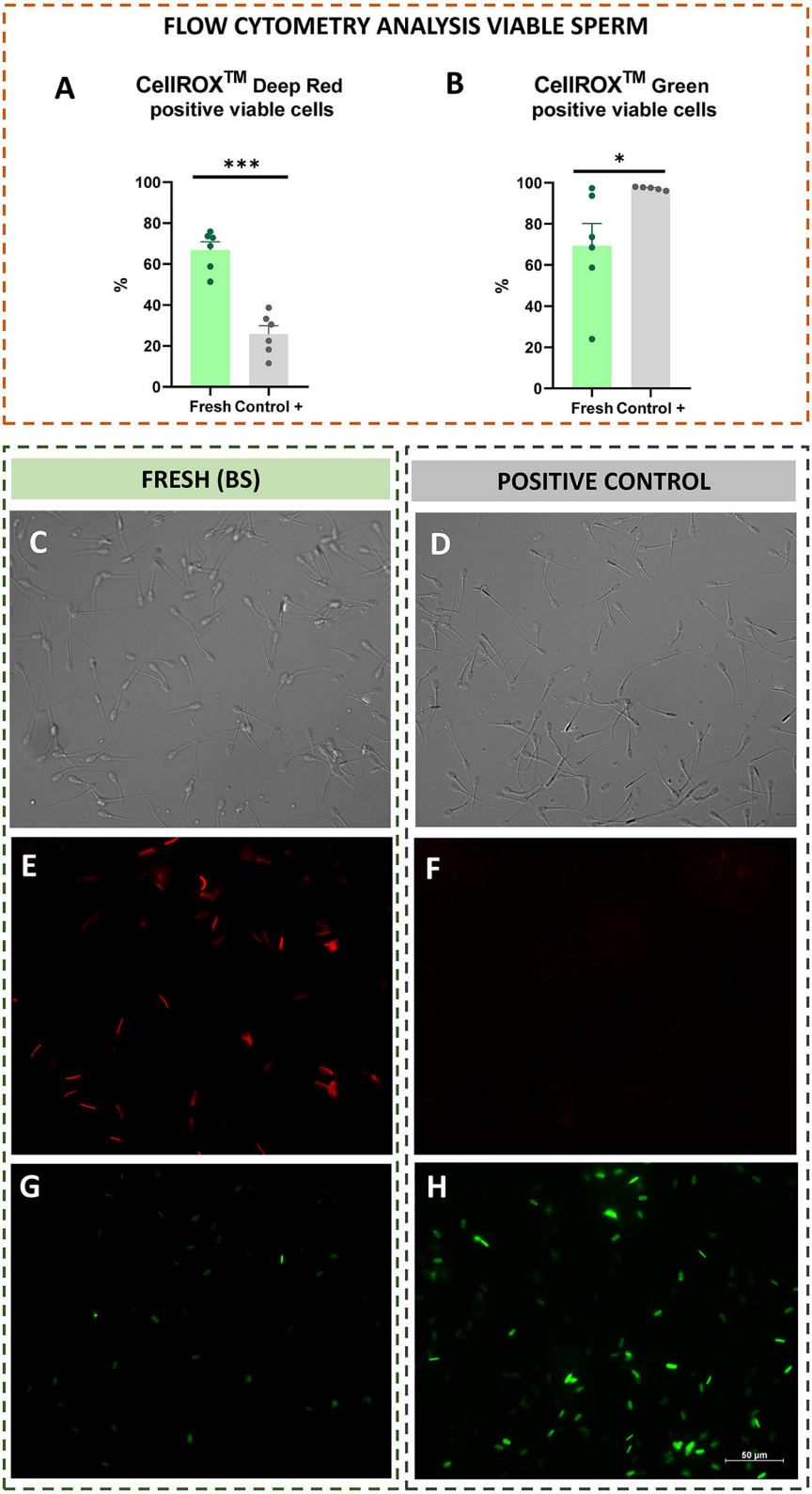

FIGURE 5
Flow cytometry analysis from viable sperm (CellROX™ Deep Red and Green), and intracellular reactive oxygen species localization by confocal microscopy in fresh (breeding season) and positive control [treated with hydrogen peroxide (300 µM)] ram sperm samples. **(A)** CellROX™ Deep Red positive viable sperm. **(B)** CellROX™ Green positive viable sperm. **(C)** Light field image of the BS sample. **(D)** Light field image of the positive control sample. **(E)** CellROX™ Deep Red fluorescent probe in the BS sample (middle piece stained red). **(F)** CellROX™ Deep Red fluorescent probe in the positive control sample (middle piece not stained). **(G)** CellROX™ Green fluorescent probe in the BS sample (nucleus not stained). **(H)** CellROX™ Green fluorescent probe in the positive control sample (nucleus stained green). The number of asterisks (*) indicates the level of significance: one asterisk (*) indicates $P < 0.05$, and three asterisks (***) indicate $P < 0.001$.

marker in stallion sperm (57, 58). Davila et al. (57) have previously included the CRDR probe in their studies, where they demonstrated that it measures superoxide production. Their studies showed that an increased concentration of superoxide anion in mitochondrial sperm was associated with intense mitochondrial activity. Moreover, these authors also observed positive correlations with motility and membrane integrity in stallion sperm. This could be explained by the fact that the blocking electron transfer caused a disruption in production of superoxide anion, which is the target molecule of CRDR. In addition, other studies have shown that 1% to 2% of superoxide anion (O_2) used in the electron transport chain is not completely reduced, generating the O_2 (68). These observations are in accordance with our results, where sperm samples affected by cryopreservation or peroxide treatment suffered a decrease in superoxide anion production, triggering a decrease in CRDR labeling. For this reason, considering that the CRDR probe mainly detects this anion, an increase in the O_2 is associated with high mitochondrial activity rather than oxidative stress. On the other hand, CRG has been described as a ROS marker in humans (69) and bulls (36, 70). Unlike CRDR, the number of CRG-positive cells was higher in the frozen-thawed and positive control samples than in the breeding season samples (Figure 2B). Moreover, some significant positive correlations were found between CRG-positive cells and negative sperm quality markers, such as apoptosis and MDA (Figure 3). Likewise, attending to the viable sperm population, a higher expression of CRG-positive cells was also observed in peroxide-treated samples compared to fresh samples (Figure 5B). Our results were in accordance with previous works that demonstrated an increase in the percentage of CRG-positive cells after exposure to an oxidative environment (36). Moreover, de Castro et al. demonstrated that this probe was a very sensitive marker in bull sperm (36). They observed a dose-dependent effect, and CRG fluorescence intensity increased with increasing concentrations of hydrogen peroxide (36). However, the CRG probe has not yet been studied in ram sperm. According to the manufacturer's instructions, and as described by Riley et al. (69) in their study, the CRG probe has weak basal fluorescence that increases as the sample becomes oxidized (Supplementary Figure 4). Based on our results, it is necessary to include all the available techniques for CellROX characterization, including intracellular location, which is a crucial factor (40). In addition to the opposite labeling pattern, our studies revealed different intracellular locations of the CellROX fluorescent probes as studied by confocal microscopy. CRDR fluorescence was located in the middle piece of the sperm (Figure 4C) as previously published by Rodrigues et al. (35). These authors found that only sperm subjected to oxidative stress showed red fluorescence. Contrarily, our study only detected red fluorescence in fresh samples (35). This inconsistency may be due to the solvent used for the oxidative stress induction agent since Rodrigues et al. (35) used TALP medium, which has antioxidants in its composition and could had had some interaction during samples incubation.

While in our study we used hydrogen peroxide, Rodrigues et al. used ferrous sulfate and sodium ascorbate (35). This could mean that the medium used by Rodrigues et al. did not induce oxidative stress as effectively, since in our study CRDR fluorescence correlated with the most parameters of seminal quality and oxidative stress. This could be due to the antioxidant effect of Tyrode's Albumin Lactate Pyruvate (TALP) medium that could neutralize the effect of the agents used by Rodrigues et al. to induce oxidative stress. The components of the TALP medium contain pyruvate and lactate, components with an antioxidant effect and promoting mitochondrial functionality as previously described (71, 72). In addition, mitochondria are located in the midpiece of the sperm, which is the location of the highest metabolic activity in viable sperm (73). Therefore, the localization of CRDR fluorescence in the midpiece of the fresh samples could be a useful indication of proper sperm functionality. On the other hand, we observed that CRG fluorescence was located in the sperm nucleus in positive control samples (Figures 4D, 5H). In accordance with our results, CRG fluorescence was observed in the nuclei of bull sperm subjected to hydrogen peroxide treatment (36).

These results demonstrated that CRG and CRDR presented an opposite labeling pattern that was corroborated by fluorescence microscopy that showed different localization of the probes. While CRDR fluorescence was observed in the middle piece of the sperm from the fresh samples with superoxide anion production (intense mitochondrial activity sperms), CRG was found in the nuclei of the sperm in the positive control samples and was associated with DNA damage. These results could be explained by the fact that CRG is a DNA dye, and upon oxidation, it binds to DNA; thus, its signal is localized primarily in the nucleus and mitochondria. This fact was previously demonstrated by in a ROS localization study in *Solea senegalensis* sperm (40). In contrast, the signals from CRDR were localized in the cytoplasm. These results could hypothesize that in sperm, the localization of ROS is a crucial factor in sperm quality determination, as in previous studies performed in fish (40). Furthermore, we can conclude from our results that CellROX probes could be more discriminatory tests for the assessment of oxidative stress than lipid peroxidation measurements or DNA fragmentation. This is because, unlike the evaluation of lipid peroxidation, the use of CRDR and CRG allowed us to establish differences between the two experimental groups (frozen-thawed and peroxide-treated samples). Furthermore, we could consider that CRDR is a more accuracy test compared to CRG according to the highest correlations observed with the semen quality parameters studied (Figure 3). In addition, differences in the significance of both tests were also observed in viable sperm: CRDR ($P < 0.05$) shows a high level of significant differences between the two extreme groups compared to CRG ($P < 0.001$). This work allowed us to characterize CRG and CRDR with fluorescence assays for use as innovative and accurate tools in seminal assessment by focusing on the redox status of ram sperm cells. In this study, we observed the relationship between intracellular ROS location and the effect of ROS on sperm cells. CRDR fluorescence was located in the sperm mitochondrial region (middle piece) in the fresh sperm samples, which could be associated with high mitochondrial activity according to viability and motility analyses performed in this work. On the other hand, CRG fluorescence was mostly located in the cell nuclei in the positive control and frozen-thawed samples, which could be related with sperm damage, due to its positive

correlation with detrimental sperm quality parameters such as MDA concentration.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by Animal Care and Use Committee of the University of León (Spain) ÉTICA-ULE-050-2022. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

CP-M: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. LA-L: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Visualization, Writing – review & editing. MA: Conceptualization, Project administration, Resources, Writing – review & editing. MN-M: Conceptualization, Investigation, Methodology, Formal analysis, Visualization, Writing – review & editing. RM-G: Conceptualization, Investigation, Methodology, Formal analysis, Visualization, Writing – review & editing. CS-Ú: Conceptualization, Resources, Writing – review & editing. PP: Conceptualization, Resources, Writing – review & editing. LA: Conceptualization, Funding acquisition, Project administration, Resources, Writing – review & editing. MR: Conceptualization, Methodology, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1342808/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Experimental groups used in the experiment: fresh samples (breeding season (BS) and non-breeding season (NBS)), frozen-thawed provided from NBS samples and positive control group provided from BS samples (oxidative stress induction by hydrogen peroxide).

SUPPLEMENTARY FIGURE 2
Emission spectra of the fluorochromes provided by the commercial manufacturer: Zombie Violet™ Fixable Viability Kit, CellEvent™ Caspase-3/7 Green Detection Reagent, CellROX™ Deep Red, and CellROX™ Green.

SUPPLEMENTARY FIGURE 3
Representative cytograms of the assays reported in the present study. **(A)** Dot plot showing the region gated corresponds to sperm. **(B)** Events gated in **(A)** are now plotted against SSC-H and SSC-A for select single cells, and this region was further used to set the remaining populations of interest. **(C)** Unstained control for Zombie Violet™, Caspase-3/7 Green, CellROX™ Deep Red, and CellROX™ Green. **(D)** FlowJo analyses for viability (Zombie Violet™). **(E)** FlowJo analyses for apoptosis (Caspase-3/7 Green). **(F)** FlowJo analyses for mitochondrial functionality (CellROX™ Deep Red). **(G)** FlowJo analyses for ROS content (CellROX™ Green).

SUPPLEMENTARY FIGURE 4
FlowJo analyses of CellROX™ Green by flow cytometry. **(A)** Sperm sample without the fluorochrome CellROX Green. **(B)** Fresh sample from the breeding season. **(C)** Sample from the positive control group (hydrogen peroxide-treated).

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Systematic review and meta-analysis of cryopreserved bovine sperm assessment: harnessing imaging flow cytometry for multi-parametric analysis

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Cryopreservation of sperm is an essential technique in assisted reproduction in cattle. The objective of the study was to systematically review and synthesize the literature on bull semen quality evaluation based on the comparison of morphological and metabolic parameters of cryopreserved bovine spermatozoa such as DNA integrity, mitochondrial status, plasma membrane alterations, total motility, and morphology (% of abnormal cells). The electronic databases PubMed, Web of Sciences, Scopus, and Google Scholar were searched up to December 2023. Studies and references were included if they reported the following parameters: DNA integrity, mitochondrial status, plasma membrane alterations, total motility, and morphological aberrations (% of abnormal cells) for conventional cryopreserved bovine spermatozoa. After an electronic search, out of 1,526 original studies, only 40 were included in the meta-analysis. Standardized mean differences (SMD) with 95% confidence intervals were estimated for the chosen studies, and a meta-analysis was performed using a random effects model. The tau-squared (tau^2) and inconsistency index (I^2) quantified heterogeneity among different studies. The regression analysis for the evaluated parameters showed a positive correlation between mitochondrial membrane potential (MMP), total motility, and abnormal morphology and a negative correlation between DNA fragmentation index (DFI) and total motility and MMP. Moreover, subgroup analysis demonstrated similar associations for dairy and non-dairy bull breeds, albeit with lower I^2 values. The presence of publication bias was confirmed by Egger's test, except for the MMP parameter. A multi-parametric analysis of morphological and metabolic parameters can address the existing limitations of cryopreserved bovine spermatozoa quality assessment. Combining imaging flow cytometry (IFC) with standardization of sperm pre-processing and optimization of the experimental protocols may help to differentiate sperm from cellular debris and cytoplasmic droplets of similar size and alleviate limitations demonstrated by conventional sperm analysis.

KEYWORDS

sperm, cryopreservation, meta-analysis, flow cytometry, bovine semen quality, imaging flow cytometry, tetramethylrhodamine methyl ester, image-based sorting

1 Introduction

There is an urgent need to increase the fertility of livestock species and improve the efficiency of food-producing animals to overcome the growing demand for food and animal protein access. Cryopreservation of sperm is an essential aspect of breeding in agriculture (1–4), allowing the storage of selected gametes at liquid nitrogen temperature and the preservation of the genetic material of the cells with their structural and functional integrity (5, 6). Artificial insemination relies on frozen-thawed bull semen and has the greatest impact on the genetic breeding of cattle (7). The sperm quality assessment is crucial for the success of artificial insemination and breeding in cattle (8). Despite early reassurances that this temperature arrests all metabolic processes and sperm quality would not be affected (9), concerns have recently arisen regarding the functionality and quality of cryopreserved sperm after long-term storage (1, 10). In general, 40–50% of the sperm does not survive the cryopreservation procedure (6). Post-thawed spermatozoa, particularly in cattle, are very sensitive to temperature changes (11) and rapidly decline in viability after post-thawing (12–15). However, conventional sperm analysis is time-consuming, and due to the absence of standardized protocol and subjectivity of the microscopic assessment, results from multiple laboratories are highly variable (16–21). The flow cytometric approach has the advantage of evaluating multiple cellular features of spermatozoa; however, it does not allow evaluation of the morphological parameters (22–24).

As of today, there is no single *in vitro* sperm quality assessment test that allows accurate evaluation of the quality of sperm and prediction of sperm fertilizing potential (25). The most promising tests available include sperm viability assessment. With the rise of new technologies such as computer-assisted sperm analysis (CASA) systems and flow cytometry, subsequent studies started to pay closer attention to combining the morphological features and metabolic parameters of sperm cells to assess sperm quality for proper fertilization. Changes in the plasma membrane integrity, mitochondrial potential, chromosome integrity, acrosome, and axoneme structures (26–29) may decrease sperm viability and lead to low fertility (30). During past decades, meta-analyses were used to investigate the possible associations between morphological sperm parameters and the overall fertility state of bulls (31, 32).

This systematic review and meta-analysis aimed to compare studies describing the major morphological and metabolic parameters of cryopreserved bovine spermatozoa to evaluate their relationship to each other and state after cryodamage. We are also discussing imaging flow cytometry as a possible hybrid technology for multi-parametric analysis, and providing an example of multi-parametric morphological and functional analysis of bovine sperm.

Abbreviations: CASA, computer-assisted sperm analysis; CI, confidence interval; DFI, DNA fragmentation index; HMMP, high mitochondrial membrane potential; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; SD, standard deviation; SDF, sperm DNA fragmentation; SE, standard error; SMD, standardized mean difference; TMRM, tetramethylrhodamine methyl ester.

2 Materials and methods

2.1 Search strategy

An electronic search of Google Scholar, MEDLINE (PubMed), Scopus, and ISI Web of Science databases was performed up to December 1, 2023. The combination of the following keywords and search terms were used: "bull," "semen evaluation," "bovine sperm" AND "parameter," "sperm," "DNA fragmentation," "morphology," "mitochondria," "viability" AND "assessment" OR "cryopreservation," and "thawed." The search was conducted by two independent reviewers, initially resulting in 1526 articles. The search process and results are depicted in the flow diagram. Additionally, the reviewers manually checked reference lists of relevant articles and reviews in search of potentially eligible studies.

2.2 Inclusion and exclusion criteria

The articles were excluded and included in the first case based on their titles and abstract contents. In the second case, full-text articles were indexed, and criteria targeted a paper's title and complete contents. First, full versions of the articles went through the exclusion criteria, which included (1) non-bovine sperm, (2) no required parameters, (3) pregnancy rates research, (4) evaluation of extender effect, (5) cryoprotectant efficiency, (6) non-cryopreserved semen, (7) cell signaling research, (8) proteomic or lipidomic analysis. Additionally, we excluded preprints and data from subgroups of treated sperm from studies that had measured post-thawing parameters after applying some treatment. Then, the remaining articles were checked according to inclusion criteria, which required (1) target parameters to have mean and standard deviation data, (2) detailed quantitative data on viability and some of the next parameters: morphology, mitochondrial potential, motility and DNA fragmentation, (3) sperm sample origin, (4) data on hours after thawing, and (5) sample sizes. Selected articles should have at least one of the parameters studied for bovine semen quality along with the viability or, in some cases, given as the plasma and acrosomal membrane integrity or plasma membrane integrity. The sample size was taken as the number of bulls whose semen was evaluated, and the mean and standard deviation values should be given for all the bulls, not for their ejaculates or batches. When the samples are analyzed and divided into groups (fertility differences, age, bulls' specialization, etc.), each subgroup should be used for the analysis as it is without further mathematical calculations for deriving common values for the groups. These measurements were undertaken to avoid biased and erroneous evaluations of collected data.

2.3 Data collection and data items

Final data were obtained from all the eligible studies, including the author's name, year of publication, sample size, bull breed, study design, description of the parameters, and measurable methods. The search results were extracted from scientific databases and uploaded in EndNote X9. Measurements of post-thawing sperm parameters [motility, DNA fragmentation index (DFI), viability, morphology,

mitochondrial membrane potential (MMP)] were extracted into the data table and grouped by their fertility rates, breeds, and locations. The second reviewer downloaded full-text articles of all search results and used system and software indexation to apply exclusion and inclusion criteria. The same parameters were then transferred to the data table. Additional information on the studies' characteristics can be found in Supplementary Tables S1, S2.

2.4 Statistics and meta-analysis

Sperm viability was taken as a control parameter since it is one of the important fertility factors, presenting the number of live cells. The viability was estimated to have a mean of 51.720 throughout 59 groups within the studies that were included in the meta-analysis. The minimum and maximum values were 26.13 and 84.43, respectively, with a 95% confidence interval (CD) from 48.466 to 54.794 inclusively. In several analyzed studies, results were given as mean and standard error (SE) for each of the bulls or their ejaculates (presented as straws or batches). Therefore, we calculated the standard deviation (SD) and the sample size (n) according to the pooled standard deviation formula (33). Data parameters were extracted from the source csv file and exported to Pandas DataFrame. Pandas v.2.0 and Matplotlib v.3.7.2 were used to study the data and check for preliminary correlations and outliers. Further, DataFrame columns with desired parameters were transformed into NumPy arrays to fit the data using matrix operations, yielding linear regression lines and planes describing the variables' relationship.

Heterogeneity between studies was evaluated using Q test and I^2 statistics (34) and tau squared. The latest was estimated through the restricted maximum likelihood procedure, which shows particular robustness in the random effects model. In each category with more than five high-quality studies, subgroup analysis and univariate regression were conducted to explore sources of heterogeneity. As $I^2 > 50\%$ was expected between studies, all analyses were performed using the random effect model (35). As a part of the statistical analysis, we conducted a traditional and multiple regression analysis (36). Both were estimated in Python v.3.8 using the NumPy v.1.22.0 and Seaborn library v.012, including plotting and scientific calculations. IBM SPSS vs. 29.0 (IBM, United States) and Python Meta package with supporting libraries for data analysis (Pandas, NumPy) were used to analyze the available data, including linear regression, multiple linear regression, estimation of publication bias, and subgroup analysis. In the calculations, we used weighting techniques to approximate the individual effects of each study.

2.5 Sensitivity and subgroup analysis

The sensitivity analysis was performed by using a leave-one-out method and re-evaluating the effect sizes of the studies. Then, outlier studies whose confidence interval values did not coincide with the confidence interval of pooled effect sizes were removed. For further investigation of heterogeneity within studies, subgroup analysis was conducted based on fertility and the origin of breed data. Cattle breeds may significantly differ in metabolic parameters (37). According to the source of breeds, bulls mainly included dairy and non-dairy groups. The dairy breeds were Holstein, Holstein Friesian, Estonian Holstein, Brown Swiss, Swedish white and red, Sahiwal, Finnish Ayrshire, and Norwegian Red. The non-dairy group included beef bulls of the following breeds: Blonde d'Acquitaine, Limousin, Red Angus, Tropical Montana, Pinzgau, Japanese Black, Belgian Blue, Hereford, and Charolais; dual-purpose included Simmental, Senepol, Nellore, and *Bos Indicus* cattle.

2.6 Publication bias

Given the difficulty of correcting and detecting publication bias, we assessed data by measuring funnel plot asymmetry. Funnel plots of primary outcomes were visually and formally evaluated with Egger's test (38) with $p < 0.05$, indicating significant publication bias.

2.7 Imaging flow cytometry of bovine sperm

Semen was collected from Kazakh White-headed breed bulls from qualifying ejaculates (motility >60%, evaluated with CEROS II sperm-analysis CASA system (Hamilton Thorne, United States); concentration > 0.5 billion/mL, evaluated by photometry using FEK-M (RF) instrument; volume > 1 mL), diluted with OptiXcell® (IMV Technologies, France). Diluted semen was cooled, equilibrated, and filled in 0.5 mL straws for cryopreservation in liquid nitrogen. OptiXcell® is advantageous compared to other yolk-free commercial sperm extenders (39). Chemicals, including tetramethylrhodamine methyl ester (TMRM), DMSO, and Hoechst 33342 dye, were purchased from Sigma-Aldrich (United States).

Imaging flow cytometry analysis was performed using Imagestream X Mark II instrument (Amnis-Cytek, United States) equipped with combination of lasers (405, 488, 561, and 640 nm) and objectives as described elsewhere (40). Shortly, a semen sample straw was transferred from −86°C and thawed quickly in a water bath at 37°C as preparation for further analysis. TMRM (Sigma-Aldrich, United States) was kept as a stock solution in dimethyl sulfoxide (DMSO) at 20 mmol/L, aliquoted and stored at −20°C. When the sample was in the water bath, TMRM was added to the sperm at the final concentration of 5–100 ng/mL, and the sample was incubated in the dark for 15 min at room temperature. Additional staining with Hoechst 33342 dye (concentration 10–100 nm) was done before the acquisition of the sperm sample with Imagestream. Debri and multiple events were excluded by sequential gating with IDEAS (Amnis-Cytek, United States) software.

3 Results

3.1 Results of search

The detailed process of the data literature search is depicted in Figure 1. Overall, 1,526 articles were identified as a result of a web search. After the duplicated removal of duplicates, only 863 studies were considered unique. Finally, exclusion and inclusion criteria were

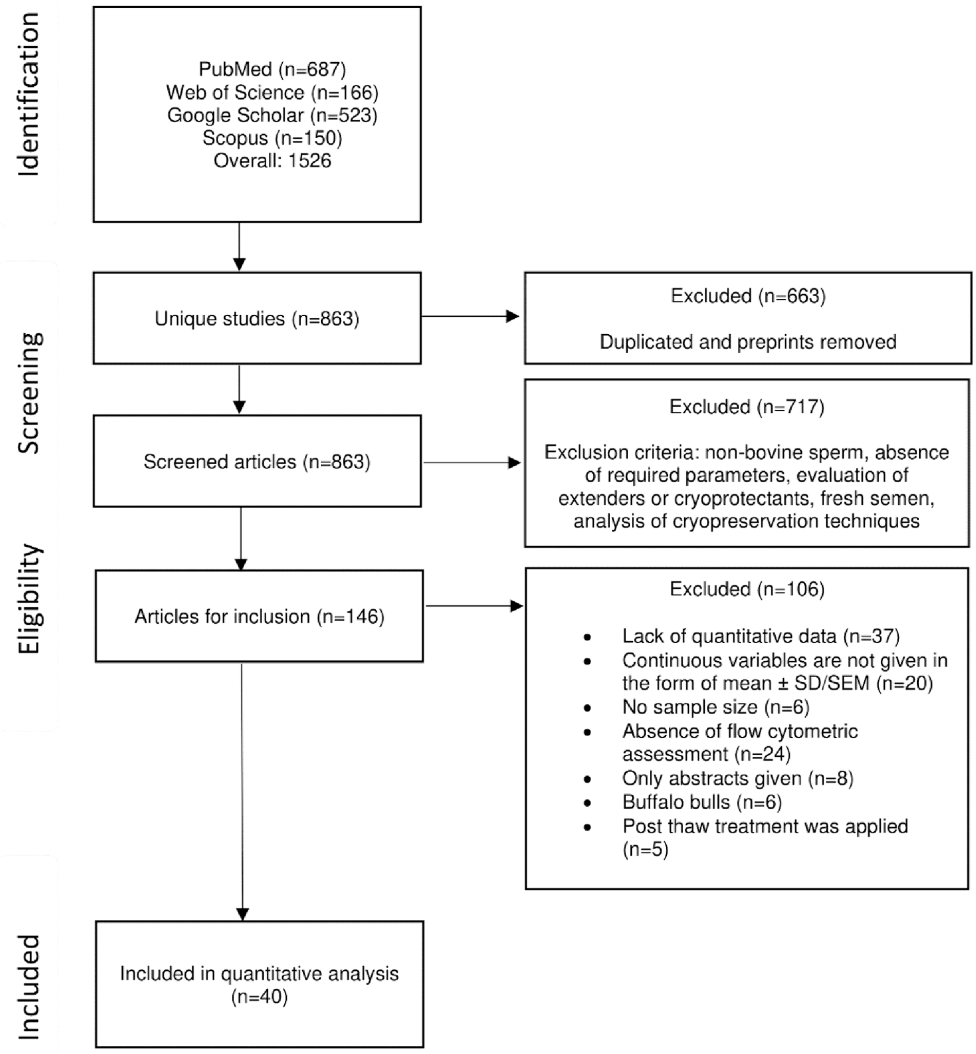


FIGURE 1
Flow diagram of study selection.

applied for a more thorough investigation. Eventually, 40 studies were found eligible and were included in our study.

3.2 Studies characteristics

In total, sperm samples obtained from 972 bulls are included in this meta-analysis from 40 original studies. The studies mainly belonged to three breed origin groups: Northern Europe, Western Europe, and others. Breeds included in the last group are Nellore (beef breed; India), Pinzgau (a triple-purpose breed, raised for meat, milk, and draught use from Austria-i.e., Western Europe), Tropical Montana (beef cattle, Brazil), Senepol (beef and dairy), American Brahman (beef cattle), and unmentioned ones. Moreover, the studies were characterized by their sperm quality assessment methods: flow cytometry and microscopy. Detailed information on methods and stains used for sperm evaluation is shown in Supplementary Table S2.

3.3 Pooled effect sizes

Overall, data was collected for the following sperm quality parameters: viability (control), abnormal morphology, total motility, DNA fragmentation index (DFI), and mitochondrial membrane potential (MMP). Heterogeneity was assessed using the I^2, tau^2, and H-statistics. Tau^2 and I^2 are our primary metrics in the assessment of heterogeneity. I^2 represents the percentage of total variation across studies due to heterogeneity (41). Tau^2 is the estimate of the variance of the effect sizes. A larger value indicates greater heterogeneity, and the greater deviation from 0 suggests the use of a random effects model and greater standard deviation in the true effect sizes. H is the square root of the tau^2 value divided by its degree of freedom (34, 42). For all parameters analyzed, the I^2 values were >78.1%, and tau^2 ranged from 0.453 to 12.491 (Table 1), which required using the random effects model.

Figure 2 presents Galbraith plots of this meta-analysis, illustrating the confidence intervals (dotted lines) of various studies alongside the

TABLE 1 Summary of the heterogeneity assessment and sensitivity analysis.

		Effect size			Heterogeneity	
Parameter		Pooled SMD	95% CI	*p*-value	I^2 (%)	Tau^2
DFI	Original	−5.699	[−7.025; −4.374]	<0.01	95.0	12.491
	No outliers	−4.103	[−4.873; −3.334]	<0.01	87.2	3.220
MMP	Original	−0.489	[−1.173; 0.195]	0.160	95.0	3.720
	No outliers	−0.401	[−0.707; −0.095]	<0.01	71.0	0.453
Morphology	Original	−3.212	[−4.052; −2.285]	<0.01	90.8	3.781
	No outliers	−2.615	[−3.250; −2.002]	<0.01	83.3	1.822
Motility	Original	0.359	[−0.750; 1.493]	<0.01	97.9	12.162
	No outliers	0.571	[0.247; 0.936]	<0.01	77.8	0.769

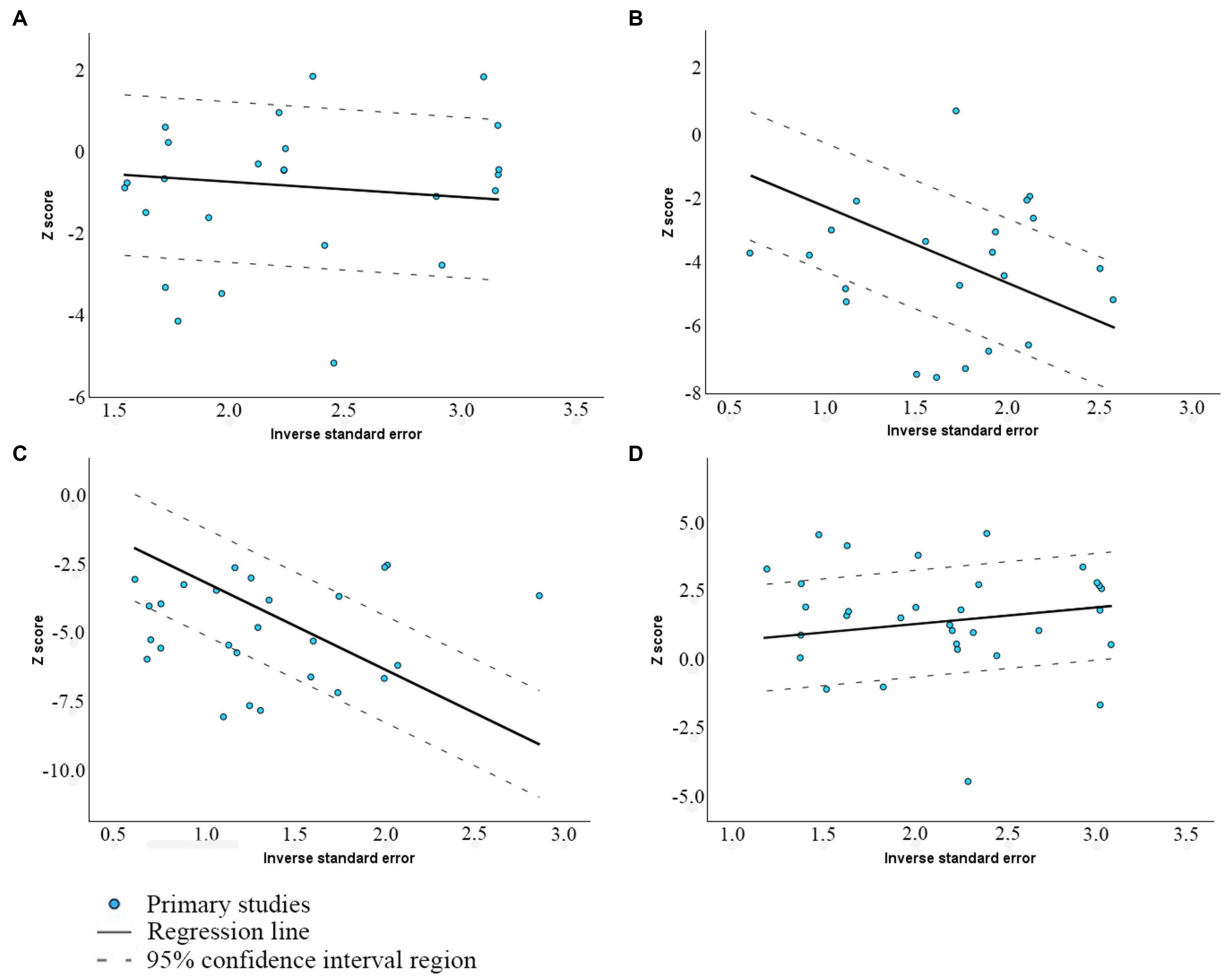


FIGURE 2
Galbraith plot of the association between viability and **(A)** MMP; **(B)** Abnormal morphology; **(C)** DFI; and **(D)** Total motility.

linear regression line, allowing for the identification of potential outliers. MMP and total motility regression lines have neutral to moderate positive slopes, suggesting that the source of the heterogeneity might come from differences at the stages of sampling, analysis, or random variations, which attests to homogeneity. The Galbraith plots for abnormal morphology and DFI show a negative trend, suggesting insufficiency of sample sizes in studies and significant standard error, increasing as the study size decreases.

3.4 Sensitivity analysis

The leave-one-out method identified studies that significantly influence the effect size estimates. Outliers were selected by both their individual and pooled confidence intervals. Removal of the outliers resulted in a decrease in the heterogeneity without affecting the overall results; however, it left the level of heterogeneity still high (>50%). Original effect sizes and ones with removed outliers are depicted in

Table 1. Corresponding graphs for the studies without outliers are provided in Supplementary Figure S1.

3.5 Subgroup analysis

A detailed subgroup analysis was performed to study the sources of heterogeneity observed in the meta-analysis. The subgroup analysis was delineated by breed type, Dairy or Beef/Mix/Dual, which provides a more specific look into the variances across different genetic backgrounds. The effect sizes and heterogeneity measures for each parameter and subgroup are comprehensively presented in Table 2. The data indicates differences between the Dairy and Non-Dairy (Beef/Mix/Dual) subgroups across all parameters, with notably distinct effect sizes and levels of heterogeneity, explainable by smaller study sizes.

3.6 Linear regression analysis

The relationship between the individual parameters was also evaluated and illustrated via linear regression plots. We used it as the first stage in assessing the value and influence of independent parameters on each other. DFI-MMP, Motility-MMP, and Morphology-MMP models yield statistically significant results, suggesting the presence of general trends even in high heterogeneity settings. Further, multiple regression provides better intuition on the relation between the parameters. The plots in Figure 3 show the association between independent parameters: MMP, morphology, DFI, and total motility. Data points are represented by mean values of parameters in %. The statistical significance of the models was evaluated using F-statistics and R^2 values (Table 3).

3.7 Multiple regression analysis

Regression analyses were conducted to identify the relationship between the independent parameters and factors influencing the heterogeneity of studies. Observations from a meta-analysis suggest that MMP and motility data are less biased and might provide additional insights into the relationship between viability and fertility. We specified one dependent variable (viability) and two independent variables (MMP, motility) to model the relationship in 3D space. It is expected that the viability will be directly proportional to MMP and motility, and multiple regression analysis helped to confirm the assumptions in the presence of strong variations across studies. We selected studies with data for required variables and estimated the regression plane using matrix operations with NumPy and Scipy. The *Z*-axis of the 2D plane was optimized using a least squares solution, and then the plane was plotted using Matplotlib. As shown in Figure 4, motility has a slightly stronger effect on viability since the plane's slope is steeper than MMP. MMP also positively correlates with viability.

3.8 Publication bias

Egger's regression-based test for publication bias yielded significant results for several sperm quality parameters, indicating potential bias ($p < 0.01$ for DFI and morphology; $p = 0.012$ for motility) (Table 4). However, for MMP, results suggest a lack of publication bias ($p = 0.088$). These interpretations are visually supported by the funnel plots with outliers presented in Figure 5 (and Supplementary Figure S2—with outliers) depicting the spread and symmetry of the studies' effect sizes against their precision.

3.9 Imaging flow cytometry analysis of thawed bull sperm

TMRE/TMRM mitochondrial staining is a specific marker of $\Delta\psi_m$ only when used at a low, non-quenching concentration (≤50 nM) (43). Below, Figure 6 provides representative images of the bovine spermatozoa stained with Hoechst 33342 for DNA (green) and TMRM dye for mitochondrial potential evaluation (purple). Evaluated morphological parameters included abnormal head, midpiece, tail, and so-called cytoplasmic "droplets" compared with morphologically normal cells (Figure 6). These morphological parameters are additive and can provide a percentage of morphologically and/or functionally aberrant spermatozoa (44).

4 Discussion

The growing interest in artificial insemination (AI) of cattle creates a need for more robust and intrinsic analyses of semen conditions. For a long time, morphological features of spermatozoa

TABLE 2 Summary of the subgroup analysis.

Parameter	Subgroup	Effect size			Heterogeneity	
		k	SMD [95% CI]	*p*-value	I^2 (%)	Tau^2
DFI	Dairy	19	−3.978 [−4.942; −3.013]	<0.01	79.1	2.455
	Beef/Mix/Dual	7	−4.473 [−5.619; −3.328]	<0.01	88.0	3.103
MMP	Dairy	23	−0.520 [−0.874; −0.166]	<0.01	79.9	0.347
	Beef/Mix/Dual	3	−0.125 [−0.643; −0.394]	<0.01	91.5	2.566
Morphology	Dairy	16	−2.780 [−3.502; −2.057]	<0.01	78.7	0.840
	Beef/Mix/Dual	7	−2.290 [−3.836; −0.722]	<0.01	94.8	3.810
Motility	Dairy	22	0.742 [0.526; 0.959]	<0.01	72.4	1.123
	Beef/Mix/Dual	14	0.342 [−0.566; 1.309]	0.41	91.0	4.427

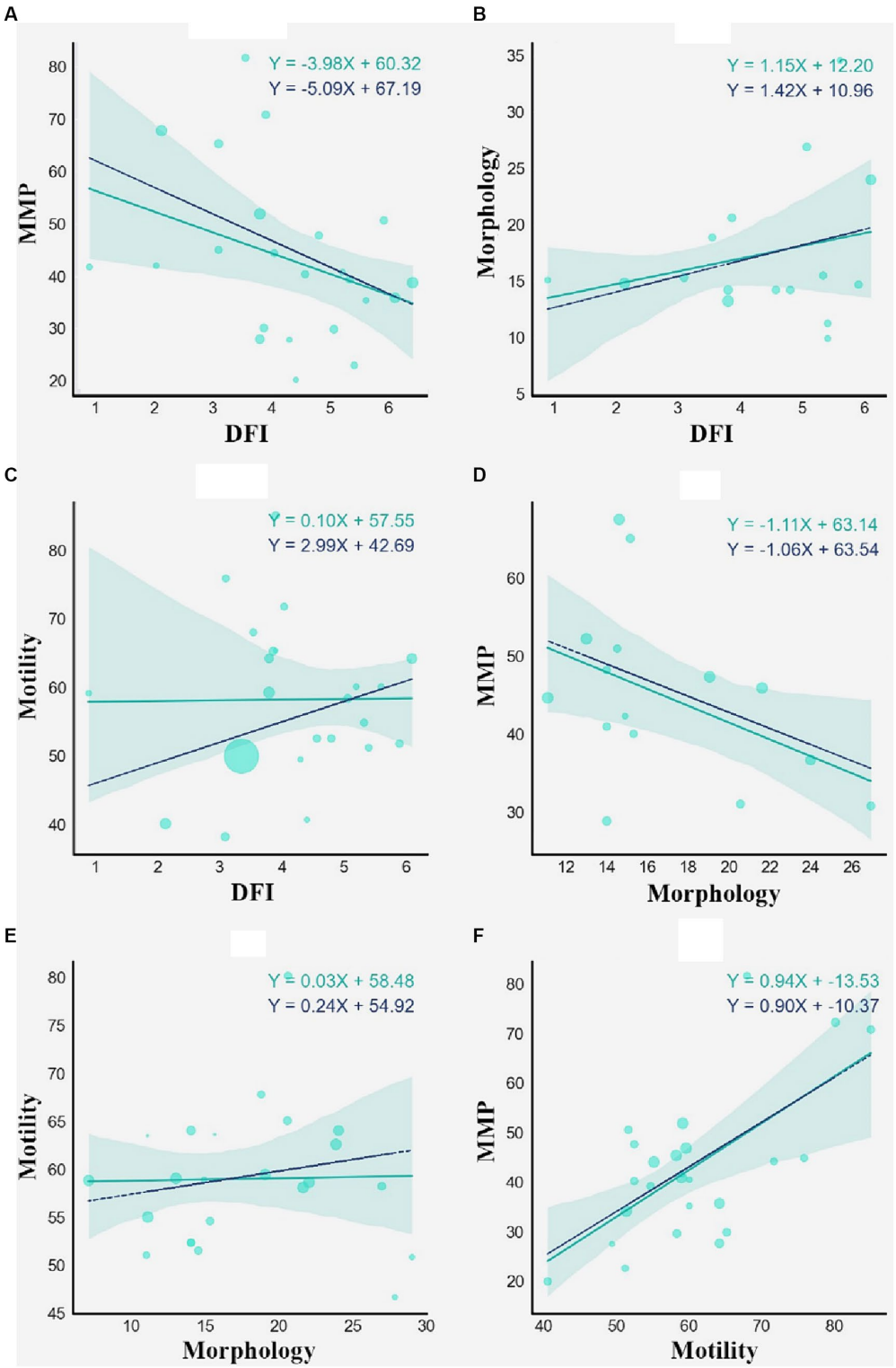


FIGURE 3
Weighted linear regression of DFI and MMP **(A)**, DFI and Morphology **(B)**, DFI and Motility **(C)**, Morphology and MMP **(D)**, Morphology and Motility **(E)**, Motility and MMP **(F)**. The cyan solid regression line represents non-weighted linear regression. The blue dashed line represents weighted linear regression, each weight associated with the sample size of a study.

(45–47) and sperm motility (48) were used as the main quality parameters to evaluate. The morphological characteristics of healthy bovine spermatozoa include an oval-shaped head, lack of defects in the midpiece and tail, and absence of cytoplasmic droplets (17, 49, 50) and were observed mainly using light microscopy (17, 51, 52). However, in the last decade, parameters related to cellular metabolism such as plasma membrane integrity, mitochondrial potential, and DNA fragmentation index (DFI) of post-thawed sperm started to be used to evaluate semen quality and predict animal fertility (53–55). This led to broader use of flow

cytometry and various fluorochromes for sperm evaluation (56). However, the flow cytometry analysis is complicated by the presence of autofluorescent "non-sperm events" originating from extender particles in sperm freezing medium, cellular or bacterial debris, and can lead to overestimating sperm populations and features (57).

Though the sperm parameters that define high fertilization potential are not fully understood, many studies indicate that sperm with defective parameters do not reach the place of fertilization.

TABLE 3 Statistical results of linear regression modeling.

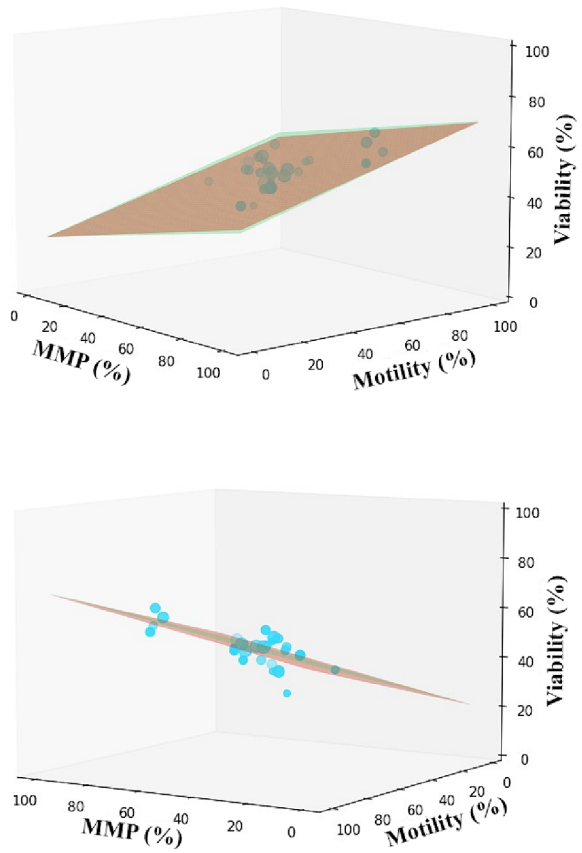
Plot	*k*	Weighted		Non-weighted	
		R^2	F-statistics	R^2	F-statistics
DFI-MMP	23	0.311	5.614	0.342	2.911
DFI-Morphology	16	0.117	1.847	0.064	0.964
DFI-Motility	23	0.099	2.309	0.001	0.002
Morphology-MMP	18	0.374	4.742	0.286	3.964
Morphology-Motility	22	0.032	0.731	0.001	0.001
Motility-MMP	25	0.378	13.96	0.409	15.93

Across the selected studies for this meta-analysis, the evaluation of thawed sperm quality parameters was mainly done on flow cytometers or microscopes. The total motility of sperm was analyzed on computer-assisted sperm analysis (CASA) for almost all studies. CASA-based systems have successfully evolved in sperm analysis over approximately 50 years measuring morphology and motility attributes of single sperm (58–60). However, interpretation of CASA data should be defined in terms of the measurement conditions, in particular, model and software version, time for tracking sample, microscope magnification and rate of image acquisition.

Some of the analyzed studies combined those methods to obtain a detailed quality assessment of bovine spermatozoa. Our meta-analysis focuses on the multiparametric evaluation of cryopreserved semen quality performed mainly through flow cytometry to characterize animal fertility. Cell viability is often taken as a control parameter of bovine sperm quality and fertilizing ability after thawing.

4.1 Sperm membrane damage and DNA fragmentation

When assessed with flow cytometry, sperm is usually stained with DNA fluorescent dyes such as propidium iodide (PI) (61), SYBR-14 (62, 63), or Hoechst 33342 (64) to evaluate sperm viability. Cells with damaged membranes allow PI to penetrate the cell and bind to DNA



k	Weighted		Non-weighted	
	R^2	F-statistics	R^2	F-statistics
27	0.473	10.522	0.492	11.628

FIGURE 4
Multiple linear regression analysis for motility, MMP, and viability. The 2D red and green planes represent unweighted and weighted models, accordingly. Data points are represented by mean values of parameters in %, scaled in size according to the corresponding sample size.

(22, 65, 66). The plasma membrane damage resulting from cryopreservation can at least in part be attributed to lipid peroxidation processes (67) and the generation of reactive oxygen species (ROS) (68). The membrane damage leads to a decline of the post-thawed bovine spermatozoa viability by an average of 50% (69, 70), which is consistent with the results obtained from our meta-analysis. Spermatozoa are viable when their plasma membrane is intact (71).

Sperm DNA fragmentation (SDF) is considered a suitable parameter for predicting fertility (72–75). DNA fragmentation index or DFI is simply a quantitative representation of SDF, calculated as a percentage of DNA fragmented sperm cells with DNA damage or abnormal protamination (74, 76, 77). Karoui and co-authors found a negative association between bull fertility and sperm DNA fragmentation using the halo test (78). DNA integrity is essential for bovine embryonic development (79), and DFI is negatively correlated with sperm binding index and conception rates (80). Interestingly, the sperm viability and motility become affected earlier than DNA, and occurrence of single-stranded DNA breaks happens in parallel with chromatin decondensation and deprotamination (81).

TABLE 4 Results of Egger's test for sperm quality parameters.

Parameter	Intercept	95% Confidence interval	*t*	*p*-value
DFI	−1.449	[−2.235; −0.663]	−2.213	<0.001
MMP	0.449	[−0.930; 1.677]	1.390	0.088
Morphology	−0.996	[−2.102; −0.062]	−2.980	<0.001
Motility	1.769	[−0.024; 3.598]	3.102	0.012

4.2 Sperm mitochondrial status

Mitochondrial status, another valuable metabolic parameter of sperm viability and, thus, fertility, is mainly characterized by mitochondrial membrane potential and shifts in mitochondrial functionality (56). The mitochondria are the "powerhouses" of the cell and are crucial for the proper motility of spermatozoa; therefore, monitoring changes in mitochondrial functionality is required to predict sperm fertility accurately (63, 82, 83). Moreover, mitochondria

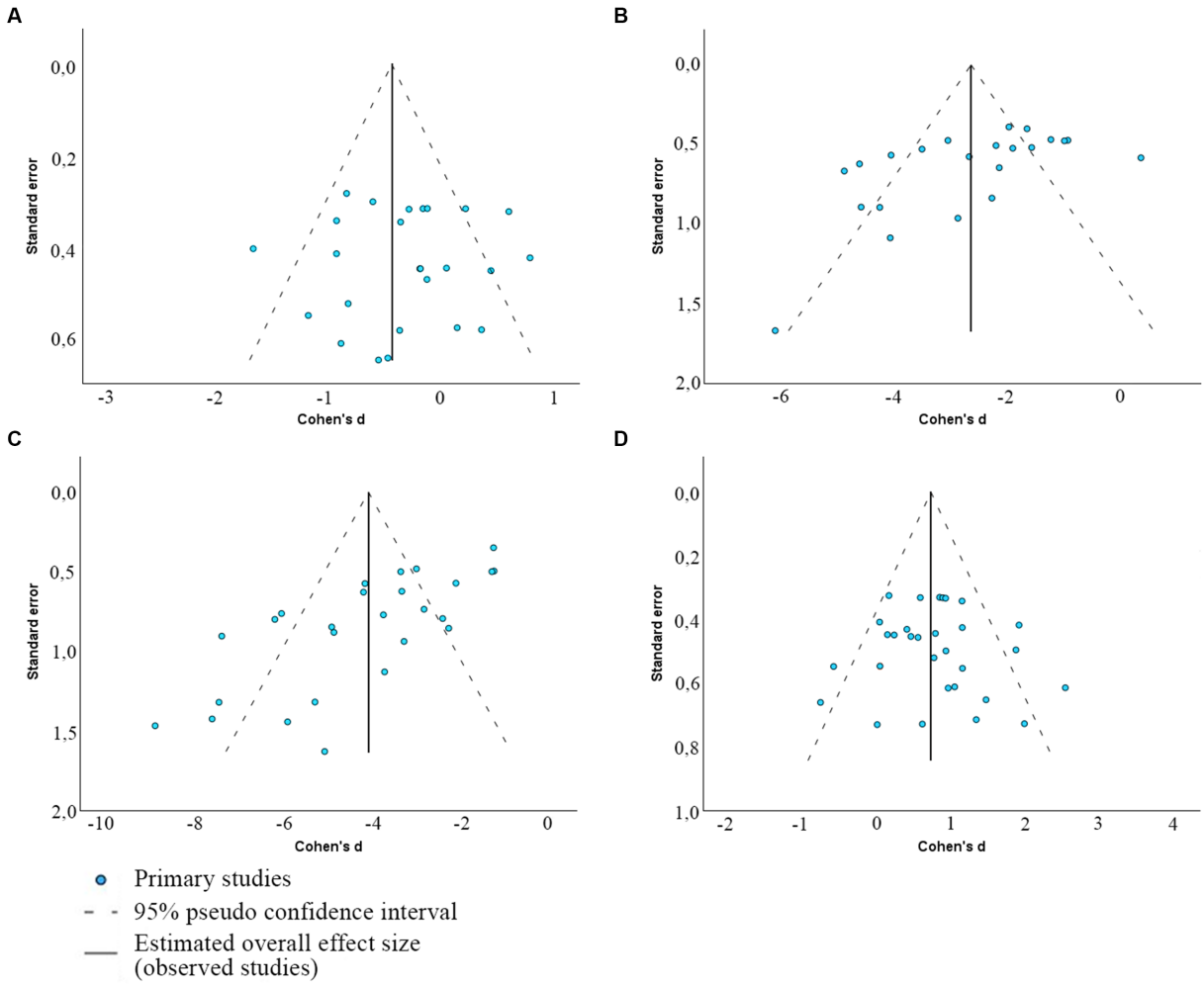


FIGURE 5
Funnel plots depicting the presence of publication bias between included articles that determine the association between viability and MMP **(A)**, morphology **(B)**, DFI **(C)**, and total sperm motility **(D)**.

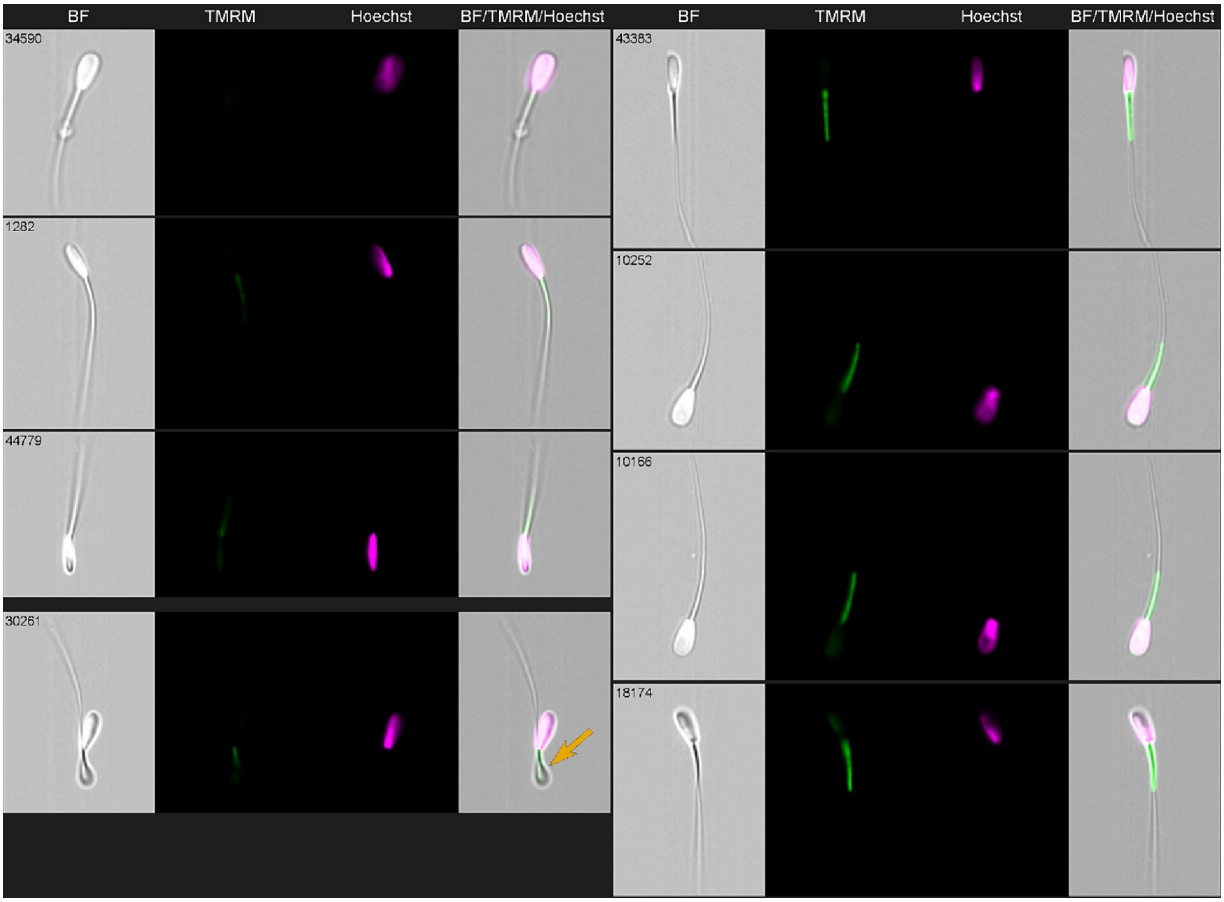


FIGURE 6
Imaging flow cytometry gallery of thawed bovine spermatozoa stained with Hoechst 33342 (green) and TMRM (purple) and acquired using ImageStream X Mark II (Amnis-Cytek, United States). Simultaneous observation of TMRM and Hoechst staining allows to differentiate normal (right row) and abnormal (left row) sperm. Top three sperm in the left—low TMRM with normal nuclei; lower left—one spermatozoa had no nucleus (arrow).

are highly prone to damage during cryopreservation, and any abnormalities in mitochondrial morphology or their functionality will decline sperm quality (84). Discrimination between high and low mitochondrial membrane potential is possible with different fluorescent dyes, including JC-1, rhodamine family dyes (rhodamine 123, TMRE, and TMRM), 3,3′-dihexiloxocarbocyanine iodide (DiOC6) and Mitotracker family dyes (85–88) and provides information about the quality of sperm (69, 84, 86, 89, 90). The uptake of most mitochondria-selective dyes, with some exceptions (acridine orange and Mitotracker Green), is dependent on $\Delta\Psi$ (91). However, some of these dyes have properties that limit their use (88, 92). Thus, JC-1, a cationic dye that is actively used in sperm research, can only detect large differences in $\Delta\Psi$ across cellular populations (93) and, therefore, subjected to artifacts (94, 95). Moreover, JC-1 double fluorescence made it challenging to use this dye combined with other fluorochromes (57) and its fluorescence can be affected by some components of the sperm freezing medium (94). A good alternative is TMRM dye, which accurately detects sperm populations displaying either high or low $\Delta\Psi$ in the conditions where JC-1 has difficulties demonstrating differences (95). We also suggest TMRM staining as a mandatory MMP part of sperm quality evaluation.

Mitochondrial potential and DFI influence were assessed relative to the control viability parameter. DFI and abnormal morphology contribute the most to heterogeneity, suggesting inconsistency at the data sampling and analysis stages. However, reported MMP values suffer less from error and are more consistent across studies relative to spermatozoa viability. Furthermore, we found that MMP is positively associated with the total motility of bovine spermatozoa, proving that high mitochondrial potential values are related to higher total motility of the sperm.

We conclude that a combination of MMP and SDF values alone cannot confidently determine the viability of spermatozoa or be a substitution for evaluating other parameters like the motility of cryopreserved sperm. However, a combination of motility, MMP, and DFI might be a better indicator. Additionally, multiple regression analysis was conducted to analyze the general trend between the parameter variables. Among MMP and motility parameters, it was determined that an increase in the motility of spermatozoa has a more significant effect on viability.

4.3 IFC as a multi-parametric approach for sperm evaluation

As stated earlier, each minor alteration in MMP is essential in overall mitochondrial status, and flow cytometry is convenient for monitoring those changes (90). Therefore, a single complex multi-parametric technique, imaging flow cytometry, capable of substituting

both flow cytometry and microscope, would be an appropriate option. As we conclude from this meta-analysis, a combination of morphological and fluorescent functional parameters may improve sperm quality evaluation. Morphometric sperm characteristics are between most important indicators of fertility (96) but are not available from conventional flow cytometry analysis. The imaging flow cytometry has already been successfully used in different laboratories for sperm evaluation (44, 97–100) and allowed the characterization of morphological and metabolic sperm parameters in thousands of spermatozoa simultaneously. The IFC is a relatively young technology that still needs to find a place in commercial applications and research, particularly in sperm characterization, where routine methods (sperm motility evaluation, etc.) have been established for decades. However, the Imagestream IDEAS software capable to differentiate sperm from cellular debris and cytoplasmic droplets of similar size solving the major problem of automatic CASA systems.

Artificial intelligence and machine learning algorithms are increasingly used to analyze IFC data (101–104). The development of IFC systems for this specific field will depend on reproducibility, usability, stability, complexity of instrument setup, and addition of artificial intelligence algorithms to remove the subjectivity and variability of analysis (105, 106). It requires improving the IFC systems to where data will be recorded, processed, and analyzed quickly in veterinary laboratories. Interestingly, current IFC systems (Imagestream and Flocyte instrument lines) are coming from a sole source (Amnis-Cytek) (107), which may simplify standardization of protocols. Recent introduction of image-based sorting instrumentation (108) capable to high-throughput sorting based on fluorescence and morphological features may prove helpful in sex-sorting of functional sperm.

4.4 Limitations of the study

First of all, the analyzed studies represented different breeds and sometimes a mix of breeds; after using subgroup analysis, the level of heterogeneity was decreased in the more homogenous dairy group of breeds. The number of studies used for each subgroup was relatively small that could result in a biased estimation of heterogeneity (I^2) (109). Next, the lack of standardization contributed to the high heterogeneity level. The methods for evaluating total motility and abnormal morphology, thawing and staining techniques, and types of fluorescent dyes are varied among the studies due to the lack of standard protocols for quality assessment of cryopreserved bovine semen. Sperm morphology needs to be better evaluated, and though CASA systems have developed protocols to analyze efficiently the kinematic parameters of animal sperm (58), their use needs to be extended to the analysis of viability, DFI and morphology (110), which considered to be vital for fertility prediction.

5 Conclusion

In this study, cryopreserved sperm research exploring different post-thaw evaluation were compared. Results of the present meta-analysis indicate a significant positive MMP correlation with both total sperm motility and viability. The percentage of morphological abnormalities and DFI was lower in sperm with high viability. The MMP and motility were the most prominent sperm quality parameters, with minor publication bias and a notable association with sperm viability.

In conclusion, a combination of morphological assays, along with an evaluation of metabolic heterogeneity, including mitochondrial status, motility, and DNA integrity of sperm, is needed for sperm analysis standardization and a proper prediction of the fertilizing ability of sperm. Multi-parametric flow cytometry has become an important method for a rapid and sensitive evaluation of functional sperm properties and in veterinary science research as well as for a routine assessment of sperm quality. However, flow cytometry does not evaluate the morphological characteristics of semen. Furthermore, using a hybrid IFC technology which combines flow cytometry and light microscopy features, can potentially lead to solving one of the major problems in sperm evaluation, the inability to test morphological and functional parameters simultaneously, and will help with to standardize protocols for semen assessment. Future studies utilizing imaging flow cytometry for sperm evaluation may be combined with image-based sex-sorting of sperm.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

AU: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. AK: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. AM: Formal analysis, Methodology, Writing – review & editing. BS: Conceptualization, Formal analysis, Supervision, Writing – review & editing. IV: Conceptualization, Funding, Supervision, Writing – review & editing. NB: Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1371586/full#supplementary-material

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Supplementation with MitoTEMPO before cryopreservation improves sperm quality and fertility potential of Piedmontese beef bull semen

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The purpose of this study was to improve the quality of frozen–thawed Piedmontese bull semen by incorporating MitoTEMPO (MT) in extended semen before cryopreservation. Semen was collected from 4 fertile bulls, using an artificial vagina, once weekly for 6 consecutive weeks. Semen samples were pooled, diluted with Bullxcell® extender, and supplemented with different concentrations of MT (0 as control, 5, 10, 20, 40, and 80 μM) before cooling, equilibration, and freezing procedures. The frozen–thawed semen was assessed for motility, vitality, acrosome intactness, plasma membrane integrity, DNA integrity, apoptosis, mitochondrial membrane potential, intracellular ROS level and *in vitro* fertilizing capability. The results showed that MT at concentrations of 10, 20, and 40 μM improved the total, progressive, and rapid motility directly after thawing while, at the highest tested concentration (80 μM), it decreased the progressive and rapid motility after 1, 2, and 3 h of incubation. The sperm kinetics including STR and LIN were noticeably increased at concentrations of 10, 20, and 40 μM directly after thawing (0 h), whereas the MT effect was variable on the other sperm kinetics during the different incubation periods. MitoTEMPO improved the sperm vitality at all tested concentrations, while the acrosomal and DNA integrity were improved at 20 μM and the mitochondrial membrane potentials was increased at 80 μM. The cleavage and blastocyst formation rates were significantly increased by using semen treated with 20 μM MT compared with controls. These findings suggest a potential use of MT mainly at a concentration of 20 μM as an additive in the cryopreservation media of bull semen to improve sperm quality.

KEYWORDS

bull semen, cryopreservation, MitoTEMPO, sperm quality, DNA, mitochondrial activity, *in vitro* embryo production

1 Introduction

The Piedmontese breed is the most numerous Italian beef breed, characterized by muscular hypertrophy due to a specific mutation in the myostatin gene (1). It is highly specialized for beef production due to the double muscling characteristic that exerts positive effects on carcass conformation, dressing percentage, and meat quality (2).

Artificial insemination is a breeding method implemented worldwide that accounts for an increasing proportion of cattle reproduction (3). Semen cryopreservation is an important factor for artificial insemination (4), as it allows for the use of cattle genetic resources by making semen of genetically superior bulls available worldwide (5). Therefore, advances in bull semen cryopreservation and sperm fertility are critical economic traits for breeding programs (6). Cryopreservation induces several biophysical and biochemical changes in the sperm membrane that markedly decrease the sperm fertility potential (7). Cryopreservation has been associated with excessive production of reactive oxygen species (ROS), which is the major reason for reduced frozen semen quality (4, 8). Furthermore, sperm cells are highly susceptible to lipid peroxidation (LPO) as they contain high levels of polyunsaturated fatty acids (PUFA), which detrimentally affect sperm motility and membrane integrity (9).

Mitochondria is one of the primary sources of sperm reactive oxygen species (ROS) (10). ROS are produced by electron leakage from the electron transport chain (ETC), which is accepted by molecules of oxygen resulting in the production of O_2^- (11, 12). Furthermore, ROS is produced by the mitochondrial apoptotic pathway, which is triggered when the phosphoinositide signaling pathway is disrupted (13). Additionally, the presence of a high level of PUFA in mitochondrial membranes, which serve as preferential ROS substrates, promotes lipid peroxidation and the production of lipid aldehydes. These bind to ETC proteins covalently, increasing mitochondrial ROS production (14, 15).

Antioxidant supplementation of extenders has been used to protect sperm from oxidative stress and improve sperm quality after thawing (4). Depending on their mechanism of action, antioxidants are classified as enzymatic or non-enzymatic substances that scavenge free radicals (16, 17). Non-enzymatic antioxidants include endogenous compounds such as amino acids, glutathione, and coenzyme Q10 as well as exogenous compounds such as polyphenols, carotenoids, vitamin E, and vitamin C. Enzymatic antioxidants include catalase (CAT), superoxide dismutase (SOD), glutathione transferase (GST), glutathione peroxidase (GPx), thioredoxins (TRX), and peroxiredoxins (PRDXs) (18). Mito-Tempo is a mitochondria-targeted superoxide dismutase mimetic that scavenges superoxide molecules by converting them into hydrogen peroxide or oxygen and then detoxifies them to oxygen and water by catalase or glutathione peroxidase (19, 20). Therefore, using mitochondria-targeted antioxidants could help to preserve the activity and fertility potential of cryopreserved sperm cells (21). Mitochondria-targeted antioxidant (MitoTEMPO, MT) is a novel antioxidant and a powerful cell-permeable ROS scavenger that protects cells from oxidative stress in a variety of conditions by scavenging superoxide anion in the catalytic cycle (22–24). MitoTEMPO contains the antioxidant piperidine nitroxide (Tempo) and the lipophilic cation, triphenylphosphonium (TPP+) and accumulates within mitochondria (25). The addition of MT to semen extender has a sperm quality protective effect in frozen–thawed human (26, 27), rooster (28), buffalo (29) and buck (30) sperm as well as chilled ram semen (24).

The influence of MT on the fertility parameters of cryopreserved bull semen has not been recorded. Therefore, the present study aims to investigate the effect of MT supplementation to semen extender before cryopreservation on post-thawing sperm motility, vitality, mitochondrial membrane potential (MMP), membrane functionality, DNA integrity, intracellular ROS level, as well as sperm *in vitro* fertilizing capability.

2 Materials and methods

The experimental design flowchart is presented in Figure 1.

2.1 Ethics approval statement

No experimental animals were used in this study. All procedures were performed in accordance with DPR 27/1/1992 (Animal Protection Regulations of Italy) in compliance with European Community Regulation 86/609.

2.2 Semen collection and extension

For semen collection, four Piedmontese bulls (1.5–2 years old) of proven fertility at the ANABORAPI center in Carrù, Piemonte region, Italy, were used. From each bull, semen was collected once weekly for 6 consecutive weeks (6 ejaculates/bull with a total of 24 ejaculates) using a bovine artificial vagina adjusted at 45°C. Samples with concentration $\geq 800 \times 10^6$/mL and motility ≥70% were used. Pooled semen samples were extended with Bullxcell® extender (IMV, France) following the manufacturer's instructions, and supplemented with MitoTEMPO (MT, SML0737, Sigma-Aldrich, Italy) at different concentrations: 0 (control), 5, 10, 20, 40, and 80 µM. The MT solution was prepared by dissolving the content of the MT vial in 1 mL TALP medium (as detailed below) followed by storage as aliquots of 70 µL at-80°C until use. The doses used in this study were determined depending on the results reported in previous publications in different species including humans (26, 27), ram (24), boar (31), rooster (28), buffalo (29), and buck (30). Extended semen containing 30×10^6 spermatozoa/mL was cooled to 5°C and then packed into 0.5 mL polyvinyl straws (Minitube, Germany) followed by equilibration for 4 h (h) and freezing using a controlled-rate freezer (SY-LAB Gerate GmbH, Neupurkersdorf, Austria). Semen freezing was performed at five predetermined rates: extended semen was cooled at a rate of-4°C/min from +4°C to-9°C. In the range of −9°C to −25°C, the freezing rate was −50°C/min. From −25°C to −100°C, the freezing rate dropped to −35°C/min, −20°C/min from −100°C to −144°C, and − 4°C/min from −144°C to −150°C. After reaching −150°C, the straws were dipped in liquid nitrogen (−196°C). At least after 24 hin liquid nitrogen, straws were thawed ($N = 4$/trial/treatment) at 37°C for 40 s for the various assessments.

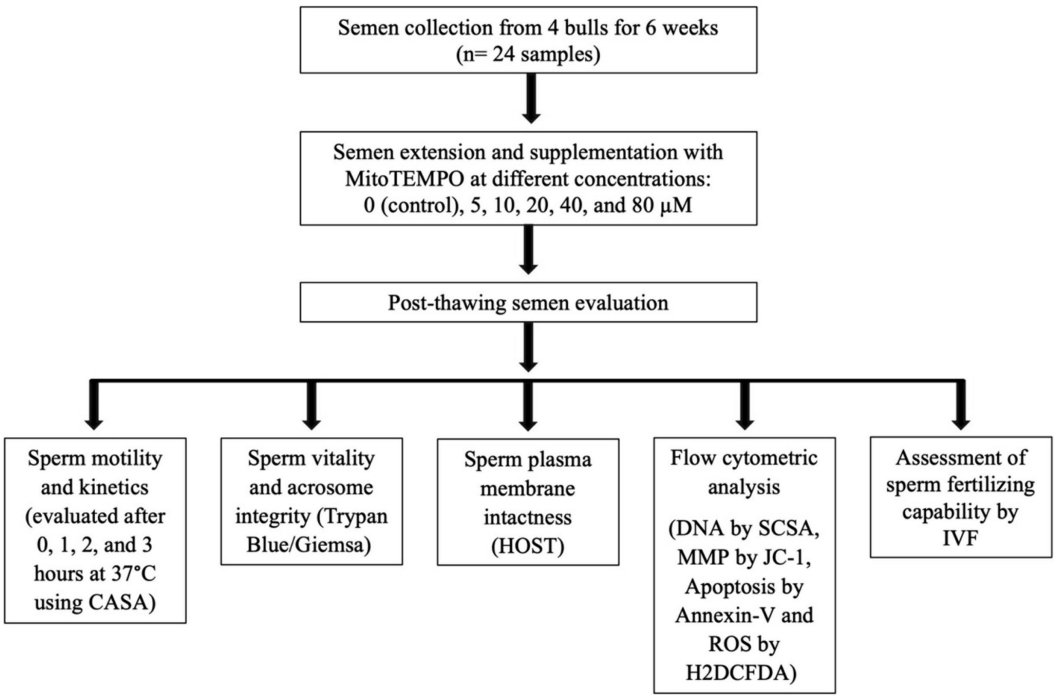

FIGURE 1
Schematic form of the experimental design. CASA, computer-assisted semen analysis; HOST, hypoosmotic swelling test; MMP, mitochondrial membrane potential; SCSA, sperm chromatin structural assay; ROS, reactive oxygen species; H2DCFDA, 2′,7′-Dichlorodihydrofluorescein diacetate; IVF, *in vitro* fertilization.

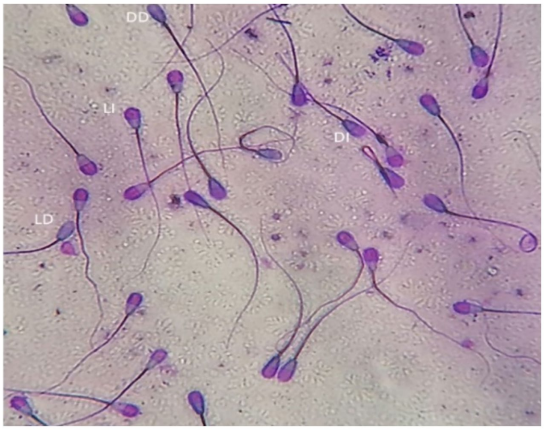

FIGURE 2
Bull spermatozoa stained with Trypan blue/Giemsa stain. Four types of stained sperm were identified: LI, live with intact acrosome, LD, live with damaged acrosome, DI, dead with intact acrosome, DD, dead with damaged acrosome.

2.3 Semen evaluation

2.3.1 Assessment of sperm motility and kinetics

The thawed sperm motility and kinetics were evaluated after different periods of incubation (0, 1, 2, and 3 h) at 37°C using the computer-assisted sperm analyzer (CASA; Hamilton Thorne, Inc., Beverly, MA, United States). In a prewarmed Mackler chamber, 10 µL semen specimens were loaded according to the manufacturer's instructions and evaluated with a bull semen-specific setup; 30 frames were obtained at a 60 Hz frame rate, with a minimum cell size of 8 pixels and a minimum contrast of 40. The VAP and VSL cutoff times were 15 µ/s and 4.4 µ/s, respectively. The speed standards were set as fast; >80 µm/s, medium; >60 µm/s, slow; >20 µm/s and static. The motility (total, progressive, and rapid, %) and velocities such as Straight linear velocity µm/s (VSL), Curvilinear velocity µm/s (VCL), Beat cross-frequency Hz (BCF), Average path velocity µm/s (VAP), Amplitude of lateral head displacement, µm (ALH), Straightness (STR, [VSL/VAP] × 100) and Linearity (LIN, [VSL/VCL] × 100) parameters, were assessed in eight randomly selected fields.

2.3.2 Assessment of sperm vitality and acrosomal integrity

The dual staining technique (Trypan Blue/Giemsa) according to Boccia et al. (32) was used to evaluate sperm vitality and acrosomal status. Equal volumes of semen and trypan blue (0.27%) were mixed and smeared on a glass slide and allowed to dry before being fixed for 4 min in 37% formaldehyde with neutral red. The fixed smears were stained overnight with Giemsa (7.5%), after which, slides were washed with distilled water and allowed to dry before being microscopically examined (Advanced Automated Research Microscope System, Nikon Eclipse E200, phase contrast at 40x and 100x magnifications, United States). Four different types of stained sperm were identified: live with intact acrosome, live with damaged acrosome, dead with intact acrosome, and dead with damaged

acrosome (Figure 2). Two-hundred sperm cells were evaluated for each experimental group.

2.3.3 Assessment of sperm plasma membrane intactness

According to Akhter et al. (33), the hypo-osmotic swelling test (HOST) was used to evaluate the intactness of the sperm plasma membrane. A 50 µL semen specimen mixed with 500 µL of prewarmed HOST solution (0.735 g sodium citrate and 1.351 g fructose dissolved in 100 mL of distilled water with an osmotic pressure of approximately 190 mOsm/kg) was incubated for 40 min at 37°C. Using a phase contrast microscope (400X), at least 200 spermatozoa/slide were assessed for the percentage of cells with intact plasma membrane (curled/swollen tails; HOST positive, Figure 3).

2.3.4 Flow cytometric analysis

A FacsStar Plus flow cytometer (Becton Dickinson Immunochemistry, San Jose, CA, United States) equipped with standard optics and an air-cooled argon laser at 488 nm and 15 mW was used to assess the integrity of sperm DNA (SCSA), mitochondrial membrane potential (JC-1), apoptosis (Annexin-V/PI binding assay), and intracellular ROS level (H2DCFDA). Using CellQuest® software (BD Biosciences, San Jose, CA, United States) and a flow rate of 200 events/s, a total number of 10.000 gated events were analyzed per sample.

2.3.4.1 Assessment of sperm DNA integrity using SCSA

The sperm DNA integrity was assessed flow cytometrically using sperm chromatin structure assay (SCSA; Figure 4) according to Evenson and Jost (34). The thawed sperm were washed in phosphate buffered saline (PBS) by centrifugation at 500 g for 10 min. The sperm pellets were diluted to a final concentration of 2 × 106 sperm/mL with TNE buffer (0.01 M Tris–Cl, 0.15 M NaCl, 1 mM EDTA, disodium pH 7.4) followed by the addition of 400 µL of acid detergent solution (0.08 N HCl, 0.15 M NaCl, 0.1% (w/v) Triton X-100, pH 1.2). After 30 s, semen was stained with 1,200 µL of Acridine Orange (AO, Sigma-Aldrich, United States) staining solution containing 600 µL AO (6 mg/mL) diluted in 100 mL staining buffer (0.037 M citric acid, 0.126 M Na2HPO4, 1.1 mM EDTA disodium, 0.15 M NaCl, pH 6.0) followed by the Flowcytometric evaluation. The cells' red (single-stranded DNA) and green (double-stranded DNA) fluorescence were recorded. The fluorescence was measured using FL1 (530/15 nm) and FL3 (650 nm) filters. The percentage of sperm with double-stranded DNA was recorded.

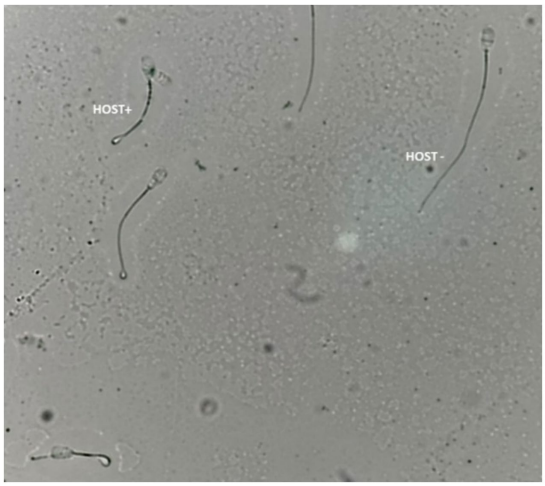

FIGURE 3
Bull sperm plasma membrane integrity assessed by the hypoosmotic swelling test (HOST). HOST+; sperm with an intact plasma membrane, HOST-; sperm with the damaged plasma membrane.

2.3.4.2 Assessment of sperm mitochondrial activity

According to Martinez-Pastor et al. (35), the sperm mitochondrial activity was assessed using the lipophilic cation JC-1 (MitoProbe™ JC-1 Assay Kit for Flow Cytometry; M34152), Fisher Scientific – Scheepsbouwersweg 1b – Postbus 4 – 1120 AA Landsmeer; Figure 5. Thawed sperm (1×10^6) were washed in 1 mL PBS by centrifugation at 500 g for 10 min. After centrifugation, the sperm pellets were resuspended in 1 mL PBS and supplemented with 10 µL of JC-1 (JC-1200 µM in DMSO), and incubated for 15 to 30 min at 37°C, 5% CO2. At the end of incubation, semen was centrifuged in 2 mL PBS, followed by resuspension in 500 µL PBS and assessed Flow Cytometrically. With a 488 nm laser, JC-1 was excited and by using the emission filters of 535 nm and 595 nm, the cells with green (JC-1 monomers) and orange (JC-1 aggregates) fluorescence were quantified. Using the frequency plots of FL1 (green) and FL2 (orange), the percentage of sperm stained green and orange was determined. The percentage of orange-stained sperm was recorded as cells of high mitochondrial membrane potential (HMMP) cells.

2.3.4.3 Evaluation of sperm for apoptosis (Annexin-V/PI-binding assay)

The sperm plasma membrane integrity and apoptosis were assessed by using Alexa Fluor 488 Annexin-V Apoptosis Kit (INVITROGEN – V13245) and Propidium Iodide (Figure 6) according to Anzar et al. (36). Thawed sperm (1×10^6) were washed in 1 mL PBS by centrifugation at 500 g for 10 min. Sperm pellets were resuspended in 100 µL of Annexin-V-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4), and supplemented with 1 µL of PI (100 µg/mL) and 5 µL of Annexin-V before being gently mixed and incubated in the dark for 15 min at room temperature. At the end of incubation, 400 µL of Annexin-V-binding buffer was added before flow cytometric evaluation which was conducted as soon as possible. By using the annexin V/PI binding assay, the orthogonal light scatter (SSC), forward light scatter (FSC), FITC fluorescence (FL1), and PI fluorescence (FL3) were assessed. To eliminate particles and restrict the analysis only to spermatozoa, an acquisition gate was used in the FSC/SSC two-dimensional histogram. The percentage of apoptotic (annexin V+ and PI+), early apoptotic (annexin V+ and PI^-), necrotic (annexin V^- and PI+), and viable (annexin V^- and PI^-) spermatozoa was recorded.

2.3.4.4 Assessment of sperm intracellular ROS levels

The 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Abcam Cellular ROS Assay Kit, ab113851) was used to measure the sperm intracellular ROS level according to Gallo et al. (37). Thawed sperm (1×10^6) were washed in 1 mL PBS by centrifugation at 500 g for 10 min. The sperm pellets were resuspended in 500 µL freshly prepared stain (10 µM H2DCFDA) and incubated at 37°C for 30 min in the dark. After incubation, the samples were analyzed flow cytometrically. The percentage of sperm ROS was recorded on the fluorescence intensity

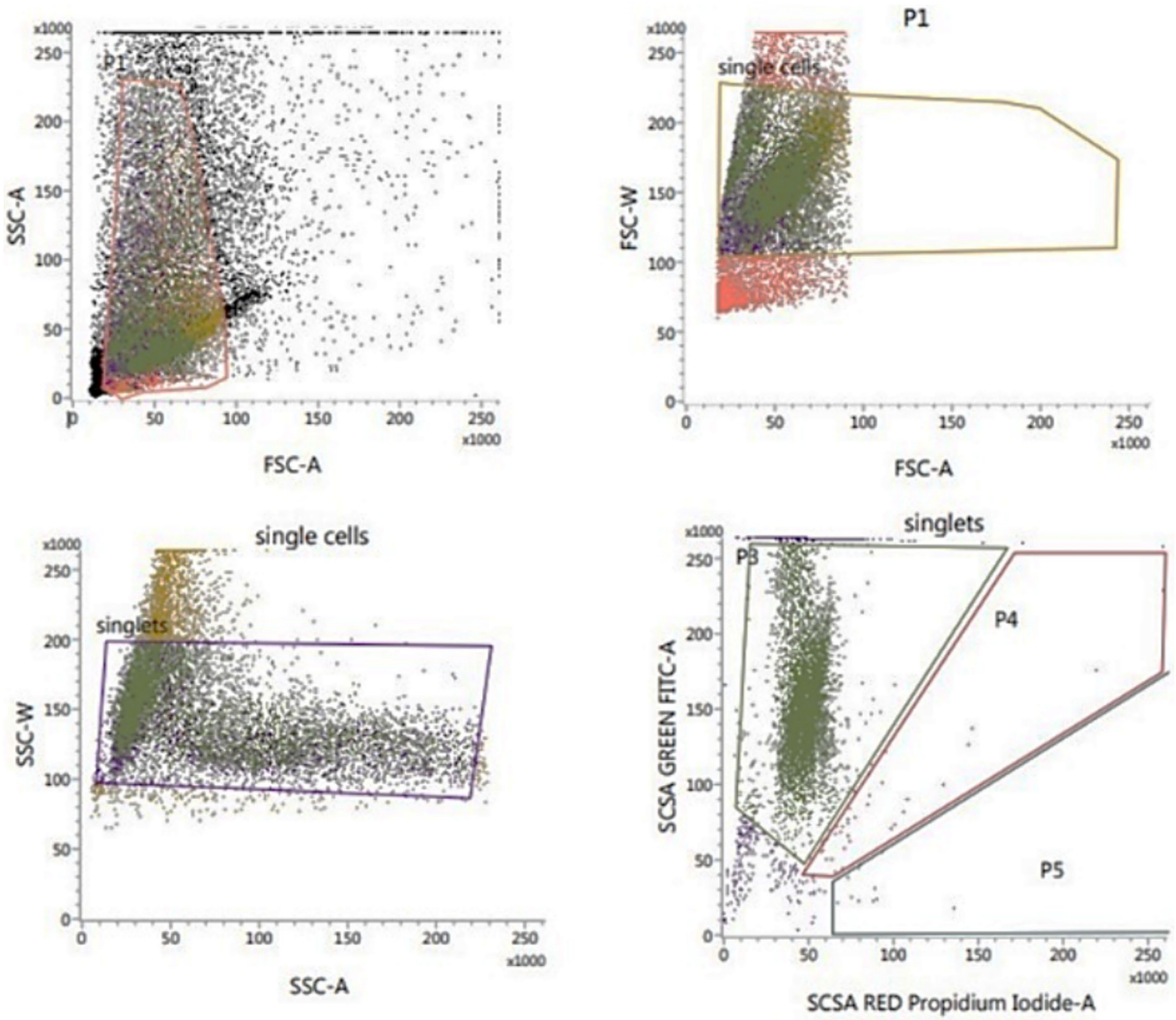

FIGURE 4
Flow cytometric evaluation of sperm DNA integrity using SCSA.

of the emission spectrum that was measured from 500 to 560 nm (typically FL1) with an excitation wavelength of 488 nm (Figure 7).

2.4 Assessment of sperm fertilizing capability

Based on the effects of MT on sperm quality parameters, the fertilizing capacity of thawed semen treated with 20 µM MitoTEMPO was evaluated *in vitro* using bovine oocytes and compared with control in the absence of MT. Briefly, Piedmontese bovine ovaries were collected from the slaughterhouse (Manzo Carni, Cuneo, Italy), washed, and transported in warm saline solution (38°C) to the laboratory within 2 h. After rinsing with warm saline, follicles 2–8 mm in diameter were aspirated using a 10 mL syringe and an 18-gauge needle under the stereo microscope (ZEISS®, Carl Zeiss Suzhou Co., Ltd. Germany). Sediments of the aspirated follicular fluid were transferred to a 100 mm dish, and cumulus-oocyte complexes (COCs) with multiple layers of intact compact cumuli were selected and washed three times in TCM-199 HEPES (supplemented with 10% FCS) and three times in the *in vitro* maturation medium. The oocyte *in vitro* maturation was performed as reported in Elkhawagah et al. (38) in TCM-199 Earle's Salt Medium supplemented with 10% FCS, 5 µg/mL follicle-stimulating hormone (FSH; Folltropin-V®, Bioniche Animal Health.), 5 µg/mL luteinizing hormone (LH; Lutropin-V®, Bioniche Animal Health, Belleville, ON, Canada.), 1 mg/mL 17β-estradiol, 0.2 mM sodium pyruvate, and 10 µg/mL gentamycin (38). The oocytes were cultured in wells containing 400 µL IVM medium covered with paraffin oil and incubated at 38.5°C in 5% CO_2. After 24 h, thawed semen was washed by centrifugation at 300 g for 30 min in a Percoll discontinuous gradient by adding 1 mL Percoll 90% under 1 mL Percoll 45% and semen was layered on the top in a 15 mL tube. At the end of the IVM culture, the oocytes underwent *in vitro* fertilization (IVF) in Tyrode's-albumin-lactate-pyruvate (TALP) medium containing 2 mM penicillamine, 1 mM hypo- taurine, 250 mM adrenaline, 20 mg mL^{-1} heparin, 114 mM NaCl, 3.2 mM KCl, 0.4 mM NaH_2PO_4, 10 mM sodium lactate, 25 mM $NaHCO_3$, 0.5 mM $MgCl_2$ x $6H_20$, 2.0 mM $CaCl_2$ x $2H_2O$, 6 mg $mL\text{-}^1$ BSA, 5 mL mL^{-1}

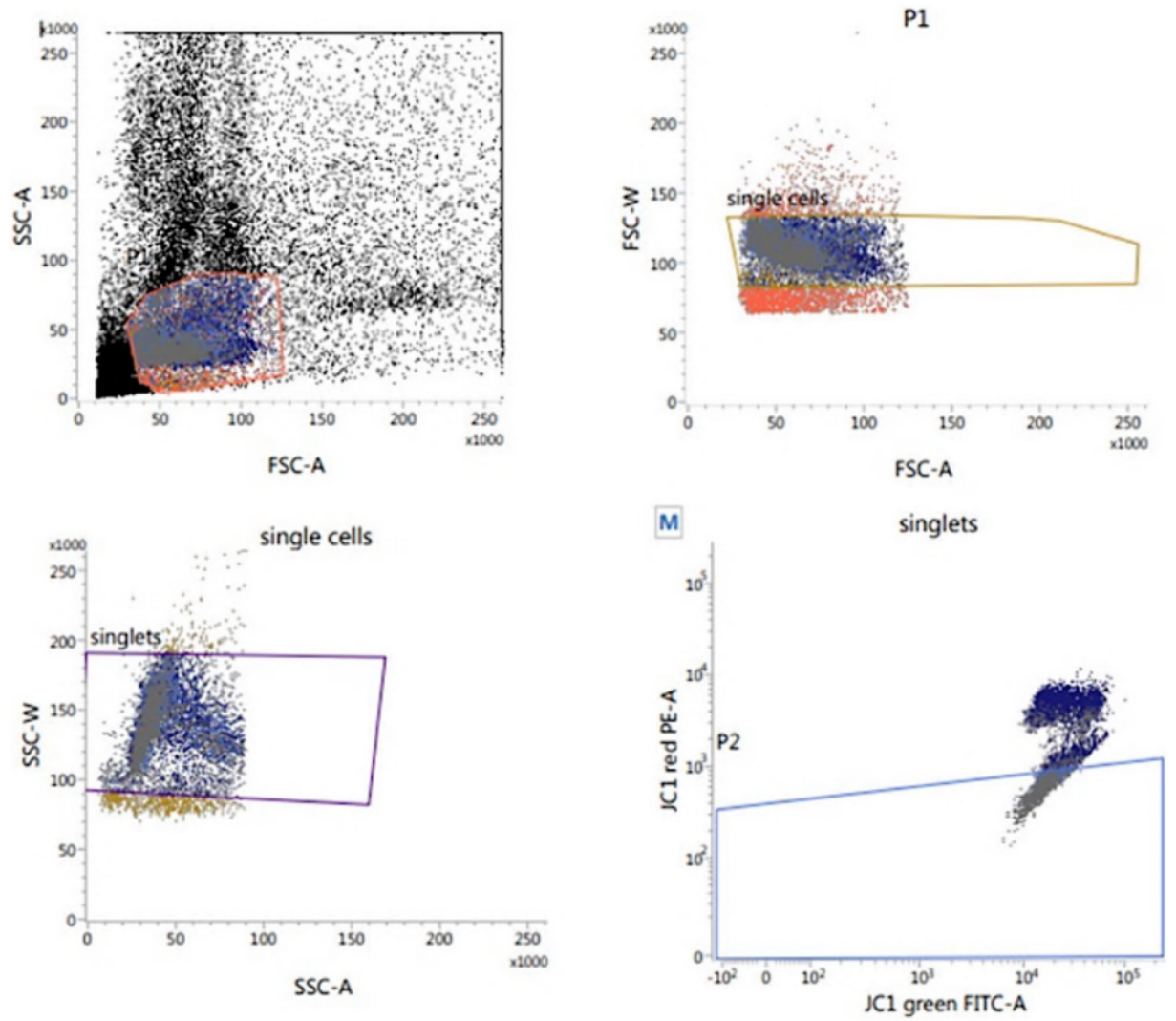

FIGURE 5
Flow cytometric evaluation of sperm mitochondrial membrane potential (MMP) using JC-1.

gentamicin and 0.2 mM sodium pyruvate (39). Specifically, the semen (with or without MT treatment) was added to IVF droplets containing the oocytes (10 µL semen/droplet) at the final concentration of 10×10^6 spermatozoa/ml. After gametes co-incubation at 38.5°C for 20 h, cumulus cells were removed, and zygotes were washed and cultured for 7 days at 38.5°C with 5% CO_2 and 5% O_2 in synthetic oviductal fluid (SOF) (40): 1.1 M NaCl, 72 mM KCl, 12 mM KH_2PO_4, 7.4 mM $MgSO_4$, 50 mM D, L-lactate, 250 mM $NaHCO_3$, 260 mM phenol red, 100 mM sodium pyruvate, 178 mM $CaCl_2$ $2H_2O$, 125 mM HEPES sodium salt, 30.8 mM glutamine, 500 mM glycine, 84.2 mM alanine, 100x minimum essential medium (MEM) non-essential amino acids, 50x basal medium Eagle (BME) amin oacid solution, 2.8 mM myoinositol, 340 mM trisodium citrate, 2% FCS, 0.005gmL^{-1} BSA, 0.2 mM sodium pyruvate and 5mLmL^{-1} gentamicin. At the end of incubation, embryos were fixed with 2% paraformaldehyde, mounted on a glass slide, and stained with Hoechst 33258. Embryos were evaluated for cleavage and blastocyst formation rates using a Nikon Eclipse TE 2000-S fluorescence microscope (Nikon®, Nikon Solutions Co., Ltd., Shinagawa, Japan) fitted with a B2A (346 nm excitation/460 nm emission) filter.

2.5 Statistical analysis

Semen data were analyzed and presented as mean ± SEM using SPSS vers. 25 software (IBM®, Rome, Italy). The general linear model (GLM) multivariate with Duncan test was used to make multiple comparisons of the means. The fertilizing potential was assessed using the Chi-squared test, considering the cleavage rate and blastocyst formation rate. Statistical significance was set for $p < 0.05$.

3 Results

3.1 Effect of MitoTEMPO on motility parameters

The mean values of sperm motility parameters evaluated by CASA analysis are shown in Table 1. Sperm samples treated with MT at 10, 20, and 40 µM showed increased total sperm motility ($p < 0.01$) when assessed directly after thawing. Similarly, sperm samples treated with MT at 20 and 40 µM, after 1 h of incubation, and those treated

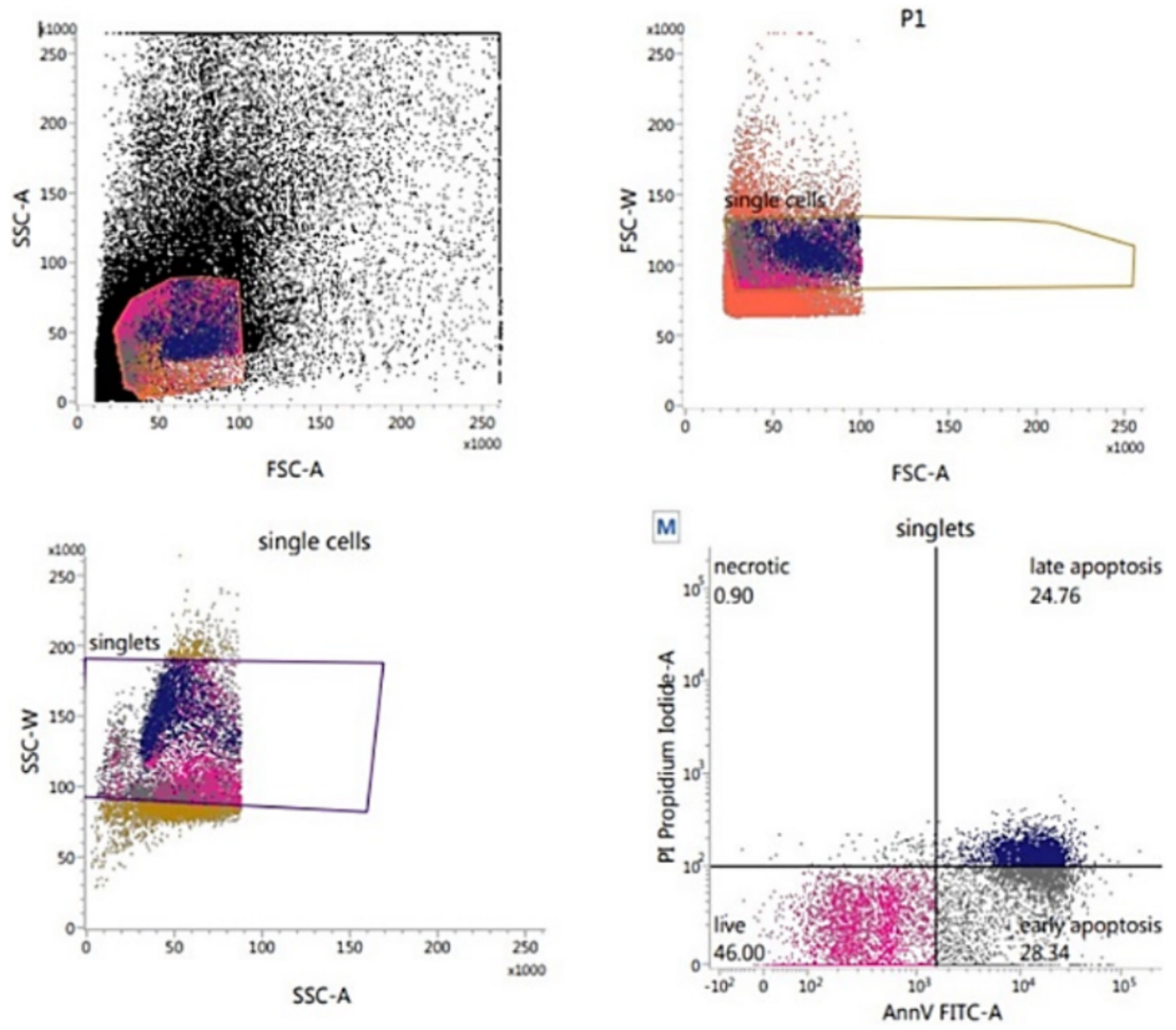

FIGURE 6
Flow cytometric evaluation of sperm apoptosis using Annexin-V apoptosis kit.

with MT at 5 and 20 μM, after 2 h of incubation, showed a significant increase in total sperm motility. On the contrary, after 3 h of incubation, a marked decrease in the total sperm motility was found when high MT concentrations (20, 40, and 80 μM) were used ($p < 0.01$), compared to controls. Interestingly, the progressive and rapid motilities were improved in all MT-treated samples when assessed directly after thawing ($p < 0.01$), with the best values recorded in the MT 20 μM group. However, a marked decrease in these parameters was recorded, after 1 and 2 h of incubation, in samples treated with MT at 80 μM, compared to the other experimental groups ($p < 0.01$). Compared to the controls, a significant decrease in the rapid sperm motility was recorded with the lowest concentration of MT (5 μM) after 3 h of incubation ($p < 0.05$).

3.2 Effect of MitoTEMPO on sperm velocity parameters

The different values of sperm velocity parameters under the effect of MT are presented in Table 2. MitoTEMPO incorporation in extender medium during cryopreservation modified several velocity parameters after thawing and incubation up to 3 h at 37°C. MitoTEMPO significantly decreased VAP values immediately after thawing in samples treated with 40 μM ($p < 0.01$), as well as, in all concentrations of MT compared to controls after 1 h of incubation. While, after 2 h, it decreased upon 5, 20, and 80 μM MT addition, and after 3 h incubation at 5 and 80 μM MT compared to the other experimental groups.

The values of VSL increased ($p < 0.01$) directly after thawing with the MT concentration of 5 μM, while decreased ($p < 0.01$) after 1 h with all concentrations of MT and after 2 h of incubation with 80 μM MT compared to controls. After 3 h of incubation, the VSL values were increased when 20 μM MT was used compared to the concentration of 5 μM.

VCL values were decreased after thawing in samples treated with 20 and 40 μM MT and after 1 h and in all MT-treated samples compared to the controls ($p < 0.01$). While after 2 h it decreased with the 5, 20, and 80 μM concentrations compared to the other groups, and after 3 h of incubation with the 5 μM concentrations compared to the controls ($p < 0.05$).

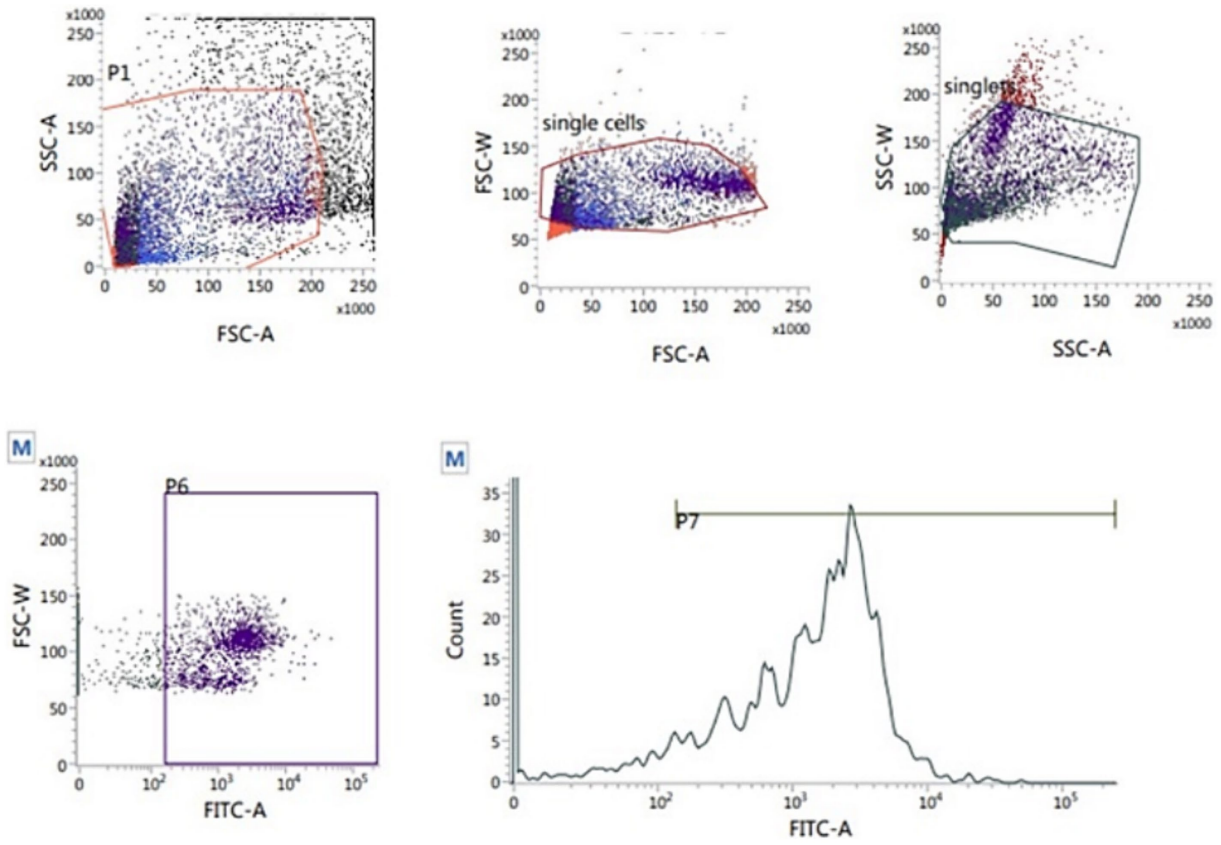

FIGURE 7
Flow cytometric evaluation of sperm ROS using T2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) staining kit.

Although ALH values decreased after thawing in samples treated with 20 µM MT compared to controls ($p < 0.05$), on the contrary, the values significantly increased in samples treated with 40 µM MT after 2 and 3 h of incubation.

BCF values decreased immediately after thawing in samples treated with 5 µM MT ($p < 0.05$), in all MT-treated samples ($p < 0.01$) after 1 h of incubation and in 80 µM MT-treated samples ($p < 0.01$) after 3 h of incubation, compared to the control group.

STR percentages increased in samples treated with 10, 20, and 40 µM MT immediately after thawing, at concentrations of 5 and 10 µM MT after 1 h of incubation, while they significantly decreased in samples treated with MT at the highest concentrations (20, 40, and 80 µM after 1 h and 40 and 80 µM after 2 h and 80 µM MT after 3 h of incubation) compared to the controls. The percentage of LIN increased ($p < 0.01$) after thawing with concentrations 10, 20, and 40 µM and after 1 h with the concentrations 5 and 10 µM, while decreased ($p < 0.01$) with the concentrations 40 and 80 µM after 1 h and with the concentrations 80 µM after 2 and 3 h of incubation compared to the controls.

3.3 Effect of MitoTEMPO on sperm vitality, acrosome, and plasma membrane integrities

As presented in Table 3, sperm vitality was increased in all MT-treated samples compared with controls ($p < 0.01$). In addition, the sperm acrosomal integrity was improved by 20 µM MT treatment ($p < 0.01$), whereas the plasma membrane integrity was not affected by MT treatments compared to controls.

3.4 Effect of MitoTEMPO on sperm apoptosis

As shown in Table 4, no statistical differences were recorded regarding the percentage of normal viable sperm and apoptotic sperm between MT-treated samples and controls, however, the group treated with 20 µM MT recorded the highest percentage of normal viable sperm and the lowest percentage of apoptotic sperm. Moreover, significant differences were recorded in the percentage of necrotic sperm between the groups treated with 20 and 40 µM MT with the lowest value recorded in the 20 µM MT samples ($p < 0.05$).

3.5 Effect of MitoTEMPO on sperm mitochondrial membrane potential, DNA integrity (SCSA) and ROS level

As presented in Table 5, a significant increase of sperm with high MMP was recorded if treated with 80 µM MT, compared to the other experimental groups ($p < 0.01$), while the DNA integrity was significantly ($p < 0.01$) improved in the 20 µM MT group compared to controls and the highest MT-tested concentration (80 µM). On the

TABLE 1 Effect of MitoTEMPO (MT) on motility parameters (Mean ± SEM) of cryopreserved Piedmontese bull semen after different incubation periods.

Parameter	Group	Post-thawing	1 h	2 h	3 h
Total motility (%)	Control	75.92 ± 1.19^{a}	63.44 ± 1.98^{ab}	42.94 ± 2.13^{ab}	37.15 ± 1.25^{a}
	MT 5 µM	77.69 ± 1.66^{abc}	65.88 ± 1.75^{abc}	48.77 ± 2.36^{b}	33.73 ± 1.36^{ab}
	MT 10 µM	81.42 ± 0.90^{c}	68.98 ± 2.19^{bc}	45.67 ± 1.84^{ab}	34.77 ± 1.35^{ab}
	MT 20 µM	81.19 ± 1.15^{c}	70.71 ± 1.81^{c}	48.40 ± 1.72^{b}	32.75 ± 1.65^{b}
	MT 40 µM	80.58 ± 1.01^{bc}	69.60 ± 1.80^{c}	42.96 ± 2.40^{ab}	30.79 ± 0.92^{b}
	MT 80 µM	77.00 ± 1.50^{ab}	61.06 ± 1.98^{a}	41.00 ± 1.60^{a}	31.54 ± 1.31^{b}
	Sig.	0.01	0.01	0.05	0.01
Progressive motility (%)	Control	39.06 ± 1.18^{a}	31.73 ± 1.35^{a}	13.63 ± 1.32^{a}	2.94 ± 0.46
	MT 5 µM	41.85 ± 0.95^{b}	31.46 ± 1.22^{a}	13.40 ± 1.06^{a}	2.06 ± 0.36
	MT 10 µM	44.46 ± 0.77^{bc}	32.63 ± 1.21^{a}	13.67 ± 0.89^{a}	2.90 ± 0.37
	MT 20 µM	46.31 ± 0.93^{c}	31.00 ± 0.83^{a}	10.77 ± 0.88^{a}	2.67 ± 0.49
	MT 40 µM	43.71 ± 0.80^{bc}	30.90 ± 1.27^{a}	10.94 ± 0.89^{a}	2.15 ± 0.28
	MT 80 µM	41.71 ± 0.82^{b}	25.96 ± 1.08^{b}	6.83 ± 0.62^{b}	2.08 ± 0.33
	Sig.	0.01	0.01	0.01	NS
Rapid motility (%)	Control	57.60 ± 1.29^{a}	42.56 ± 1.81^{a}	22.69 ± 1.70^{a}	11.79 ± 0.80^{a}
	MT 5 µM	60.94 ± 1.35^{b}	41.44 ± 1.57^{a}	22.04 ± 1.71^{a}	7.92 ± 0.89^{b}
	MT 10 µM	63.50 ± 0.74^{bc}	43.02 ± 1.58^{a}	23.85 ± 1.32^{a}	10.46 ± 0.68^{a}
	MT 20 µM	65.40 ± 1.00^{c}	44.31 ± 1.32^{a}	20.90 ± 1.12^{a}	9.40 ± 1.10^{ab}
	MT 40 µM	62.56 ± 0.92^{bc}	44.23 ± 1.58^{a}	21.69 ± 1.39^{a}	9.33 ± 0.59^{ab}
	MT 80 µM	60.73 ± 1.14^{b}	36.94 ± 1.46^{b}	16.75 ± 1.09^{b}	10.69 ± 0.82^{a}
	Sig.	0.01	0.01	0.01	0.05

Six replicates were performed. Sig, significance; NS, non-significant. The different superscripts within the same column indicate significant differences.

contrary, no significant differences have been found concerning intracellular ROS levels among the different experimental groups.

3.6 Effect of MT-treated semen on developmental potential of bovine embryos

On the basis of the results obtained on semen quality assessment, MT at a concentration of 20 µM was chosen to test the effect of MT-treated semen on the fertilizing potential of *in vitro*-produced embryos (Figure 8). A total of 193 cumulus-oocyte complexes (COCs) were cultured in six replicates (Table 6). Among these, 103 were fertilized with MT-extended semen, whereas 90 formed the control group. As shown in Table 6, the cleavage rate was significantly improved by using MT-treated spermatozoa compared with controls (46.6% vs. 38.9%; $p < 0.05$) and the blastocyst formation rates resulted in a significant increase in the MT group compared with controls (37.5% vs. 31.4%; $p < 0.05$).

4 Discussion

Semen cryopreservation aims to prolong spermatozoa life span and maintain their fertilizing capacity (41). Nevertheless, the cryopreservation process is known to induce detrimental changes in sperm cells including membrane disruption, considerable reactive oxygen species (ROS) production, lipid peroxidation, decreased sperm motility and mitochondrial activity as well as increased damage of DNA with lowered sperm fertility either *in-vitro* or *in-vivo* (42–46). In spermatozoa, the origin of intracellular ROS is mainly mitochondrial (44, 45) and in stressful condition such as cryopreservation, sperm is exposed to oxidative damage resulting from the uncontrolled production of ROS by the cryo-injured mitochondria (47). Therefore, the use of mitochondria-targeting antioxidants (e.g., MitoTEMPO, MT) in bull semen extender could improve post-thaw sperm fertility parameters.

The sperm motility and kinetics are associated with the sperm transportation capacity to the site of fertilization and are commonly used as semen quality evaluation criteria (48, 49). Mitochondria play an important role in preserving normal sperm function and energy balance through oxidative phosphorylation and ATP synthase (50). Indeed, cryogenic mitochondrial damage has been shown to exert a detrimental effect on sperm motility through impaired ATP transport processes (51). In the present study, MT incorporation in bull semen extender improved sperm motility parameters and sperm kinetics, including STR and LIN. In agreement with our results, MT was found to improve motility and velocity parameters of frozen–thawed human (26, 27), boar (31), rooster (28), buffalo (29), and buck (30) sperm, as well as ram-chilled sperm (24). In this study, the improvement of the sperm motility, observed immediately and even up to 2 h after thawing, could be attributed to the ameliorative action of the MT in preserving sperm viability in terms of mitochondrial membrane potential, acrosome integrity and reduction of cellular necrosis.

TABLE 2 Effect of MitoTEMPO (MT) on velocity parameters (Mean ± SEM) of cryopreserved Piedmontese bull semen after different incubation periods.

Parameter	Group	Post-thawing	1 h	2 h	3 h
VAP (μm/s)	Control	77.18 ± 0.48^{ab}	65.93 ± 0.53^{a}	54.53 ± 0.76^{a}	48.25 ± 0.44^{a}
	MT 5 μM	77.51 ± 0.30^{a}	62.74 ± 0.53^{b}	51.63 ± 0.88^{b}	45.50 ± 0.47^{b}
	MT 10 μM	76.32 ± 0.33^{ab}	62.70 ± 0.54^{b}	55.78 ± 0.61^{a}	47.46 ± 0.46^{a}
	MT 20 μM	76.07 ± 0.45^{bc}	62.49 ± 0.36^{b}	51.64 ± 0.64^{b}	48.10 ± 0.51^{a}
	MT 40 μM	75.03 ± 0.42^{c}	63.83 ± 0.52^{b}	54.29 ± 0.41^{a}	48.34 ± 0.37^{a}
	MT 80 μM	76.60 ± 0.37^{ab}	60.79 ± 0.51^{c}	50.99 ± 0.73^{b}	50.65 ± 0.45^{c}
	Sig.	0.01	0.01	0.01	0.01
VSL (μm/s)	Control	62.23 ± 0.34^{ac}	55.18 ± 0.48^{a}	43.33 ± 0.96^{ab}	33.88 ± 0.78^{ab}
	MT 5 μM	63.26 ± 0.34^{b}	53.01 ± 0.47^{b}	41.69 ± 0.84^{bc}	32.11 ± 0.69^{b}
	MT 10 μM	62.86 ± 0.29^{ab}	53.01 ± 0.44^{b}	44.56 ± 0.67^{a}	33.86 ± 0.67^{ab}
	MT 20 μM	62.64 ± 0.24^{ab}	51.85 ± 0.32^{b}	40.49 ± 0.69^{cd}	34.07 ± 0.67^{a}
	MT 40 μM	61.69 ± 0.28^{c}	52.53 ± 0.47^{b}	42.10 ± 0.58^{bc}	33.30 ± 0.54^{ab}
	MT 80 μM	62.63 ± 0.31^{ab}	50.27 ± 0.42^{c}	39.44 ± 0.69^{d}	32.92 ± 0.70^{ab}
	Sig.	0.01	0.01	0.01	0.05
VCL (μm/s)	Control	134.37 ± 0.88^{a}	112.65 ± 1.00^{a}	95.81 ± 1.04^{a}	85.25 ± 1.17^{a}
	MT 5 μM	134.29 ± 0.56^{a}	105.34 ± 0.95^{b}	90.22 ± 1.29^{b}	81.52 ± 1.08^{b}
	MT 10 μM	130.38 ± 0.64^{bc}	104.92 ± 0.78^{b}	97.07 ± 0.92^{a}	83.60 ± 1.12^{ab}
	MT 20 μM	129.45 ± 0.87^{c}	105.40 ± 0.51^{b}	90.74 ± 0.85^{b}	85.55 ± 0.76^{a}
	MT 40 μM	128.14 ± 0.81^{c}	108.11 ± 0.74^{c}	94.59 ± 0.69^{a}	83.98 ± 1.18^{ab}
	MT 80 μM	132.42 ± 0.88^{ab}	103.75 ± 0.88^{b}	89.92 ± 1.28^{b}	85.96 ± 1.41^{a}
	Sig.	0.01	0.01	0.01	0.05
ALH (μm)	Control	5.74 ± 0.03^{ab}	5.44 ± 0.03^{a}	5.72 ± 0.13^{ab}	4.76 ± 0.43^{a}
	MT 5 μM	5.82 ± 0.03^{a}	5.29 ± 0.03^{b}	5.44 ± 0.08^{b}	5.91 ± 0.50^{b}
	MT 10 μM	5.66 ± 0.03^{bc}	5.35 ± 0.03^{bc}	6.06 ± 0.06^{ac}	5.67 ± 0.40^{ab}
	MT 20 μM	5.62 ± 0.04^{c}	5.29 ± 0.03^{b}	5.52 ± 0.12^{b}	4.98 ± 0.33^{ab}
	MT 40 μM	5.69 ± 0.03^{bc}	5.50 ± 0.04^{a}	6.19 ± 0.15^{c}	5.98 ± 0.46^{b}
	MT 80 μM	5.73 ± 0.04^{ab}	5.41 ± 0.04^{ac}	5.47 ± 0.26^{b}	5.42 ± 0.45^{ab}
	Sig.	0.01	0.01	0.01	0.05
BCF (Hz)	Control	23.50 ± 0.17^{a}	21.69 ± 0.15^{a}	17.98 ± 0.39^{a}	15.35 ± 0.49
	MT 5 μM	23.15 ± 0.11^{b}	20.86 ± 0.12^{b}	18.05 ± 0.37^{a}	14.53 ± 0.37
	MT 10 μM	23.49 ± 0.14^{a}	20.81 ± 0.11^{b}	18.10 ± 0.27^{a}	15.19 ± 0.29
	MT 20 μM	23.64 ± 0.14^{a}	20.50 ± 0.16^{bc}	17.94 ± 0.33^{a}	14.91 ± 0.33
	MT 40 μM	23.54 ± 0.07^{a}	20.27 ± 0.12^{cd}	18.22 ± 0.23^{a}	14.64 ± 0.22
	MT 80 μM	23.35 ± 0.09^{ab}	19.94 ± 0.12^{d}	16.79 ± 0.42^{b}	14.59 ± 0.31
	Sig.	0.05	0.01	0.01	NS
STR (%)	Control	81.98 ± 0.26^{a}	84.31 ± 0.19^{a}	79.92 ± 0.80^{abc}	70.85 ± 1.15^{a}
	MT 5 μM	82.35 ± 0.16^{ac}	85.13 ± 0.11^{b}	81.27 ± 0.39^{a}	71.69 ± 0.98^{a}
	MT 10 μM	83.08 ± 0.16^{b}	85.15 ± 0.18^{b}	80.63 ± 0.44^{ab}	72.29 ± 0.94^{a}
	MT 20 μM	83.08 ± 0.30^{b}	83.48 ± 0.15^{c}	79.29 ± 0.50^{bc}	71.69 ± 0.97^{a}
	MT 40 μM	82.96 ± 0.22^{bc}	83.02 ± 0.17^{c}	78.58 ± 0.68^{c}	69.79 ± 0.84^{a}
	MT 80 μM	82.40 ± 0.21^{ac}	83.13 ± 0.23^{c}	78.50 ± 0.56^{c}	65.88 ± 0.88^{b}
	Sig.	0.01	0.01	0.01	0.01

(Continued)

TABLE 2 (Continued)

Parameter	Group	Post-thawing	1 h	2 h	3 h
LIN (%)	Control	48.79 ± 0.33[a]	50.71 ± 0.23[a]	46.31 ± 0.69[ab]	40.46 ± 0.65[ab]
	MT 5 µM	49.15 ± 0.23[a]	51.83 ± 0.13[b]	47.27 ± 0.47[a]	40.35 ± 0.54[ab]
	MT 10 µM	50.33 ± 0.16[b]	52.08 ± 0.16[b]	47.27 ± 0.42[a]	41.44 ± 0.53[a]
	MT 20 µM	50.54 ± 0.22[b]	50.77 ± 0.18[a]	45.90 ± 0.48[ab]	40.42 ± 0.60[ab]
	MT 40 µM	50.25 ± 0.22[b]	50.20 ± 0.14[c]	46.08 ± 0.51[ab]	40.44 ± 0.43[ab]
	MT 80 µM	49.33 ± 0.17[a]	49.94 ± 0.21[c]	45.48 ± 0.46[b]	39.04 ± 0.33[b]
	Sig.	0.01	0.01	0.01	0.05

Six replicates were performed. Sig, significance; NS, non-significant; VAP, average path velocity; VSL, straight linear velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, straightness ([VSL/VAP] × 100); LIN, linearity ([VSL/VCL] × 100). The different superscripts within the same column indicate the statistically significant differences.

TABLE 3 Effect of MitoTEMPO (MT) on cryopreserved Piedmontese bull semen vitality, acrosome, and plasma membrane integrities (Mean ± SEM).

Experimental groups	Sperm vitality (%)	Acrosome integrity (%)	Plasma membrane integrity (%)
Control	90.92 ± 0.87[a]	89.00 ± 0.90[a]	60.17 ± 1.75
MT 5 µM	92.92 ± 0.38[b]	90.50 ± 0.79[ab]	63.00 ± 1.78
MT 10 µM	93.17 ± 0.37[b]	90.75 ± 0.73[ab]	62.25 ± 1.80
MT 20 µM	93.08 ± 0.61[b]	91.83 ± 0.65[b]	61.50 ± 1.43
MT 40 µM	92.92 ± 0.58[b]	90.58 ± 0.85[ab]	61.50 ± 1.41
MT 80 µM	93.25 ± 0.52[b]	90.75 ± 0.84[ab]	60.92 ± 1.39

Six replicates were performed. Values with different superscripts within the same column differed significantly at $p < 0.01$.

TABLE 4 Effect of MitoTEMPO (MT) on cryopreserved Piedmontese bull semen apoptosis (Mean ± SEM).

Group	Normal viable sperm (%)	Necrotic sperm (%)	Apoptotic sperm (%)
Control	47.86 ± 1.60	2.23 ± 0.02[ab]	50.16 ± 2.18
MT 5 µM	46.93 ± 1.17	2.11 ± 0.36[ab]	50.96 ± 1.10
MT 10 µM	46.01 ± 0.85	2.52 ± 0.10[ab]	51.47 ± 0.80
MT 20 µM	50.44 ± 0.95	1.94 ± 0.30[b]	47.62 ± 0.79
MT 40 µM	47.00 ± 3.02	2.72 ± 0.33[a]	50.28 ± 2.76
MT 80 µM	46.25 ± 2.94	2.60 ± 0.10[ab]	51.19 ± 3.02

Six replicates were performed. Values with different superscripts within the same column differed significantly at $p < 0.05$.

TABLE 5 Effect of MitoTEMPO (MT) on cryopreserved Piedmontese bull sperm mitochondrial membrane potential (mean ± SEM).

Groups	HMMP (%)	DNA integrity (%)	ROS (%)
Control	49.25 ± 1.08[a]	93.80 ± 0.29[bc]	22.47 ± 1.56
MT 5 µM	48.62 ± 0.75[a]	94.80 ± 0.58[ab]	21.99 ± 1.35
MT 10 µM	48.93 ± 0.87[a]	95.06 ± 0.71[ab]	22.33 ± 1.30
MT 20 µM	49.37 ± 1.10[a]	96.05 ± 0.86[a]	23.07 ± 1.72
MT 40 µM	49.62 ± 0.36[a]	94.28 ± 0.30[ab]	20.19 ± 1.82
MT 80 µM	52.52 ± 1.28[b]	92.48 ± 0.73[c]	21.10 ± 1.88

Six replicates were performed. HMMP, high mitochondrial membrane potential. Values with different superscripts within the same column differed significantly at $p < 0.01$.

TABLE 6 Effect of MitoTEMPO-treated semen at 20 µM before cryopreservation on developmental potential of *in vitro*-produced bovine embryos.

Group	Evaluated COCS	Cleavage (%)	Blastocyst (%, cleaved)
Control	90	35 (38.9)[b]	11 (31.4)[b]
MitoTEMPO 20 µM	103	48 (46.6)[a]	18 (37.5)[a]

Six replicates were performed. Chi-square test: a, b: $p < 0.05$; COC, cumulus-oocyte complex.

Other studies reported that the main cause of decreased semen motility after thawing was related to the oxidative stress produced by the generation of ROS during the freezing and thawing processes (52). Precisely to overcome this limit, MT has been recently proposed as an antioxidant as it combines Tempo with triphenylphosphonium, capable of rapidly passing through the membranes of the lipid bilayer and accumulating in the mitochondria, where it is known to carry out its activity of elimination of mitochondrial superoxide anions (20, 53). In has been showed that the ROS scavenging ability of MT protected the sperm cells against oxidative stress, reduced lipid peroxidation, and maintained sperm membrane functionality and vitality (24, 26, 27). However, in the present study, MT did not reduce the intracellular ROS levels although a marked improvement in sperm quality was observed. We cannot exclude that other parameters such as lipid lipoperoxidation or the activity of specific antioxidant enzymes were influenced by MT, as demonstrated or exensively discussed in other species (24, 26–30, 54).

It has been reported that ROS and heat shocks decrease mitochondrial function, and induce mitochondrial fission, aggregation, and malfunction (51, 55, 56). Moreover, the imbalance between the production and elimination of free radicals creates oxidative stress, resulting in sperm apoptosis and DNA damage (57, 58). MitoTEMPO is a SOD mimetic antioxidant that preserves normal sperm integrity (59), and can be targeted to mitochondria for scavenging the superoxide anion and protecting against selective mitochondrial oxidant stress (60). In the present study, 20 µM MT improved DNA integrity and reduced the number of necrotic cells, however this result did not correlate with the improvement of mitochondrial activity, which was observed only after treatment with 80 µM MT.

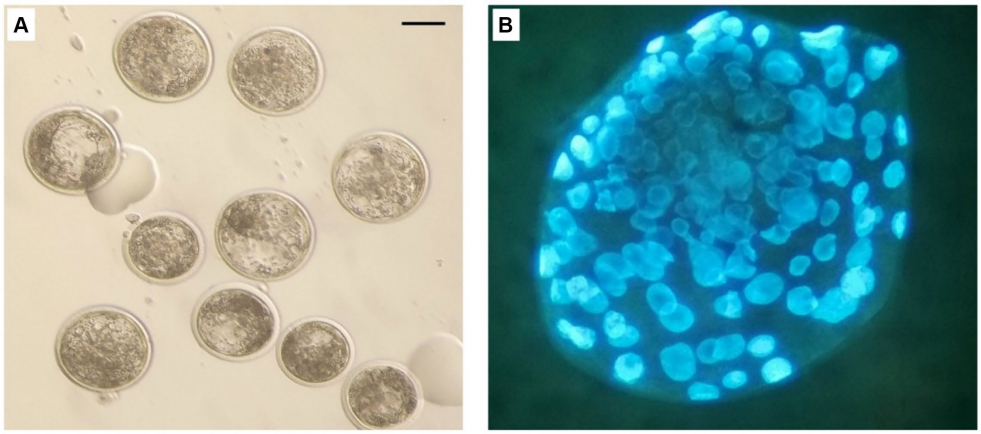

FIGURE 8
In vitro produced bovine blastocysts. **(A)** Normal blastocyst under stereo microscope. **(B)** blastocyst stained with Hoechst 33258. Scale bar: 100 μm.

MitoTEMPO has been reported to maintain mitochondrial function and viability during the freezing–thawing process through inhibition of mitochondrial Bax translocation (20), which is a Bcl-2 family pro-apoptotic member, that inserts into mitochondrial membranes upon cell death induction (61). In addition, MT inhibits the excessive generation and overflow of oxygen-free radicals caused by the sperm freezing–thawing process through its hydroxylamine-like structure (27). It has been stated that Glucose-6-phosphate isomerase (GPI), is an important glycolytic pathway enzyme, that loosely binds to mitochondria and is closely related to sperm quality (62). The sperm cryo-damage and stress lead to excessive release of GPI into the extracellular matrix (62, 63). MT has been reported to control the reduction of glucose-6-phosphate isomerase (GPI) activity and consequently leads to improved sperm quality (26, 28).

To our knowledge, there are no previous reports on the effect of MT on cryopreserved bull semen. In this study, MT-treated semen improved the developmental potential of bovine embryos as higher cleavage and blastocyst formation rates were obtained. Similarly, MT has been stated to improve the sperm *in vivo* fertilizing capacity of chilled (24) and frozen–thawed rooster (28) sperm. In addition, MT has been reported to improve the *in vitro* maturation and the blastocyst formation rates of bovine MT-treated oocytes (64). The improving effect of MT on the cleavage and formation rate of blastocysts could be attributed to its role in improving sperm motility and kinetic parameters which increased up to 2 h after thawing. We hypothesize this is due to the protective effect determined by MT during the cryopreservation process as it preserved cell viability in terms of acrosome and DNA integrity, reduction of cell necrosis which could explain the increase in sperm fertilizing capacity. In another study, MT has been stated to improve the quality and developmental potential of embryos through the regulation of mitochondrial functions by reducing the effect of superoxide (65). Further studies are needed to assess the fertilizing ability of sperm under the influence of MT using natural insemination and to evaluate conception and pregnancy rates.

In conclusion, the incorporation of MitoTEMPO (mainly at a concentration of 20 μM) in bull extended semen before cryopreservation has a positive protective and improving effect on different sperm parameters including motility and kinetics as well as sperm vitality, acrosomal intactness, DNA integrity, and sperm *in vitro* fertilizing capacity. Depending on these results, clinical application of MitoTEMPO could be profitable for bull frozen semen production centers.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because no experimental animals were used in this study. All procedures were performed in accordance with DPR 27/1/1992 (Animal Protection Regulations of Italy) in compliance with European Community Regulation 86/609. Please see file "Declaration" in the section "Additional files."

Author contributions

AE: Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. AR: Investigation, Writing – review & editing. AB: Investigation, Writing – review & editing. MP: Formal analysis, Investigation, Writing – review & editing. TN: Investigation, Writing – review & editing. GD: Formal analysis, Investigation, Writing – review & editing. LV: Conceptualization, Investigation, Supervision, Writing – review & editing. NM: Conceptualization, Formal analysis, Investigation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publisher's note

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Effect of cryopreservation and semen extender on extracellular vesicles isolated from bull semen

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Introduction: Semen cryopreservation is the most popular practice for semen production for artificial insemination and *in vitro* fertilization in cattle. The Seminal plasma contains extracellular vesicles (spEVs) which modulate sperm viability and function during oocyte fecundation. The study of spEVs in frozen-thawed semen doses may yield novel indicators for predicting bull fertility, but the presence of the semen extender may hinder molecular profiling of spEVs. The aim of this study was to provide extensive characterization of EVs isolated from seminal plasma before and after the cryopreservation process and the addition of a commercial animal protein-free semen extender to understand the potential influence of EVs originating from the extender in hindering the use of spEVs derived biomarkers for assessment of bull fertility.

Methods: EVs were isolated from the seminal plasma (with or without the extender), from the cryopreserved straw devoid of spermatozoa, and from the extender using two different methods, ultracentrifugation (UC) and size exclusion chromatography (SEC), and characterized for their structure and composition.

Results: Physical characterization of EVs showed that size and particle numbers were related to the method of isolation. spEVs were larger but less abundant (UC: 168.9 nm, $n = 2.68 \times 10^9$; SEC: 197.0 nm, $n = 6.42 \times 10^9$) compared to extender EVs (UC: 129.0 nm, $n = 2.68 \times 10^{11}$; SEC: 161.8 nm, $n = 6.47 \times 10^{11}$). Western blotting analysis (WB) confirmed the presence of typical EV markers in spEVS: the membrane bound CD9 (25 kDa) and the luminal markers Alix (96 kDa) and TSG101 (48 KDa). Although Transmission Electron Microscopy confirmed the presence of a lipid bilayer structure in all preparations, no specific EV markers were detected in the vesicles isolated from extender when the Single Molecule Array (SiMoa) was used. A total of 724 Bos taurus miRNAs were identified in at least one preparation. The percentage of miRNAs identified in EVs from the extender (0.05%–0.49% of the total reads) was lower than in the preparation containing spEVs (10.56%–63.69% of the total reads). Edge-R identified a total of 111 DE-miRNAs between EVs isolated from the extender by two methods. Among them, 11 DE-miRNAs (bta-miR-11980, bta-miR-11987, bta-miR-12057, bta-miR-1246, bta-miR-125b, bta-miR-181b, bta-miR-2340, bta-miR-2358, bta-miR-2478, bta-miR-2898, and bta-miR-345-3p) were also abundant in EVs isolated from seminal plasma preparations with extender.

Conclusion: This study clearly demonstrates that the presence of the extender does not prevent the characterization of spEVs in cryopreserved semen.

However, the molecular profiling of spEVs can be influenced by the isolation method used and by the presence of some miRNAs from the extender. Therefore, in such studies, it is advisable to characterize both spEVs and the vesicles isolated from the extender.

KEYWORDS

extracellular vesicles, seminal plasma, extender, cryopreservation, miRNA, bull

1 Introduction

In cattle farming, male infertility is closely related to production efficiency and is a major cause of economic losses. Bulls have a high inter-individual variability in fertility, hence assessment of sperm quality and sire fertility rate is important for breeding plans (1). In some cases, despite the use of bulls with high genetic value, full-term pregnancies are not obtained (2). This is also the case when employing high-merit bulls, based on their spermatozoa motility and morphology (3, 4), since this characteristic doesn't necessarily indicate the absence of molecular defects of the spermatozoa, which might affect fertilization or contribute to abnormal embryo development (5).

To optimize reproductive success many cattle breeders make use of assisted reproductive technologies such as sperm cryopreservation, artificial insemination (AI) and *in vitro* fertilization (IVF). AI strategies vary around the world. Far Eastern and some European countries favor the use of fresh semen, but the use of cryopreserved semen remains the most popular practice worldwide (6).

The use of cryopreserved semen allows breeders to estimate the bull potential fertility rate, by the assessment of specific events such as non-return rate (NRR) or estimated relative conception rate (ERCR). Several advanced technologies can be used to examine the quality of spermatozoa, such as computer-assisted semen analysis (CASA) and flow cytometry (FCM), which can provide accurate and objective evaluation of sperm function. The combination of kinetic semen parameters originating from CASA and DNA analysis based on FCM seems able to classify fertility levels in bulls even in the high-fertility range (7). Moreover, to better predict bull fertility, a combined approach that integrates semen quality evaluation with advanced technologies and sperm molecular characterization using different modern approaches can be advantageous (8). In recent years, several studies combining methods traditionally used in spermatology with techniques of molecular biology that target sperm microRNAs (miRNAs) (9) or integrating multi-omics approaches like transcriptomic, proteomic and metabolomic analysis (10), were able to identify molecules potentially influencing bull fertility.

Although some specific molecular markers were found to be associated with different sperm quality traits, like sperm motility, acrosome, plasma membrane and DNA integrity, their potential for large-scale employment is still limited (11).

These studies strictly evaluated molecular composition of sperm, but it is known that several other molecules present in the ejaculate can support oocyte fertilization and enhance embryo development.

Seminal plasma (SP) plays a key role in modulating fertility by modulating sperm viability and function, interacting with the various compartments of the female genital tract and serving as a carrier of signals regulating the female immune system (12, 13).

SP is a heterogeneous composite fluid which contains inorganic ions, specific hormones, proteins, cholesterol and extracellular vesicles that interact with the various compartments of the tubular genital tract preparing for an eventual successful pregnancy (13). Extracellular vesicles (EVs) are membrane-enclosed microparticles originating from outward budding of plasma membranes by active secretion that that mediate cell to cell communication in proximity to, or distant from, the cells of origin. EVs are usually isolated from complex body fluids by different methods such as ultracentrifugation, density gradient centrifugation, precipitation with polymers and size exclusion chromatography (14).

Seminal plasma contains a very high number of EVs compared to other biological fluid (15). EVs contained in the seminal plasma (spEVs) are released by the testis, epididymis and male accessory glands, such as prostate and vesicular glands (16). Accordingly, spEVs exchange active molecules with mature sperm and endometrial epithelial cells and regulate sperm motility, capacitation, and acrosome reaction and also facilitate the safe transit of spermatozoa through the female genital tract (17). Supplementation of spEVs was observed to improve fertilizing capacity of bulls in *in-vitro* experiments (18). Alteration of SP and spEV molecular composition was also reported in different andrological diseases. For example, proteomic characterization of seminal plasma collected from infertile men with unilateral varicocele showed dysregulation of the exosome associated protein (19), while miRNA profiles of spEVs in human patients with oligoasthenozoospermia was altered. In cattle, different studies reported potential seminal plasma biomarkers for bull fertility. Metabolomic characterization of SP identifies specific metabolites whose abundance potentially correlates with bull fertility (20). Recently, seminal plasma small RNA profiling in high- and low-fertile Holstein bulls revealed alteration in miRNAs targeting genes potentially regulating sperm function and structure, fertilization, and placental and embryo development (21).

However, all these studies reported molecular characterization of spEVs isolated from fresh ejaculate. In order to optimize production and preserve individuals of high genetic value, natural mating has been passed over to make way for AI, which involves cryopreservation of semen with extender. A preliminary study is needed to clarify whether the vesicular component present in the extender can influence the detection of fertility markers in EVs.

This study aimed to characterize EVs isolated from seminal plasma before and after extender addition and

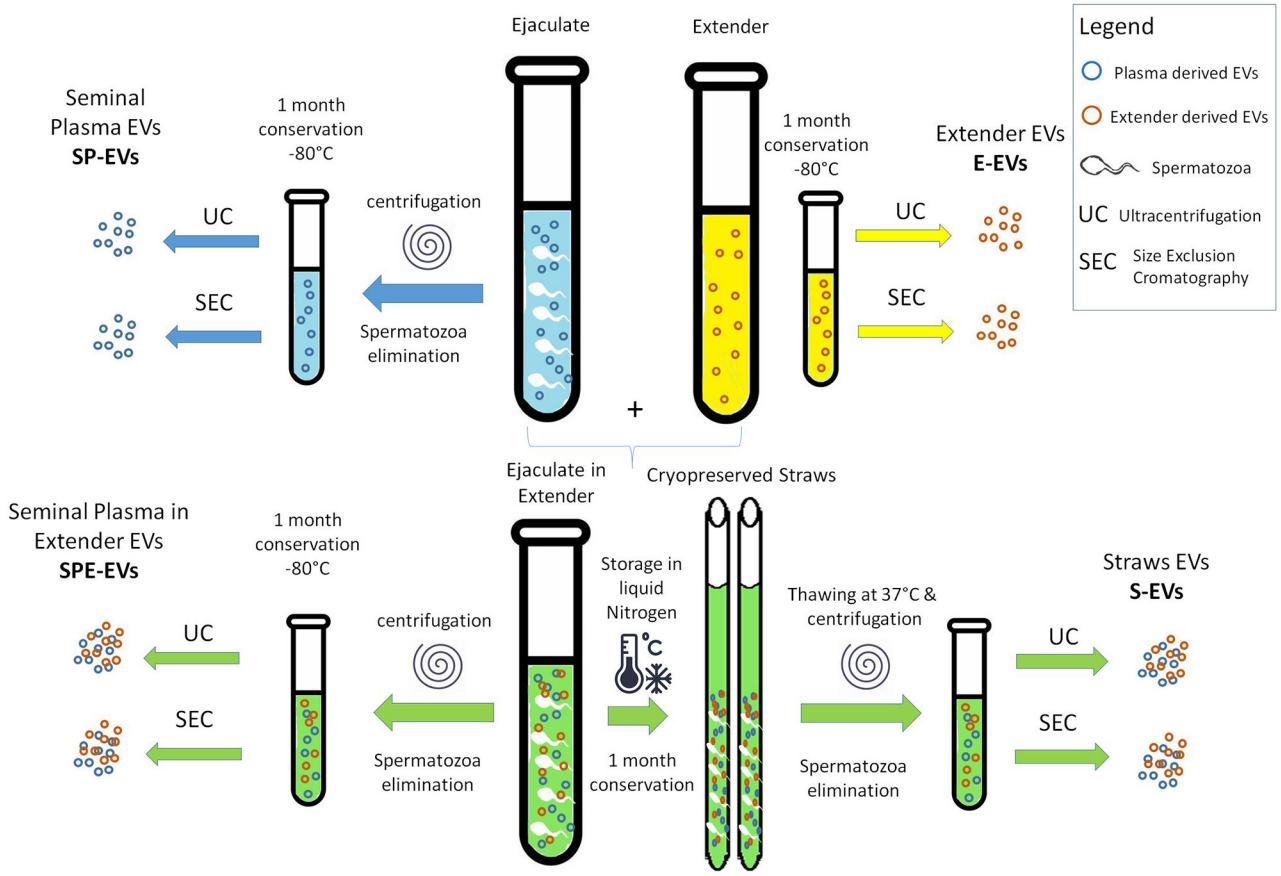

FIGURE 1
Scheme of the experimental design.

cryopreservation, to evaluate the potential use of spEV-derived biomarkers from cryopreserved semen straws in assessing bull fertility.

2 Materials and methods

2.1 Isolation of EVs

2.1.1 Biosample collection and semen quality

The experimental plan is reported in Figure 1. Five fresh ejaculates from five proven and fertile Italian Holstein bulls were collected at an Artificial Insemination (AI) center (GB GENETICS COFA SRL, Cremona, Italy) using an artificial vagina (Supplementary Table S1). Semen evaluation was performed by the personnel of the AI center on fresh samples. In particular semen concentration was evaluated with a Accucell photometer (IMV Technologies) and sperm motility was evaluated subjectively using a phase contrast microscope. Total motility and sperm kinetics parameters were assessed on post-thaw semen by CASA system (ISAS®v1, Proiser, R+D S.L., Paterna, Spain) combined with a phase contrast microscope (Nikon Optiphot) equipped with a negative phase contrast 10× objective and integrated warmer stage and connected to a video camera (Proiser 782M, Proiser R+D).

2.1.2 EV preparation

EVs were isolated as follows. Immediately after semen collection each ejaculate was divided (i) half was centrifuged (6,000 g for 5 min) to remove spermatozoa, and seminal plasma and then stored –at 80°C (SP). A second half was extended with a commercial animal protein-free semen extender (BioXcell: IMV, L'Aigle, France) and (ii) devoided of spermatozoa (6,000 g for 5 min) and the seminal plasma with extender stored at −80°C (SPE), or (iii) used to prepare straws for cryopreservation in liquid nitrogen (S). (iv) An aliquot of pure semen extender was also stored at −80°C (E). After 1 month, the cryopreserved straws (S) were thawed at 37°C for 1 min and centrifuged (6,000 g for 5 min) to remove the spermatozoa. SP, SPE, and E samples stored at −80°C were also thawed.

A pool of five bull's samples, was created from each (SP, SPE, S, and E) sample five that was split into three aliquots to obtain three biological replicates ($n = 3$). Samples underwent two sequential centrifugations at 4°C (600 g for 20′ and 4,000 g × 20′), and the supernatants were used for EV isolation by ultracentrifugation (UC) and size exclusion chromatography (SEC) to obtain the different preparations (SP-EVs_UC, SP-EVs_SEC, SPE-EVs_UC, SPE-EVs_SEC, S-EVs_UC, S-EVs_SEC, E-EVs_UC, and E-EVs_SEC) as reported in Table 1.

Ultracentrifugation was performed at 100,000 g (Beckman Coulter OptimaX, Milan, Italy), at 4°C for 1 h. The pellet was resuspended in a serum-free medium with 1% dimethylsulfoxide and stored at −20°C. SEC was performed on pEV10 column (IZON, Medford, MA, USA), following the manufacturer's instruction.

2.2 EV characterization

EVs isolated from each preparation were characterized according to the MISEV2023 guidelines (22).

2.2.1 EVs nanoparticle tracking analysis

Number, dimension and quantity of isolated particles were determined by Nanoparticle tracking analysis (NTA) using a NanoSight NS300 system (Malvern Technologies, Malvern, UK) configured with 532 nm laser, according to the manufacturer's instruction and previously reported method (23). Each EV preparation was diluted to achieve a final volume of 1 ml in filtered PBS to obtain the ideal particle per frame (about 20–100 particles/frame). Samples were injected with a constant flow and three videos of 60 s were captured and analyzed with Malvern NTA software version 3.2. Particle size and concentration were expressed in nanometer (nm) and in particles/mL.

2.2.2 Western blotting

EV proteins were evaluated by western blotting, 8 uL of reduction buffer (Laemmli buffer) was added to 32 uL of each EV preparation and the sample boiled for 5 min at 95°C; western blot analysis was performed according to method in previously published paper (24). All preparations were separated by SDS-PAGE (4%−20%, Mini-Protean TGX Precast protein gel, Bio-Rad) and blotted onto a nitrocellulose membrane (BioRad, Trans-Blot Turbo). To saturate non-specific sites a blocking step for 1 h with 5% (w/v) BSA in T-TBS (tris-buffered saline: 150 mM NaCl, 20 mM TrisHCl, pH 7.4, and 0.5% Tween 20) was performed. Primary antibody incubation with anti-CD9 (1:1,000, BD Pharmingen), anti-Alix (1:1,000, Santa Cruz, CA, USA), and anti-TSG101 (1:1,000, Novus Bio, Centennial, CO, USA) was performed overnight at 4°C. The next day membranes were washed three times with T-TBS, and incubated with the secondary antibodies horseradish peroxidase-conjugated (Jackson ImmunoResearch, Tucker, GA, USA) diluted 1:3,000 for 1 h. After final washing, the Bio-Rad Clarity Western ECL Substrate (Bio-Rad) was added, and signal detected using a Chemidoc XRS + (BioRad).

2.2.3 Single molecule array

SiMoA beads conjugation was performed according to Quanterix Homebrew kit instructions using the recommended buffers (25). In pan-tetraspanin three-step assay, beads solution was prepared at the concentration of 2×10^7 beads/ml in Bead Diluent. The detector antibody (biotinylated CD9, CD63, and CD81 antibodies by Ancell) solutions (0.3 µg/ml) were diluted in Homebrew Sample Diluent (Quanterix), while the EV preparations were diluted 1:4 in Homebrew Sample Diluent (Quanterix). Twenty five µl of beads were transferred into a 96 microwell plate and 100 µl of diluted sample added, incubated for 30 min at 25°C at 800 rpm. After incubation, beads were washed with an automatic plate-washer and then incubated for 10 min with 100 µl of detector antibody. Then, beads were washed and incubated for 10 min with a 150 pM SBG solution (in SBG Diluent, Quanterix). After SBG incubation step, final washes were performed and the plate was inserted into the Quanterix SR-X instrument for analysis where

TABLE 1 The table reports the extracellular vesicle (EV) preparations obtained from different sources using two isolation methods: ultracentrifugation (UC) and size exclusion chromatography (SEC).

Source	Replicates	EVs isolation method	EVs preparation name
Seminal plasma	Pool 1	UC	SP-EVs_UC_1
Seminal plasma	Pool 2	UC	SP-EVs_UC_2
Seminal plasma	Pool 3	UC	SP-EVs_UC_3
Seminal plasma	Pool 1	SEC	SP-EVs_SEC_1
Seminal plasma	Pool 2	SEC	SP-EVs_SEC_2
Seminal plasma	Pool 3	SEC	SP-EVs_SEC_3
Seminal plasma in extender	Pool 1	UC	SPE-EVs_UC_1
Seminal plasma in extender	Pool 2	UC	SPE-EVs_UC_2
Seminal plasma in extender	Pool 3	UC	SPE-EVs_UC_3
Seminal plasma in extender	Pool 1	SEC	SPE-EVs_SEC_1
Seminal plasma in extender	Pool 2	SEC	SPE-EVs_SEC_2
Seminal plasma in extender	Pool 3	SEC	SPE-EVs_SEC_3
Cryopreserved straws	Pool 1	UC	S-EVs_UC_1
Cryopreserved straws	Pool 2	UC	S-EVs_UC_2
Cryopreserved straws	Pool 3	UC	S-EVs_UC_3
Cryopreserved straws	Pool 1	SEC	S-EVs_SEC_1
Cryopreserved straws	Pool 2	SEC	S-EVs_SEC_2
Cryopreserved straws	Pool 3	SEC	S-EVs_SEC_3
Extender	Pool 1	UC	E-EVs_UC_1
Extender	Pool 2	UC	E-EVs_UC_2
Extender	Pool 3	UC	E-EVs_UC_3
Extender	Pool 1	SEC	E-EVs_SEC_1
Extender	Pool 2	SEC	E-EVs_SEC_2
Extender	Pool 3	SEC	E-EVs_SEC_3

RGP is automatically added. Data were analyzed and processed by Reader Software SiMoA 1.1.0.

2.2.4 Transmission electron microscopy

For EV analysis, 10 μl of each vesicle suspension were placed on Parafilm (Bemis, Neenah, WI, USA). Formvar-coated copper grids (Electron Microscopy Sciences, Hatfield, PA, USA) were placed on top of the drops with the coated side facing the suspension. The EVs were adsorbed onto the grid for 1 h at room temperature in a humidity chamber. The grids were then briefly washed in 0.1 M phosphate buffer saline (PBS), pH 7.3, rinsed with distilled water and contrasted with 2% uranyl acetate (Electron Microscopy Sciences). The grids were observed under a Philips EM 208 microscope equipped with a digital camera (University Centre for Electron and Fluorescence Microscopy, CUMEF, Perugia, Italy).

2.3 miRNA profiling of EVs

2.3.1 RNA isolation

RNA was extracted from all isolated EVs with TRIzol (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. After centrifugation (12,000 g 15′, 4°C), upper aqueous solution containing RNA was cleaned-up with the NucleoSpin miRNA kit (Macherey–Nagel, Germany), following the protocol in combination with TRIzol lysis with small and large RNA in one fraction (total RNA). Concentration and quality of RNA were determined using RNA 6000 Pico Kit for 2100 Bioanalyzer (Santa Clara, CA, USA). The isolated RNAs were stored at −80°C until use.

2.3.2 Library preparation and sequencing

In total, 24 small RNA libraries were obtained from EVs isolated by different methods ($n = 2$), different preparations ($n = 4$), and replications ($n = 3$). Small RNA libraries were prepared using QIAseq miRNA Library Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Concentration and profile of libraries were determined by High Sensitivity DNA kit for Agilent 2100 Bioanalyzer. Libraries were pooled and sequenced on a single lane of Illumina Novaseq X (San Diego, CA, USA).

2.3.3 Data analysis

Illumina raw sequence analysis was carried out with the nf-core/smrnaseq pipeline, v2.2.4 (26). The pipeline performs a number of steps, encompassing sequence quality control and trimming, and reads alignment to *Bos taurus* miRNAs available at miRBase (http://www.mirbase.org/). MiRNA identification and quantification is performed by MiRDeep2 and its modules. The Bioconductor edgeR package (version 2.4) was used to identify statistically significant differential expression between groups of samples (false discovery rate [FDR] < 0.05) (27). MicroRNA cluster analysis was performed with Genesis (version1.8.1) (28). Venn diagrams were produced with InteractiVenn (29). Statistics of SiMoA data was performed by GraphPad PRISM 9.0 (La Jolla, California); *t*-test analysis was used to evaluate the significant different, p-value < 0.05 was considered significant.

3 Results

3.1 Biosamples collection, semen quality and isolation of EVs

All the data about semen quality are available in the Supplementary Table S2.

Semen volume was between 3.16 ml and 5.14 ml, and sperm concentration ranged from 575 to 1,188 × 106 sperm/ml. The subjective motility evaluated on fresh semen at the AI center was between 77% and 80%. On post-thaw samples the motility evaluated by CASA system ranged from 65.3% and 77.8%.

EVs were collected from different preparations (Figure 1):

- EVs from seminal plasma devoid of spermatozoa (SP-EVs).
- EVs from seminal plasma devoid of spermatozoa, with addition of the extender (SPE-EVs).
- EVs from cryopreserved sperm, devoid of spermatozoa (S-EVs).
- EVs from the extender (E-EVs).

All EV preparations were obtained in triplicate by combining a pool of seminal plasma from five different bulls, using two different isolation methods: Ultracentrifugation (UC) and Size Exclusion Chromatography (SEC). Table 1 reports all EV preparations analyzed in this study.

3.2 EV characterization

EVs isolated from each preparation were characterized according to the MISEV2023 guidelines (22). In order to evaluate the size distribution and the particle concentration, Nanoparticle Tracking Analysis (NTA) was performed on each sample. Overall, some statistically significant differences in terms of EV recovery yield and size comparing UC and SEC isolation methods and different preparations, were noted (Figure 2). A significantly higher number of larger particles were recovered from the SEC preparations SP-EVs (SEC: 197.0 nm ± 14.9; UC: 168.9 nm ± 7.6) and E-EVs (SEC: 161.8 nm ± 7.3; UC: 120.0 nm ± 4.2), except for SPE-EVs (SEC: 153.7 nm ± 23.5; UC: 137.2 nm ± 7.6), and S-EVs (SEC: 158.8 nm ± 16.7; UC: 133.8 nm ± 7.8) for which differences in size were not significant, compared to UC preparations (Figure 2A).

For both isolation methods, preparations containing the extender: SPE-EVs (SEC, $n = 2.38 \times 10^{11}$ nm; UC, $n = 1.20 \times 10^{11}$ nm), S-EVs (SEC, $n = 4.88 \times 10^{11}$ nm; UC, $n = 1.69 \times 10^{11}$ nm), and E-EVs (SEC, $n = 6.47 \times 10^{11}$ nm; UC, $n = 2.68 \times 10^{11}$ nm) showed a significantly higher number of particles when compared to SP-EVs (SEC, $n = 6.42 \times 10^{9}$ nm; UC, $n = 2.68 \times 10^{9}$ nm) (Figure 2B). In addition, E-EVs were significantly smaller when compared to SP-EVs and affected the size distribution of SPE-EVs and S-EVs (Figure 2A).

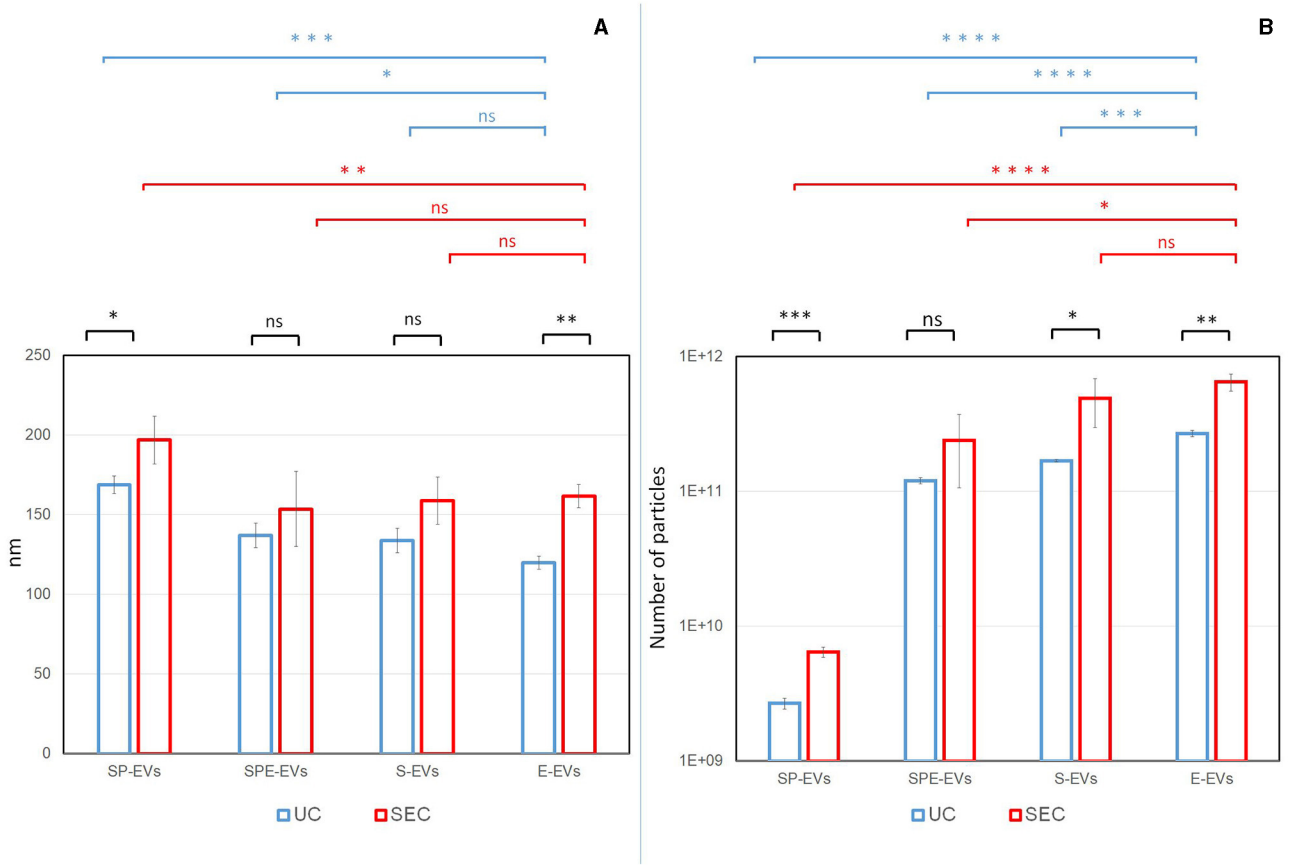

FIGURE 2
Particle size distribution **(A)** and concentration **(B)** were assessed by Nanoparticle Tracking Analysis (NTA). For each preparation (SP-EVs: EVs from seminal plasma devoid of spermatozoa, SPE-EVs: EVs from seminal plasma devoid of spermatozoa with addition of the extender, S-EVs: EVs from cryopreserved sperm devoid of spermatozoa, and E-EVs: EVs from the extender) and isolation methods (UC: Ultracentrifugation, SEC: Size Exclusion Chromatography), the replicates average for size and particle number were reported. Statistical differences were calculated: between two methods (in black), between different separation for UC (in blue) and between different separation for SEC (in red). *P*-value: (*) < 0.05, (**) < 0.01, (***) < 0.001, (****) < 0.0001.

It is worth noting that NTA particle count is not specific for EVs and that protein aggregates, lipoproteins and other non-vesicular material could contribute to the particle count providing an overestimation of EV concentration. These issues can be addressed by performing additional analyzes at the molecular level.

Transmission electron microscopy (TEM) showed the presence of EVs in all preparations containing plasma and in the extender for both isolation methods (Figure 3). Western blotting analysis (WB) was performed on all SP-EV preparations isolated by UC and SEC, to confirm the presence of typical EVs' markers: the membrane bound CD9 (25 kDa) and the luminal markers Alix (96 kDa) and TSG101 (48 KDa) (Figure 4A). UC derived SP-EVs give rise to more intense signals, suggesting a higher recovery. A quantitative analysis of EVs in three different pools of each preparation was performed by Single Molecule Array (SiMoa), according to recently developed protocols for ultra-sensitive pan-tetraspanin detection (25) (Figure 4B). In this bead-based immune-assay, antibodies against tetraspanin (CD9, CD63, and CD81) conjugated onto paramagnetic beads are used to capture EVs, while detection is based on the use of biotinylated anti-tetraspanin antibodies. Exploiting SiMoA technology, we were able to detect tetraspanin signals that is directly proportional at the amount of EVs in each preparation. Signal is expressed in Average Enzyme per Bead (AEB) (Supplementary Table S3). Statistical significate was evaluated comparing all EVs samples with diluent preparation; it was observed that all UC preparations have a significantly different compare to extender, the same was observed for the SEC preparations but excluding SP-EVs that do not show a statistically different with E-EVs. Overall, a clearly trend is evident, SP-EVs gave a high signal, while SPD-EVs and S-EVs were less intensive but higher then E-EVs. The most striking result is the absence of tetraspanin signal in E-EVs (AEB signal is similar to the negative control).

3.3 miRNA profiling in EVs

About 23.73 ± 12.49 million reads were sequenced for EVs isolated from all the preparations (Supplementary Table S4). The proportion of sequences classified as miRNAs was variable in the different datasets, depending on the type of preparation and isolation method. Overall, a greater proportion of miRNA

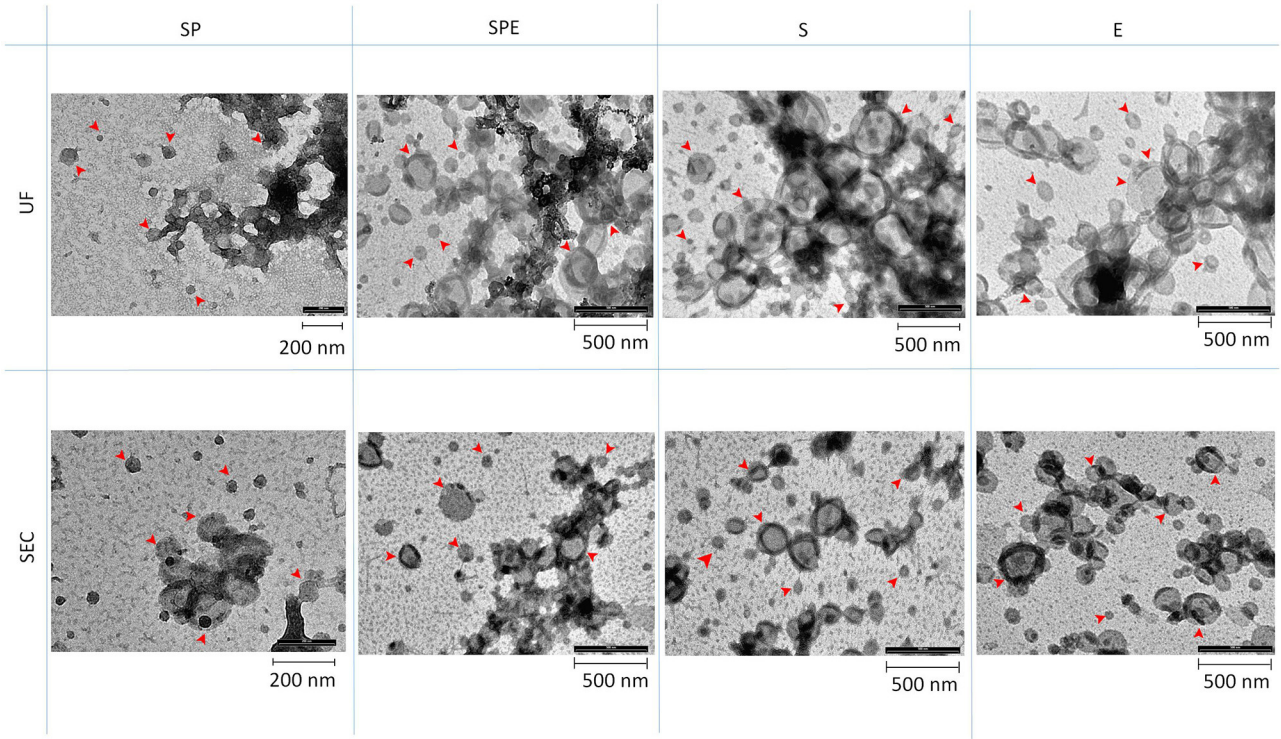

FIGURE 3
Transmission Electron Microscopy (TEM) for different preparations: SP, SPE, S, and E, isolated by Ultracentrifugation (UC) and Size Exclusion Chromatography (SEC). Arrows indicate EVs.

sequences among total sequences was observed in datasets derived from the UC isolation method. In agreement with EV characterization, miRNA profiling showed a very low content of miRNAs in the E-EV samples (0.05% with UC, 0.10% with SEC), whereas the proportion of miRNAs in total sequences in other preparations was about 23.89%, with the exception of SP-EVs obtained by UC, showing the highest percentage of assigned miRNAs (59.31%). A total of 724 *Bos taurus* miRNAs was detected in at least one sample (Supplementary Table S5). Principal component analysis of the 103 miRNAs counted at least once in all 24 samples clearly separates E-EVs vs. other samples on Principal Component 1, explaining 33.86% of the variance, indicating that the expression of miRNAs in the extender was different from SP-EVs (Figure 5A). Seminal plasma EVs from different preparations (SP, SPE, and S) cluster closely and are not distinguishable, bur their miRNA content seems, in part, to be influenced by the methods used for vesicle isolation. Observing PCA results on the 20 most abundant miRNAs present in the extender, it is evident that EVs from the extender differ from those from seminal plasma, but it is also evident that 5 out of 6 SP samples group apart from samples from other two preparations (SPE and S) containing the extender (Figure 5B).

PCA of miRNAs detected in the three EV preparations containing seminal plasma, excluding the extender, showed a partial separation between EVs isolated with UC and SEC (Figure 5C).

The comparison between EVs from the extender and EVs from other preparations containing seminal plasma showed 48 and 94 differentially expressed miRNAs (DE-miRNAs) for UC and SEC isolation methods, respectively (Supplementary Table S3) of which 31 DE-miRNAs were identified for both isolation methods. DE-miRNAs calculated between SP, SPE and S groups in EVs isolated by both UC and SEC methods show no variations among preparations containing the extender (Supplementary Table S6). On the contrary, SP showed 8 (UC) and 19 (SEC) DE-miRNAs and 6 (UC) and 26 (SEC) DE-miRNAs when compared to SPE and S, respectively (Figure 5D). Considering, all the 111 DE-miRNAs (E-EVs vs. other, by UC or SEC) and 36 DE-miRNAs (SP-EVs vs. SPE-EVS or S-EVS, by UC and SEC) we found 11 DE-miRNAs (bta-miR-11980, bta-miR-11987, bta-miR-12057, bta-miR-1246, bta-miR-125b, bta-miR-181b, bta-miR-2340, bta-miR-2358, bta-miR-2478, bta-miR-2898, and bta-miR-345-3p) that were over-represented in the EVs isolated from extender and were found abundantly in spEVS with added extender.

4 Discussion

To the best of our knowledge this is the first work describing spEV characterization in fresh ejaculate and after semen extender addition and cryopreservation. For this study, EVs were isolated using the two gold standard methods, UC and SEC. When compared with the UC method, SEC showed an increase of separation yield and a different distribution of oversized particles. A previous work evaluating the impact of isolation methods on human spEV characterization using alternative methods based on

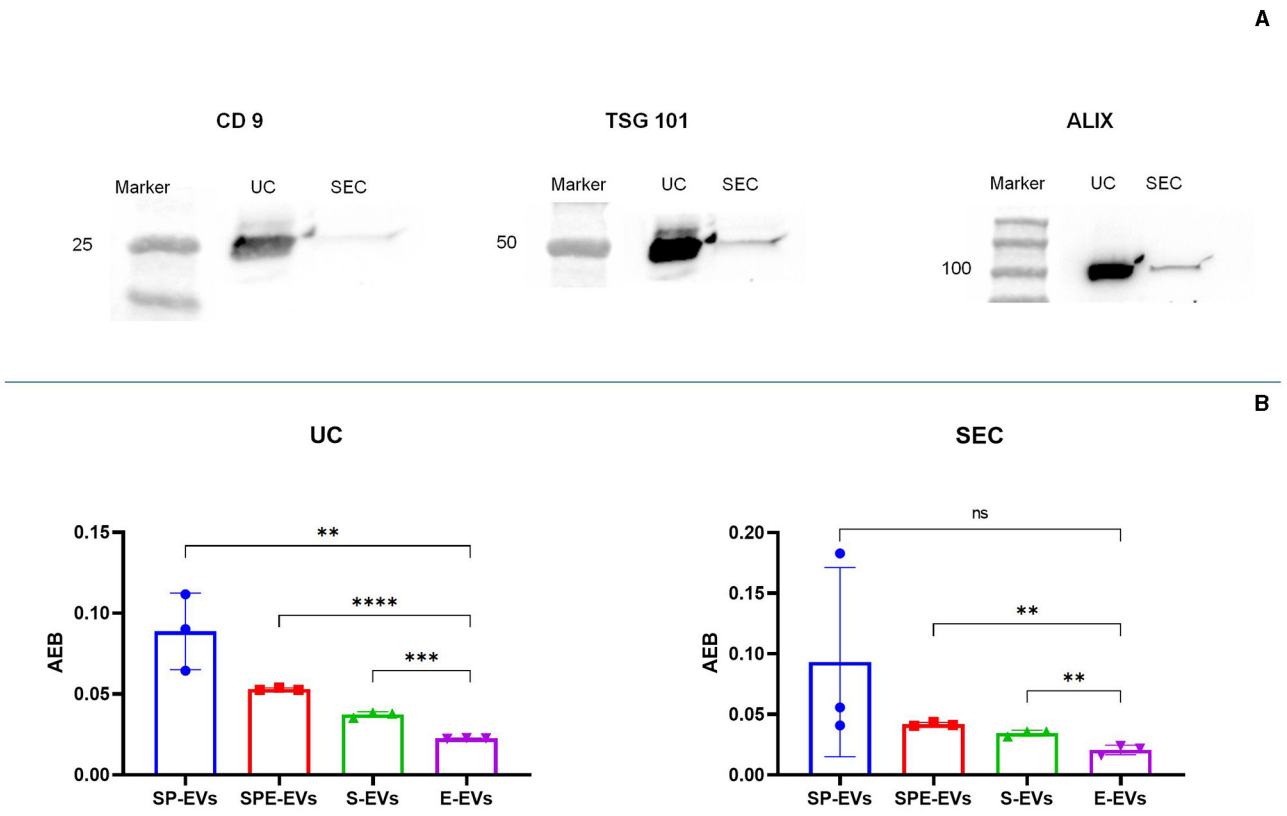

FIGURE 4
Characterization of vesicular protein markers using: **(A)** western blot characterization of sp-EV preparation using a membrane marker (CD9) and internal markers (TSG 101 and Alix), **(B)** single molecule array (SiMoa) was performed on all preparations in order to evaluate tetraspanin expression; signal is reported in AEB (Avarege Enzyme per Bead), intensity is proportional at the amount of tetraspanin (CD9, CD63 or CCD81) expressed on EVs' surface; in this way AEB signal allowed us to quantify the amount of EVs in each sample. Statistical difference was calculated by *t*-test analysis, (p value < 0.05, significant). P-value: (**) < 0.01, (***) < 0.001, (****) < 0.0001.

precipitation reagents, showed that different EV isolation methods displayed different size profiles, including size mean and mode, when compared to the ones obtained with the UC method (30). Although we did not find any other studies reporting application of SEC and UC methods in isolating spEVs, both methods were applied for isolating EVs from plasma and cell culture conditioned medium, giving contrasting results. NanoFCM analyzes of plasma EVs showed that a higher number of particles with similar size were isolated by SEC compared to UC (31), but SEC isolated fewer EVs particles in conditioned medium (32). Interestingly, in agreement with our results, the plasma miRNAome differed between UC and SEC isolation methods (31).

In our work, there was a noticeable difference using NTA between EVs isolated from seminal plasma and extender for both isolation methods. spEVs were oversized particles but less abundant, while extender particles were smaller but found in a higher number. Preparations containing both seminal plasma and extender showed particles of an intermediate size and an EV abundance comparable with the samples containing extenders. NTA is a robust method, able to accurately measure the size distribution and the total concentration of the EV preparation being studied, but it is not specific for EVs, detecting also other particles, such as protein aggregates, lipoproteins and cellular debris (33). The extender BioXcell contains vegetable components, and its chemical composition reports the presence of soy lecithin (34). This substance is able to form vesicles with a size similar to that of the EVs collected from the extender (35). TEM analysis confirmed the presence of EVs in all preparations including the extender, conversely SIMOA analysis affirmed the presence of tetraspanin: CD9, CD63, and CD81 markers, in all preparations except for the extender. It is plausible that the extender contains vesicles derived from soy lecithin. In fact, when we characterized the miRNA content in all preparations, only a very small proportion of the total reads obtained from extender samples identified miRNAs, and expression of these was very different from other preparations.

Some of the most abundant miRNAs found in E (bta-miR-11980, bta-miR-11987, bta-miR-12057, bta-miR-1246, bta-miR-125b, bta-miR-181b, bta-miR-2340, bta-miR-2358, bta-miR-2478, bta-miR-2898, and bta-miR-345-3p), were also found significantly highly expressed in SPE or S samples compared to SP and are derived from extender addition. To the best of our knowledge these miRNAs have not previously been reported to be associated with sperm fertility. Finally, cryopreservation seems not to alter miRNAs composition. Changes in miRNA composition between sperm isolated from fresh ejaculate and after cryopreservation were previously observed in mice and human (36) and bull sperm (37). To the best of our knowledge seminal

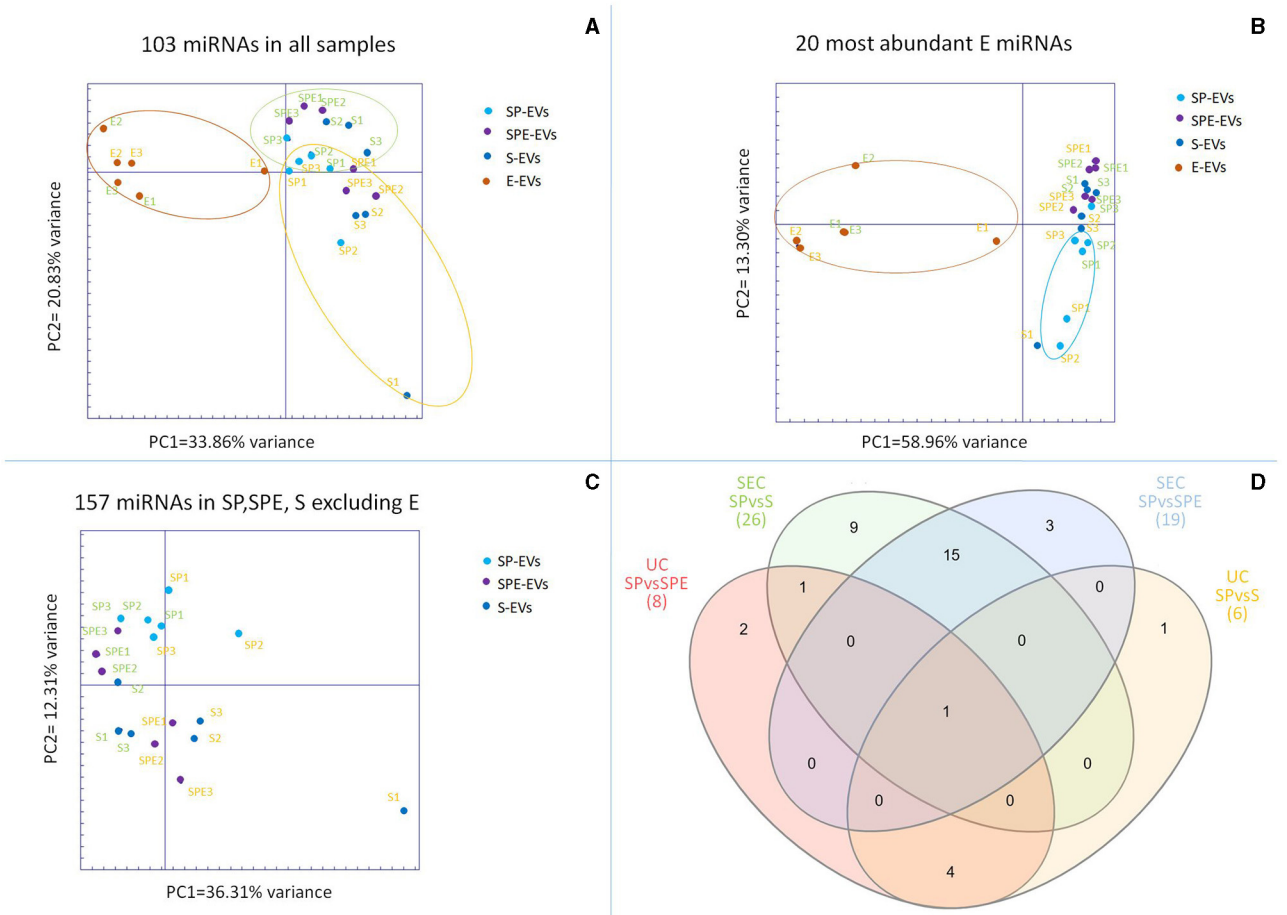

FIGURE 5
(A) Principal component analysis (PCA) of the 103 miRNAs present in all samples, **(B)** PCA for the 20 most abundant miRNAs in the extracellular vesicles (EVs) isolated from the extender (E-EVs), **(C)** PCA for miRNAs present in EVs isolated from seminal plasma preparations (SP-EVs, SPE-EVs, and S-EVs). In green EVs isolated by SEC and in orange by UC. **(D)** Venn diagram of shared differentially expressed miRNAs (DE-miRNAs) found in EVs across seminal plasma preparations, with (SPE and S) or without (SP) the extender using Ultracentrifugation (UC) and Size Exclusion Chromatography (SEC).

plasma alteration after cryopreservation was previously assessed exclusively by metabolomics studies, showing a strong difference between fresh seminal plasma and seminal plasma isolated from cryopreservation straws but without considering the effect of the extender addition (38).

5 Conclusion

In conclusion, the method used for EV isolation can influence the size and the quantity of spEVs isolated from preparations with and without extender. The commercial animal protein-free extender may contain phospholipid compounds, like soya lecithin, that form vesicle-like structures that can interfere with the correct spEV identification and characterization. The extender showed a lower miRNA cargo compared to sp-EVs preparation but several abundant miRNAs were present that were found significantly overexpressed in the S-EVs and SPE-EVs compared to spEVs. Although the EV profiles were influenced by the presence of the extender in S and SPE preparations, the miRNAs profile seems to be quite constant in the different preparations containing EVs. Nevertheless, in future studies involving the use of spEVs isolated from cryopreserved semen to evaluate potential markers of male fertility, it is advisable also to characterize the vesicles isolated from the extender used for straw preparation.

Data availability statement

The data presented in this study are deposited in the NCBI Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra) repository, accession number PRJNA1101695.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because the study analyzed bull ejaculate collected from bovine semen production center.

Author contributions

EC: Conceptualization, Data curation, Methodology, Writing – original draft. RF: Methodology, Writing – review & editing. BL: Data curation, Writing – review & editing. FT: Methodology, Writing – review & editing. GG: Methodology, Writing – review & editing. LP: Methodology, Writing – review & editing. AS: Supervision, Writing – review & editing. ALC: Writing – review & editing. FP: Conceptualization, Writing – review & editing. MC: Conceptualization, Funding acquisition, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1437410/full#supplementary-material

SUPPLEMENTARY TABLE S1
Progeny data and production, functionality, and type (PFT) index of bulls of proven fertility.

SUPPLEMENTARY TABLE S2
Characteristics of semen of proven bulls on pre-freeze and post-thaw samples.

SUPPLEMENTARY TABLE S3
SiMoA analysis reporting the average number of enzymes per bead (AEB), and the relative p-value for the comparison between the different preparations (SP-EVs, SPE-EVs, and S-EVs) and the extender (E-EVs) for UC and SEC isolation methods.

SUPPLEMENTARY TABLE S4
Sequencing statistics.

SUPPLEMENTARY TABLE S5
miRNA counts identified by MiRDeep2 and its modules.

SUPPLEMENTARY TABLE S6
Differentially expressed miRNA (DE-miRNAs) identified comparing different preparations. Value of Logarithmic Fold Change (logFC), Logarithmic Count Per Million (logCPM), *P*-Value, and False Discovery Rate (FDR) were reported.

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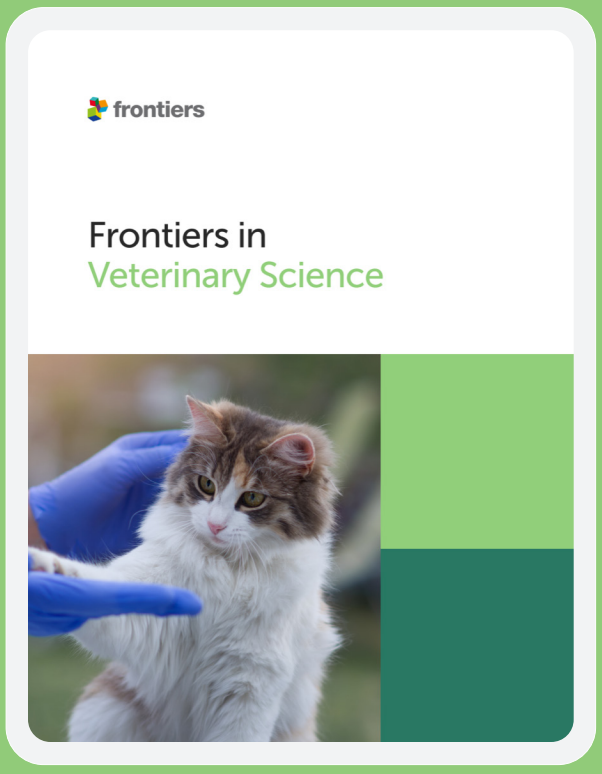

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