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ORIGINAL PAPER

Effect of initial ejaculate quality on post-thaw parameters in boar semen

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ABSTRACT. The initial ejaculate sperm quality influences its subsequent freezability, which varies significantly between individuals. The objective of this study was to determine whether non-viable, static, or morphologically altered sperm affect the freezability of motile, viable sperm with normal morphology. Semen samples were collected from five sexually mature boars (≥3 ejaculates per animal) and allocated to four treatment groups based on the proportion of viable, motile, morphologically normal sperm: 100% (control), 75%, 50%, and 25%. Samples were diluted in TRIS-egg yolk extender, cooled to 5 °C, then further diluted with a glycerol-containing freezing medium and loaded to 0.5-ml straws. The cryopreservation protocol involved two controlled cooling stages, followed by storage in liquid nitrogen for seven days. After thawing at 37 °C for 20 s, sperm quality was evaluated using computer-assisted semen analysis (CASA) for motility and flow cytometry for membrane and acrosome integrity with triple fluorescent staining (Hoechst 33342, propidium iodide, and PNA-FITC). Assessments were carried out before freezing and at 30 and 150 min post-thaw. The percentage of non-viable sperm in the ejaculates prior to freezing significantly affected post-thaw sperm quality ($P < 0.05$), reducing motility and structural integrity in proportion to their initial presence.

Introduction

Long-term sperm preservation plays a critical role in achieving economic gains, conserving valuable germplasm, maintaining genetic diversity, and improving reproductive efficiency in animals (Bolton et al., 2022; Yánez-Ortiz et al., 2022; Engdawork et al., 2024). However, boar spermatozoa are more sensitive to cryopreservation-induced damage compared to other species, e.g., cattle (Valverde et al., 2016; Paschoal et al., 2021; Shepherd et al., 2024). During the freezing and thawing process, cellular damage can occur in the plasma membrane and organelles due to osmotic stress, heat shock, and intracellular ice crystal formation (Johnson et al., 2000). These stressors compromise post-thaw sperm viability, suggesting the presence of additional, yet unidentified, factors that require further studies (Caamaño et al., 2021; Valverde et al., 2021).

Currently, cryopreservation remains the only reliable method for the indefinite sperm preservation (Pomeroy et al., 2022), playing a pivotal role in modern animal production and genetic management. This technology enhances productivity in both

commercial farms and genetic selection centres by supporting advanced breeding programmes (Schulze et al., 2019; Crowe et al., 2021; Araya-Zúñiga et al., 2025; Halaweh et al., 2025). The economic benefits are substantial, as frozen semen increases the market value of elite breeding stock (Krupa et al., 2020), improves international trade through the import and export of genetic materials (Flowers, 1997), and supports the development of germplasm banks (Chicaiza-Cabezas et al., 2023; de Andrade et al., 2023; Góngora et al., 2024). These repositories are particularly valuable in times of limited supply caused by disease outbreaks or other health-related challenges (Borate and Meshram, 2022).

The international trade of cryopreserved semen helps circumvent complications associated with animal health regulations and border restrictions. In addition, gene banks also play an important role in helping preserve animal breeds that are at risk of extinction (Ren et al., 2025), while offering protection against market disruptions caused by disease outbreaks; they also mitigate reproductive challenges caused by rising temperatures and climate variability (Godde et al., 2021).

Despite the advantages of cryopreserved semen in pathogen control and genetic dissemination, its adoption in commercial swine production remains limited (Mellagi et al., 2023), primarily due to its lower fertility rates compared to fresh semen or natural mating (Johnson et al., 2000). Until recently, the use of cryopreserved semen in artificial insemination (AI) was mostly limited to selection centres or research institutions (Capra et al., 2024). However, its application has shown promising results in recent studies (Pezo et al., 2019; Wiebke et al., 2022) and is gaining popularity due to advantages, such as reduced risk of pathogen transmission (Goldberg et al., 2013) and efficient dissemination of desirable genes, resulting in genetic improvements. Despite these benefits, cryopreserved semen is still not widely adopted in commercial swine production (Bolarin et al., 2024).

Several factors determine the quality of thawed semen and the overall efficiency of the porcine sperm cryopreservation process (Mazur, 1977). Research has demonstrated significant individual variation in boar sperm response to cryopreservation (Khan et al., 2021), affecting post-thaw viability, motility, and *in vivo* fertility (Valverde et al., 2018; 2021). This variability has led to the classification of boars as either 'good' or 'bad' sperm freezers, based on the resilience of their sperm to cryopreservation (Holt et al., 2005).

The underlying causes of this variation remain unclear, prompting efforts to optimise freezing protocols and to exclude poor-freezing boars from AI programmes. Recently, genetic factors have been proposed as a possible explanation for differences in sperm cryotolerance (Thurston et al., 2002), introducing a potential new set of criteria for evaluating an individual's suitability for cryopreservation based on genetic markers.

The freezing potential of boar semen is determined by its initial ejaculate quality. Studies indicate that ejaculates containing more than 20–30% non-viable sperm, characterised by immotility, abnormal morphology, or compromised membranes, should be discarded (Thurston et al., 2001; Jovičić et al., 2020; Al-Kass and Morrell, 2024). However, preserving high-value genetic material, particularly from endangered breeds, often necessitates processing suboptimal ejaculates. While the detrimental impact of poor-quality ejaculates on freezability is recognised (Cheng et al., 2022), the specific role of non-viable sperm in compromising viable cells remains unclear. Emerging evidence suggests that cryodamaged sperm may release harmful factors (e.g., reactive oxygen species or proteolytic enzymes) that impair intact cells during freezing (Khan et al., 2021). The present research directly investigated whether non-viable spermatozoa influence the cryopreservation resilience of functional spermatozoa, addressing a critical gap in the optimisation of semen freezing protocols for genetic conservation and increased reproductive efficiency.

Material and methods

Ethical approval

The study was conducted in compliance with the national regulations governing the use of live animals in research in Costa Rica. All procedures involving animals were performed with due care to minimise stress and ensure animal welfare throughout the study. Ethical approval was granted by the Committee of the Tropical Sustainable Agriculture Research and Development Center (CIDASTH-IT-CR), according to Section 08/2023 and Article 5.0 of DAGSC-075-2023, and CIE-206-2023. The study also complied with the ARRIVE guidelines (https://arriveguidelines.org/) for animal research reporting standards.

Study site

All experimental procedures were conducted at the Animal Reproduction Laboratory of the Costa Rica Institute of Technology's Agronomy School.

Animals

All ejaculates used in the study were collected from five adult boars of the Duroc, Pietrain, and Large White breeds, with proven fertility and routinely used for artificial insemination. The boars were housed individually in temperature-controlled pens maintained at 23 ± 2 °C. They were fed a standard breeder mix prepared on-site, consisting of maize-soybean meal supplemented with minerals and salt (2.5 kg/day). Water was provided *ad libitum*. Semen collection was performed twice weekly using standard procedures.

Semen extraction

A total of 15 ejaculates were collected (three per animal) using the manual method. The pre- and post-sperm fractions were discarded, and only the sperm-rich fraction was retained in a pre-warmed container. Directly after extraction, the ejaculates were placed in a water bath at 37 °C and diluted at a 1:2 (v/v) ratio using Beltsville Thawing Solution (BTS). The diluted semen was transferred into 50 ml Falcon tubes, preheated to 37 °C, protected from direct light, and stored at 17 °C. The samples were transported to the Animal Reproduction Laboratory in sealed polystyrene boxes with continuous temperature monitoring using a miniature data logger (Gemini Data Loggers, Ltd., Chichester, UK), After 5–6 h, the ejaculates arrived at the laboratory, where sperm concentration and quality were assessed before preparing sperm mixtures for further treatments.

Sperm concentration

Sperm concentration was assessed using a sperm nucleus counter (NucleoCounter® SP-100™ system AN-101, Chemometec, Allerød, Denmark). After determining the appropriate dilution factor for the sample concentration range, 5.0 µl of the sample was mixed with 1000 µl of reagent solution (Reagent S100, Chemometec). The mixture was gently agitated, and a sample was loaded into a disposable cassette (SP1-Cassette, Chemometec) by pressing the piston to draw in the-fluid. The results were recorded and expressed in millions of sperm per ml ($\times 10^6$ spz/ml).

Motility assessment

Sperm motility and kinetics were analysed using a Computer-Assisted Semen Analysis (CASA) system with the ISAS® v1 software (Proiser SL, Valencia, Spain). Sperm samples were gradually diluted to a final concentration of 30×10^6 spz/ml. A 5 µl aliquot of each diluted sample was placed in a pre-warmed Makler chamber (Sefi-Medical Instruments, Haifa, Israel), maintained at 37 °C on a thermal plate under a phase contrast microscope (UOP, Model UB 200 I Series, China) with a 10× negative-phase contrast objective (AN 0.25) and recorded using a video camera (ISAS 782 M). For each sample, six image sequences were captured to analyse a minimum of 600 spermatozoa per sample. For each field, 25 frames were acquired at a rate of 25 Hz, with a resolution of 768 × 576 pixels. CASA settings were configured for a particle size range of 10–80 µm^2 and a connectivity threshold of 11 µm, and motility classifications based on progressive movement (straightness ≥45% and average path velocity ≥25 µm/s). Total motility (%) and progressive motility (%) were recorded, with progressive motility defined as spermatozoa moving rapidly forward in a straight trajectory.

Analysis of sperm viability and acrosomal integrity

Sperm viability and acrosomal integrity were assessed simultaneously using a triple fluorescent staining technique adapted for porcine spermatozoa. The analysis determined the percentage of sperm with intact plasma and acrosomal membranes. The staining protocol involved three fluorochromes: Hoechst-33342 (H-42; Sigma-Aldrich, Darmstadt, Germany), diluted 1:100 (v/v) from a 5 mg/ml stock, to count spermatozoa (blue staining); propidium iodide (PI) to stain sperm with compromised membrane integrity (red fluorescence); and fluorescein-isothiocyanate-conjugated peanut lectin (PNA-FITC) to label sperm with acrosomal damage (green fluorescence). Sperm were gradually diluted in BTS to a concentration of $25–30 \times 10^6$ spz/ml. To 100 µl of this dilution, 2.5 µl of H-42, 2.0 µl of PI, and 5.0 µl of PNA-FITC were added. Samples were incubated for 10 minutes at 38 °C in a MIR 153 incubator (Sanyo, Gunma, Japan). Following incubation, the samples were analysed using flow cytometry (BD FACS Canto II™, Becton, Dickinson and Company, San Jose, CA, USA), with 10000 spermatozoa evaluated per sample. Viable sperm were defined as those exhibiting Hoechst-33342 staining (intact nuclei) while lacking PI (intact plasma membrane) and PNA-FITC (intact acrosome) signals. The results were expressed as the percentage (%) of viable sperm with normal acrosomes.

Chemical reagents and media used

The reagents for media and diluent preparation were weighed using a precision balance (Gram Precision Series ST Mod. ST-120; A.R.W.T, Barcelona,

Spain) and diluted with Milli-Q® purified double-distilled water (Advantage A10® System; Millipore™, Concord Road, Billerica, MA, USA). The dilutions were thoroughly mixed using a magnetic stirrer (Mod. 234 P Agimatic; Selecta, Barcelona, Spain). Following preparation, the pH (Mod. GLP 21; Crison, Barcelona, Spain) and osmolarity (Mod. 5520, Vapro®, Vapor Pressure Osmometer; Wescor Inc., South Logan, UT, USA) of each medium were verified.

Fresh semen samples were diluted at a 1:2 ratio (v/v) using BTS. In addition, this diluted sample was subsequently used to assess post-thaw sperm quality. BTS was prepared under sterile conditions in a laminar flow hood (Micro-R, Spain), and its pH and osmolarity were verified to ensure compliance with the required specifications. The prepared BTS was aliquoted into 15 ml or 50 ml Falcon tubes, depending on the required volume, and stored at 5 °C until use. Phosphate-buffered saline (PBS) was used as the diluent for fluorochromes applied during viability assessment of both fresh and post-thawed semen samples.

Preparation of fluorochromes

For the triple fluorescent staining used in sperm viability and acrosomal integrity assessment, all reagents were prepared and stored according to standardised protocols. A stock solution of bis-benzimide trihydrochloride (Hoechst-33342, B2261; Sigma-Aldrich, Darmstadt, Germany) was in double-distilled water to a final concentration of 5 mg/ml (w/v) and stored at 4 °C in 1000 µl aliquots. Prior to use, this stock was diluted 1:100 (v/v) in PBS (i.e., 10 µl Hoechst-33342 + 990 µl PBS). Propidium iodide (PI), used as component B of the LIVE/DEAD® Sperm Viability Kit (L-7011; Molecular Probes Europa, Leiden, Netherlands), was prepared from a 2.4 mM commercial stock by dilution in purified water to a final concentration of 1 mg/ml. Directly before use, this solution was further diluted 1:2 (v/v) with PBS to obtain a working concentration of 0.5 mg/ml (50 µl PI + 50 µl PBS). For acrosomal integrity evaluation, fluorescein-isothiocyanate-conjugated peanut agglutinin (PNA-FITC, Sigma L-7381) was prepared as a 200 µg/ml stock solution and aliquoted into 250 µl volumes. All fluorochrome solutions were maintained at 4 °C in light-protected conditions until use. Results were expressed as percentages (%).

Dilution media for sperm preservation

Two types of diluents were used for semen cryopreservation. The first was the basic TRIS-egg yolk (TRIS-Y) medium, which was added at 17 °C after sample centrifugation and supernatant removal. Egg yolk was obtained from fresh eggs collected under strict aseptic conditions. Before handling, egg surfaces were cleaned with ethanol, and yolks were carefully separated from the albumen using filter paper. The yolks were placed in sterile test tubes and stored at 4 °C until processing. Once prepared, the TRIS-Y medium was centrifuged at 4300 g for 30 min at 5 °C to separate three distinct coloured fractions. The top and bottom fractions were discarded, and only the middle layer was retained, transferred to sterile 15 ml Falcon tubes, and stored at −20 °C. The second diluent, TRIS-egg yolk-glycerol (TRIS-Y-G), was prepared fresh on freezing day by supplementing TRIS-Y medium with glycerol (cryoprotectant) and Equex STM detergent (Nova Chemical Sales Inc., Scituate, MA, USA). This solution was introduced during the second temperature reduction phase when samples reached 5 °C.

Non-viable sperm and 24-h incubation

To prepare treatments, 50 ml aliquots of the sperm-rich ejaculate fraction was collected at 17 °C and immersed in liquid nitrogen at −196 °C for nine minutes. The samples were then immediately thawed in a 37 °C water bath for 3 min, followed by complete thawing in an 80 °C water bath. Fluorescence staining confirmed complete sperm non-viability following this thermal shock protocol.

Experimental sample design with viable and non-viable spermatozoa

Spermatozoa were considered viable if they were motile, showed normal morphology, and maintained intact plasma and acrosomal membranes. Non-viable sperm were defined as those with morphological abnormalities, lack of motility, or damage to the plasma membrane or acrosome. Only ejaculates meeting the following criteria were selected for the experiment: a minimum concentration of 200×10^6 spz/ml, at least 85% sperm with normal morphology, ≥75% motile sperm, and ≥85% viable sperm. Four experimental treatments were established based on the proportion of viable and non-viable sperm: a Control Treatment with 100% viable sperm (corresponding to ≥85% viability based on the inclusion criteria); Treatment 1 (T1) with 75% viable sperm and 25% non-viable sperm; Treatment 2 (T2) with 50% viable sperm and 50% non-viable sperm; and Treatment 3 (T3) with 25% viable sperm and 75% non-viable sperm.

Sperm cryopreservation

The diluted sperm-rich fraction was re-evaluated and centrifuged at 2400 g for 3 min in

a refrigerated centrifuge at 17 °C (Heraeus Sepatech Megafuge 1.0R, Hanau, Germany). After discarding the supernatant, the sedimented spermatozoa were resuspended in TRIS-Y to achieve a concentration of 1.5×10^9 spz/ml. The resuspended samples were then cooled to 5 °C for 150 min. At this temperature, TRIS-Y-G diluent was added to adjust the final concentration to 1×10^9 spz/ml. The aliquotes were manually dispensed into 0.5 ml straws (Minitübe, Tiefenbach, Germany) and sealed at 5 °C using an automatic sealer (Ultraseal 21™; Minitübe). First, straws were placed 4 cm above liquid nitrogen in vapor phase for 20 min using a freezing apparatus consisting of a polystyrene container with a stainless steel tray supporting a perforated aluminium platform to ensure uniform cooling. Straws were arranged in a single layer on the platform. For the second stage, straws were directly immersed in liquid nitrogen (−196 °C) until reaching thermal equilibrium. Frozen straws were stored in liquid nitrogen tanks and evaluated after a minimum 7-day storage period, with thawing conducted in a 37 °C water bath for 30 s.

Analysis of sperm quality after thawing

Thawing was performed in a thermostatic bath (Huber Polystat cc1; LabWrench, Midland, ON, Canada) maintained at 37 °C for 20 s. For each analysis, one straw per treatment was thawed simultaneously, then immediately mixed and diluted in BTS (1:2, v/v; 37 °C). The diluted samples were incubated in an oven at 37 °C for 150 min. Sperm quality was assessed for motility and membrane integrity at 30 and 150 min. The percentage of sperm recovery was calculated as: (Post-thaw sperm quality × 100) / Pre-freeze sperm quality.

Statistical analysis

The assumptions of normal data distribution and homoscedasticity were evaluated using normal probability plots and Levene's test, respectively. Differences in sperm quality variables between boars and between incubation times post-thaw were analysed using mixed-effects models. A one-way analysis of variance (ANOVA) was performed to determine variations in sperm quality parameters at different incubation times prior to freezing for each boar. Additionally, separate ANOVAs were performed to evaluate differences in seminal characteristics at 30 min and 150 min post-thaw, as well as differences between boars for the same variables.

All analyses used a significance threshold of $P < 0.05$, with post-hoc pairwise comparisons conducted using the Tukey-Kramer test. Results are presented as mean ± standard error of the mean (SEM). All data were analysed using IBM SPSS, v23.0 (SPSS Inc., Chicago, IL, USA).

Results

The sperm quality characteristics of ejaculates diluted (1:2 v/v) in a commercial extender were evaluated upon arrival at the Animal Reproduction Laboratory (0 h) and after 24 h of incubation, representing the 100% viable sperm group. Significant differences ($P < 0.05$) were observed between boars across sperm quality parameters (Table 1). Boar 2 had the highest sperm concentration but a significantly lower percentage of morphologically normal sperm compared to the other boars ($P < 0.05$). It also showed reduced total motility after 24 hours of storage. Boar 3, on the other hand, exhibited the highest overall sperm quality at both evaluation times. The effect of conservation time between 0 and 24 h, was significant in all animals ($P < 0.05$), although the impact varied among boars and did not affect all sperm parameters equally. In general, a decline in sperm quality was observed after 24 h of storage. Nonetheless, the percentage of normal morphology

Table 1. Sperm quality parameters of five boar ejaculates at 0 and 24 h post-incubation

Parameter	Boar				
	1	2	3	4	5
Concentration, M/ml	256.5 ± 21.0^{a}	428.7 ± 49.8^{b}	248.2 ± 11.0^{a}	313.8 ± 12.2^{ab}	237.8 ± 32.7^{a}
TM 0h, %$^{\alpha}$	84.3 ± 2.3	84.3 ± 2.7^{x}	85.3 ± 0.3	79.3 ± 4.7	86.7 ± 0.7
TM 24h$^{\beta}$, %	81.0 ± 2.0	74.67 ± 2.7^{y}	83.3 ± 0.4	69.0 ± 4.2	82.7 ± 1.2
PM 0 h, %$^{\alpha}$	51.7 ± 4.8^{x}	46.7 ± 3.0	41.3 ± 1.9	45.0 ± 8.0	51.3 ± 8.6
PM 24 h$^{\beta}$, %	40.67 ± 4.6^{y}	41.67 ± 5.0	54.67 ± 6.0	54.0 ± 6.7	49.3 ± 5.8
Integrity$^{\chi}$ 0 h$^{\alpha}$, %	90.0 ± 1.0	92.0 ± 2.3^{x}	89.7 ± 0.9	90.0 ± 2.3	89.0 ± 1.2^{x}
Integrity$^{\chi}$ 24 h$^{\beta}$, %	89.7 ± 0.5^{a}	$80.3 \pm 1.5^{b,y}$	85.0 ± 1.6^{ab}	84.5 ± 2.2^{ab}	$83.3 \pm 0.8^{ab,y}$
Normal morphology, %	97.0 ± 2.5^{a}	90.0 ± 0.6^{b}	95.0 ± 1.7^{a}	93.7 ± 2.2^{a}	98.0 ± 0.5^{a}

TM – total motility, PM – progressive motility; $^{\alpha}$ – parameters measured upon arrival at laboratory. $^{\beta}$ – incubation time of viable sperm with non-viable sperm (100% viable treatment group); $^{\chi}$ – plasma and acrosomal membrane integrity, assessed using SYBR-14, IP and PNA-FITC fluorochromes. ^{ab} – values within the same row with different letters are significantly different at $P < 0.05$, ^{xy} –values within the same column and parameter differ significantly between 0 h and 24 h ($P < 0.05$), data are presented as mean values ± SEM

remained above 90% across all boars, indicating that this parameter was least affected by storage time.

Sperm quality assessed at 30 and 150 min post-thaw revealed significant differences ($P < 0.05$) between boars at both time points (Table 2), with a general decline observed between 30 and 150 min post-thaw. Boars 1, 3, and 4 showed the highest values for total motility and integrity of the plasma and acrosomal membranes. However, when progressive motility was analysed separately, boars 1 and 3 consistently recorded higher values compared to the others at both evaluation times.

Analysis of the percentage of total motile sperm recovered at 30 and 150 min post-thaw indicated clear differences between boars. Samples containing 100% viable sperm prior to freezing consistently showed the highest recovery percentages for all boars, while those with 50 and 25% viable sperm had the poorest recovery rates. Moreover, the differences recovery percentages were more pronounced at 150 min than at 30 min post-thaw (Figure 1). Significant differences ($P < 0.05$) were recorded between the high-viability groups (100 and 75% viable sperm) and low-viability groups (50 and 25% viable sperm). In the initial 30-min post-thaw period, >80% of sperm remained motile in the 100% and 75% viability groups. While motility declined over time, the 100% viability group maintained superior performance even at 150 min post-thaw. For instance, boar 1 demonstrated this pattern clearly – despite an overall decrease in motility by 150 min, the 100% viability group still showed significantly higher motility ($P < 0.05$) compared to other treatments.

Significant differences were found between boars in the percentage of progressively motile sperm recovered at both 30 and 150 min post-thaw. In samples with 100% viable sperm before freezing, the highest recovery rates were consistently observed in all boars at both 30 time points.

Table 2. Sperm quality parameters at 30 and 150 min post-thaw in control samples with 100% viable sperm, by boar

Parameter, %	Boar						SEM
	min	1	2	3	4	5	
TM	30	$71.6^{a,x}$	$44.9^{b,x}$	$73.0^{a,x}$	$59.6^{ac,x}$	$53.0^{bc,x}$	4.2
	150	$50.5^{a,y}$	$31.7^{b,y}$	$54.5^{a,y}$	$45.3^{ab,y}$	$34.9^{b,y}$	3.9
PM	30	$60.1^{a,x}$	$35.1^{b,x}$	$55.0^{a,x}$	40.0^{b}	$37,2^{b,x}$	3.3
	150	$45.9^{a,y}$	$25.3^{bc,y}$	$46.5^{a,y}$	36.8^{ac}	$25.7^{bc,y}$	3.6
Plasmatic and acrosomal membrane integrity	30	$70.2^{a,x}$	$54.1^{b,x}$	$71.7^{a,x}$	$59.5^{ab,x}$	$52.8^{b,x}$	4.8
	150	$63.4^{a,y}$	$48.8^{b,y}$	$62.1^{ac,y}$	$52.4^{bc,y}$	$43.9^{b,y}$	2.6

TM – total motility, PM – progressive motility; ^{abc} – values within the same row with different superscripts are significantly different between boars ($P < 0.05$), ^{xy} – values within the same column and parameter are significantly different between 30 and 150 min post-thaw ($P < 0.05$), data are presented as mean values ± standard error of the mean (SEM)

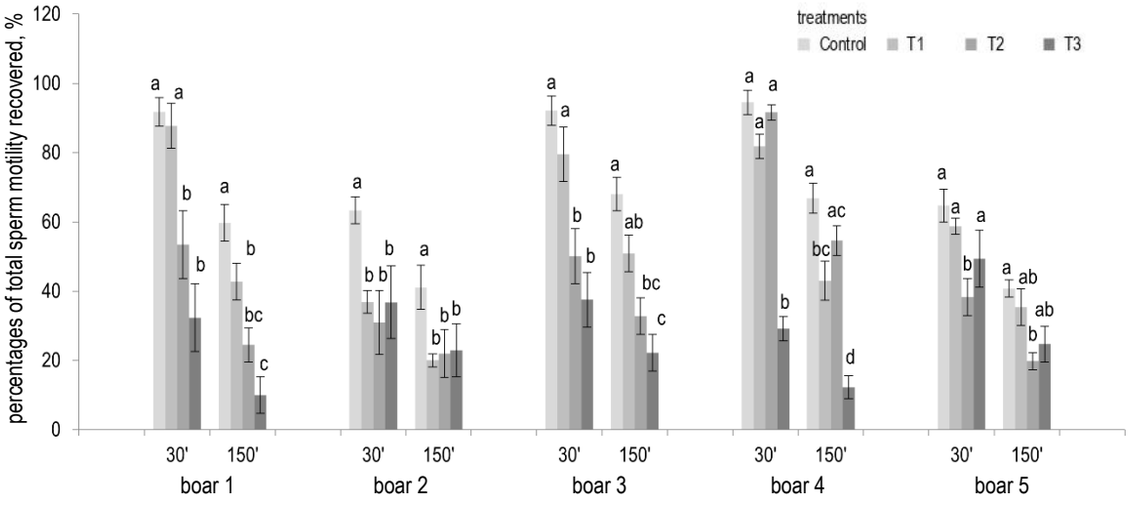

Figure 1. Total motile sperm expressed as recovery percentages (% ± SEM), at 30 min and 150 min post-thaw in different treatment groups, by boar. Recovery was calculated using the formula: (% sperm quality post-thaw × 100) / (% sperm quality pre-cryopreservation). Control (100% viable sperm before freezing), T1 (75% viable + 25% non-viable), T2 (50% viable + 50% non-viable) and T3 (25% viable + 75% non-viable); $^{a–d}$ – different superscript letters indicate statistically significant differences for each boar and analysis time ($P < 0.05$)

However, in certain cases, no significant differences ($P > 0.05$) were observed between treatments, specifically in boar 2 at 150 min post-thaw and in boar 5 at 30 min post-thaw. For all other boars and post-thaw incubation times, sperm samples with 50% and ($P > 0.05$) were detected at either time point for boars 3, 4, and 5. For the two boars where treatment effects were observed, the highest recovery percentages were recorded in samples with 100% viable sperm before freezing (Figure 3).

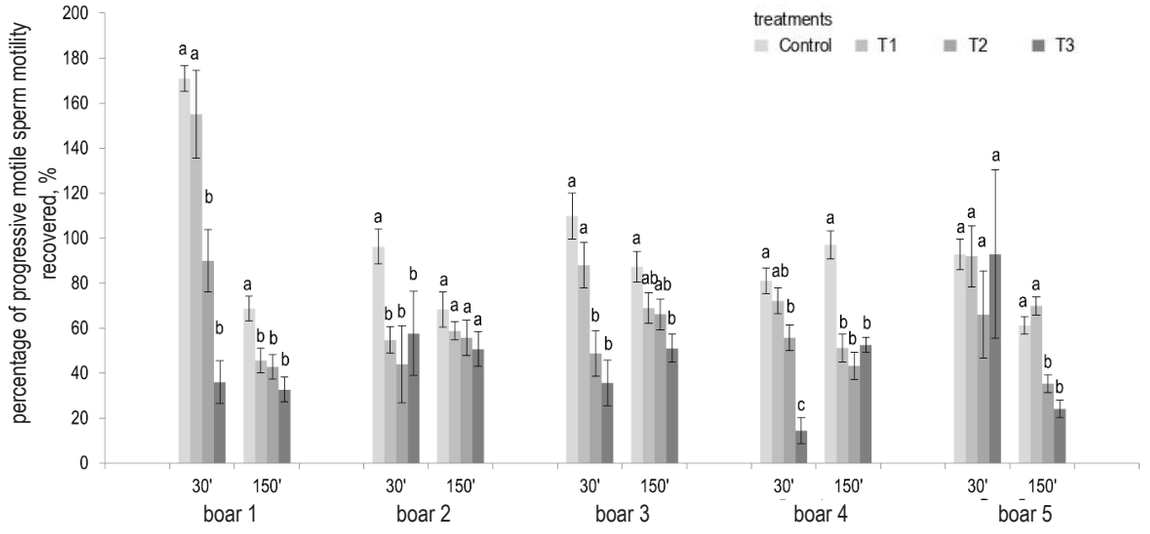

Figure 2. Progressive motility expressed as percentage recovery ± standard error of the mean indicated in bars of frozen-thawed sperm at 30 min and 150 min, according to boar in sperm samples with different treatments. Recovery was calculated using the formula: (% sperm quality post-thaw × 100) / (% sperm quality pre-cryopreservation). Control (100% viable sperm before freezing), T1 (75% viable + 25% non-viable), T2 (50% viable + 50% non-viable) and T3 (25% viable + 75% non-viable); [abc] – different letters indicate statistically significant differences for each boar and analysis time ($P < 0.05$)

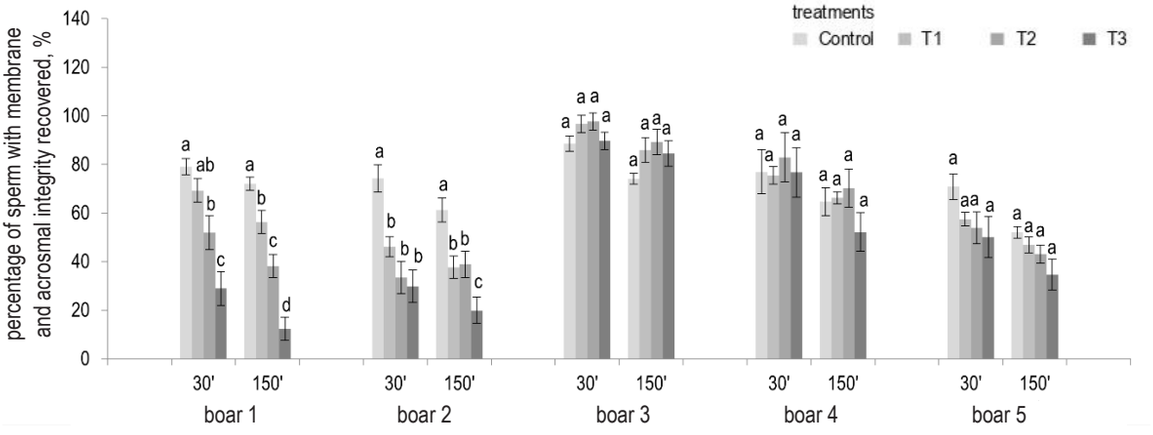

Figure 3. Spermatozoa with intact plasma membrane and acrosomal integrity in frozen-thawed semen samples at 30 min and 150 min post-thaw, by boar and treatment groups. Recovery was calculated using the formula: (% sperm quality post-thaw × 100) / (% sperm quality pre-cryopreservation). Control (100% viable sperm before freezing), T1 (75% viable + 25% non-viable), T2 (50% viable + 50% non-viable) and T3 (25% viable + 75% non-viable). Data are expressed as mean recovery percentages ± SEM, shown as bars; [a–d] – different letters indicate statistically significant differences for each boar and analysis time ($P < 0.01$)

25% viable sperm before freezing showed the lowest recovery percentages after thawing (Figure 2).

Differences ($P < 0.01$) between treatments in the percentages of sperm recovered with intact plasma and acrosomal membranes were significant. Among animals, boars 1 and 2 differed markedly ($P < 0.01$) between treatments at both post-thaw incubation times. In contrast, no significant differences

Discussion

This study demonstrates the negative influence of non-viable spermatozoa on the freezability of viable sperm in pigs. In humans, the detrimental effects of morphologically abnormal sperm and lipid peroxidation in non-viable sperm on sperm quality, particularly motility, is well-documented

(Krzastek et al., 2020; Cheng et al., 2022; Walke et al., 2023; Sciorio et al., 2024). Additionally, several studies have shown that the presence of morphoanomalies negatively affects sperm cryotolerance (Mańkowska et al., 2020; Ďuračka et al., 2023). The extent of this impact appears to be proportional to the percentage of sperm with morphological abnormalities or compromised membrane integrity (Alahmar, 2019).

Protection mechanisms against oxidative stress vary between individuals and may be influenced by the biochemical composition of the initial ejaculates (Jakop et al., 2022; Fraser et al., 2025). Clusters of non-viable spermatozoa present in the ejaculates prior to freezing not only affect the freezability of viable sperm but also exacerbate oxidative stress (Sabeti et al., 2016; Alahmar, 2019; Dutta et al., 2019). This variability among boars directly impacts cryotolerance and indicates the need for developing improved sperm selection methods prior to freezing (Yeste, 2015). Understanding the factors that affect boar sperm freezing processes is essential for developing more effective cryopreservation protocols and contributes directly to improving reproductive efficiency in the pig industry (Bolarin et al., 2024)

Under normal conditions, sperm production centres report that it is uncommon for animals to produce ejaculates with a high proportion of non-viable spermatozoa (Henning et al., 2022). On the other hand, various studies have described sperm subpopulations in ejaculates, demonstrating how variations in kinematic and swimming patterns influence overall sperm quality (Barquero et al., 2021). The present findings demonstrate that increased levels of non-viable sperm negatively affect the freezability of viable sperm, and that this detrimental effect is proportional to the percentage of non-viable sperm present in the ejaculate (Ďuračka et al., 2023). However, evaluating non-viable sperm in routine semen samples is challenging, as boar ejaculates used in artificial insemination programs generally have a relatively high sperm quality (Wolf and Smital, 2009). Consequently, obtaining ejaculates with naturally high proportions of non-viable sperm is difficult and requires experimental induction of non-viability for controlled studies. Interestingly, even genetically superior boars selected for production traits (e.g., lean yield, backfat thickness) may occasionally produce poor-quality ejaculates. Understanding the negative effects of non-viable sperm in such cases is important for developing sperm selection methods to eliminate defective sperm before cryopreservation (Tanga et al., 2021; Bang et al., 2022).

Despite working with genetically selected boars undergoing standardised artificial insemination protocols designed to minimise variation, significant inter-boar differences in post-thaw sperm quality were still observed. These findings align with previous porcine studies demonstrating inherent individual variability in cryopreservation success (Jovičić et al., 2020). Furthermore, the present results demonstrate that the negative impact of non-viable spermatozoa on the cryotolerance of viable sperm also varies between individual boars.

The existence of ‘bad freezer’ boars is well-documented (Holt et al., 2005) and our results confirm this phenomenon, showing that some boars had a poor post-thaw semen quality even under optimal conditions (100% viable spermatozoa group). Interestingly, the same boars demonstrated higher recovery rates of plasma and acrosomal membrane integrity in samples containing substantial proportions of non-viable spermatozoa (75, 50, and 25%). This suggests that, despite being poor freezers overall, the viable spermatozoa from these individuals may possess greater resistance to the detrimental effects of non-viable sperm in their environment. Conversely, other boars that exhibited good post-thaw seminal quality under standard conditions showed lower recovery rates for membrane integrity as the proportion of non-viable spermatozoa increased. This indicates that their viable spermatozoa are more vulnerable to damage caused by non-viable cells. Importantly, these findings demonstrate that good freezing ability does not necessarily confer protection against the negative impact of non-viable spermatozoa in the ejaculate. The variation observed between boars in the extent to which non-viable sperm affect viable sperm suggests that individual sensitivity to this negative interaction differs. This is evidenced by the higher recovery percentages of viable sperm seen in certain boars, even in treatments with high proportions of non-viable spermatozoa. Such variability may be linked to differences in the biochemical composition of the sperm plasma membrane among boars. Previous studies have reported variability in the content of polyunsaturated fatty acids in the membranes of both fresh and frozen-thawed porcine spermatozoa (Iaffaldano et al., 2016; Monteiro et al., 2022), as well as in the composition of seminal plasma (Juyena and Stelletta, 2012), and the activity of antioxidant enzymes such as superoxide dismutase or glutathione peroxidase (Valverde et al., 2021). The observation that some boars maintain high sperm quality even in the presence of increasing levels of non-viable sperm highlights a potential protective role of the

membrane composition or antioxidant capacity. These findings provide a strong rationale for further research into the structural and biochemical properties of boar sperm membranes.

Cryopreservation significantly reduces boar sperm quality, as shown in previous studies (Jovičić et al., 2020; Monteiro et al., 2022). In the present work, freezing and thawing decreased total and progressive motility by 40–50% and viability by 30–40%. The extent of quality loss depended on initial sperm quality, with poorer quality samples (50% and 25% viable sperm) showing particularly low recovery rates for motility, plasma membrane integrity, and acrosomal membrane integrity. These results contrast with reports finding no correlation between pre- and post-freeze quality (Woelders et al., 1995), a discrepancy that may be explained by their use of exclusively high-quality ejaculates before cryopreservation.

Sperm motility is highly dependent on the function of cytoplasmic organelles, particularly mitochondria, which supply ATP required for flagellar movement. These organelles are among the most sensitive structures to cryopreservation-induced damage (Nowicka-Bauer and Szymczak-Cendlak, 2021; Costa et al., 2023; Vahedi Raad et al., 2024). This is of particular importance, as sperm motility has been positively correlated with *in vivo* fertility (Hirai et al., 2001). Mitochondrial activity generates reactive oxygen species that disrupt electron transport (Halliwell and Gutteridge, 1990), while the coupling between electron transport and oxidative phosphorylation maintains the membrane potential necessary for ATP formation (Li et al., 2016). Porcine sperm counteract oxidative stress, through high levels of superoxide dismutase activity at both mitochondrial and cytoplasmic levels (Guthrie and Welch, 2006). The sperm cell plasma membrane is most damaged during cryopreservation, although the outer acrosomal membrane and mitochondrial membranes are also affected (Watson, 1995). These structural alterations result in the leakage of intracellular enzymes and ionic imbalances, leading to loss of selective membrane permeability and disruption of both aerobic metabolism and glycolysis. This cascade ultimately compromises all energy-dependent cellular functions, including motility (Tsujii et al., 2006). The results of this study show that increasing proportions of non-viable sperm progressively reduced post-thaw total and progressive motility. This suggests that a lower number of metabolically active sperm cells were capable of sustaining normal mitochondrial function under these conditions. Additionally, the reduced recovery of total and progressive motility demonstrated the negative influence of non-viable spermatozoa on these critical sperm quality parameters.

Conclusions

The presence of non-viable sperm negatively influenced the cryoresistance of viable spermatozoa. Furthermore, the degree of this detrimental effect was proportional to the percentage of non-viable spermatozoa present in the sample.

Non-viable spermatozoa significantly impair post-thaw sperm quality, reducing both total and progressive motility while compromising plasma membrane and acrosomal integrity. The detrimental effects increase proportionally with the percentage of non-viable sperm present in the sample. These findings demonstrate that non-viable spermatozoa directly influence the cryotolerance of viable sperm populations.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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