veterinary sciences

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Protective Effects of Seminal Plasma Supplemented in Boar Frozen Semen Thawing Media: Sperm Quality, Proteome, and Reproductive Performance

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Simple Summary

Cryopreservation improves the utilization of elite boar semen in pig production, but severe structural and functional damage to thawed sperm limits its large-scale industrial application. Seminal plasma contains abundant protective bioactive molecules, but its effective supplementary ratio and core protective mechanisms remain unclear. In this study, seminal plasma from freeze-tolerant and non-freeze-tolerant boars was added to thawing medium at four gradient ratios to assess sperm performance. The 20% addition group exerted the most stable protective effects, with broadly similar outcomes between the two seminal plasma sources. Ultracentrifuged soluble fractions alone produced weaker, less durable protection than complete seminal plasma. Quantitative proteomics identified three core metabolic pathways linked to sperm function, which were further supported via Western blot analysis. Artificial insemination trials revealed that this selected medium requires approximately 2 billion sperm per insemination, with no statistically significant difference in litter performance detected relative to fresh semen. Historical farm records suggest that conventional frozen semen typically requires a higher dosage. This work establishes a practical, easy-to-operate thawing protocol and systematically elucidates the molecular basis of seminal plasma-mediated sperm protection during cryopreservation.

Abstract

Boar semen cryopreservation enables elite genetic utilization, yet thawing-induced cryodamage impairs sperm quality. While seminal plasma shows promise as a natural cryoprotective additive, its effective concentration and molecular mechanisms remain unclear. This study aimed to screen for an effective seminal plasma concentration for thawing media and to elucidate its protective mechanisms in frozen–thawed boar sperm. Seminal plasma from freeze-tolerant (FT) and non-freeze-tolerant (NFT) boars was added to thawing media at gradient concentrations. Sperm quality was assessed post-thawing, followed by quantitative proteomics, functional enrichment, Western blot analysis, and artificial insemination (AI) fertility trials. Supplementation with 10–30% seminal plasma significantly improved post-thaw sperm motion parameters, and 20% showed the highest numerical values for most parameters among the tested concentrations. FT and NFT seminal plasma conferred similar protective effects, with no statistically significant difference detected between the two phenotypes. Proteomics identified 2965 sperm proteins, with only 11 differentially expressed proteins (DEPs) differing between FT and NFT groups, indicating substantial

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Academic Editor: Lian Li

Received: 2 September 2026
Revised: 27 October 2026
Accepted: 29 October 2026
Published: 31 October 2026

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similarity in their sperm proteome profiles under the applied analysis. DEPs were significantly enriched in three core pathways: lysosome, complement and coagulation cascades, and sphingolipid metabolism. Western blot supported the proteomic expression trends for LGMN, PLS1, SERPINA1 and CLU. In field AI trials, frozen–thawed semen supplemented with 20% seminal plasma produced litter sizes and live piglet numbers that did not differ significantly from those of fresh semen; however, no contemporaneous conventional frozen-semen control group was included. Addition of 20% FT or NFT seminal plasma to thawing medium improves post-thaw sperm quality and fertilization capacity, possibly through the co-regulation of three core pathways. These findings provide a practical, operationally feasible protocol for commercial frozen boar semen production.

Keywords: cryopreservation; freezability; artificial insemination; CASA; litter size; cryodamage

1. Introduction

Cryopreservation of boar semen is a core livestock technology for exploiting elite boar genetic resources, cutting breeding costs and enabling cross-regional semen transport and germplasm conservation. Yet porcine sperm suffer severe motility and fertility loss after thawing, a prominent technical barrier compared with cattle and horse cryopreservation [1,2]. Boar sperm are highly sensitive to cold shock; ice crystal formation [3,4] and oxidative stress [5,6] trigger irreversible damage to sperm membranes and intracellular functions. Cryopreservation-derived ROS accumulation [7] degrades membrane lipids and mitochondrial proteins, while rapid post-thaw warming further raises membrane permeability and cytoplasmic leakage, continuously reducing sperm motility [8,9]. Conventional cryoprotectants and BTS thawing media only partially relieve cryoinjury, failing to meet large-scale pig production demands. Thus, novel protective additives are urgently required [2].

Adding bioactive substances to thawing media has gained research interest, with seminal plasma attracting particular attention for its native protective effects. As the main liquid fraction of semen, seminal plasma contains ions, proteins, metabolites and extracellular vesicles that regulate sperm motility [10], capacitation [11], the acrosome reaction and uterine fertilization microenvironments [12]. In routine cryopreservation, seminal plasma is retained during liquid storage but removed via centrifugation to concentrate sperm [13]. Resuspending thawed sperm in artificial diluents rather than native seminal plasma largely explains inferior fertilization relative to fresh semen or natural mating [14,15], and plasma absence is a key contributor to variable insemination efficiency [16,17].

Multiple studies confirm that seminal plasma supplementation improves post-thaw boar sperm quality, enhancing metabolic activity [18], restoring surface protein function [19] and alleviating plasma membrane cryoinjury [20]. Experiments have shown that seminal plasma components improve in vitro fertilization (IVF) of porcine oocytes [20], relieve cryocapacitation and elevate intracervical insemination efficiency [21], and raise pregnancy rates and litter sizes of thawed epididymal sperm [22]. However, the 50% plasma concentration used in previous trials is impractical for large-scale breeding [23], and the molecular basis of low-dose protection lacks systematic research.

This study aimed to screen for an effective concentration of boar seminal plasma for supplementation in thawing media and to compare the protective effects of seminal plasma from freeze-tolerant (FT) and non-freeze-tolerant (NFT) boars on sperm quality after thawing. Additionally, data-independent acquisition (DIA)-based quantitative proteomics was used to identify and characterize the core molecular pathways mediating the cryoprotective

effects of seminal plasma. Finally, artificial insemination trials were conducted to validate the selected supplementation ratio for commercial production of frozen semen.

2. Materials and Methods

2.1. Study Design

This study adopted a completely randomized design to evaluate how seminal plasma phenotypes and supplementation concentrations affect the quality, proteome, and reproductive performance of frozen–thawed boar sperm. Healthy Landrace boars with normal reproductive performance were used as experimental animals and raised under standardized feeding and management. Two seminal plasma phenotypes were tested: freeze-tolerant (FT) and non-freeze-tolerant (NFT). Four volumetric supplementation ratios (0%, 10%, 20%, 30%, v/v) were set for the thawing medium. For sperm quality experiments, the experimental unit was the individual boar semen sample ($n \geq 15$ per treatment). For the proteomic experiment, 15 frozen straws from 15 independent boars were pooled in groups of five to yield three pooled semen samples, which served as biological replicates (n = 3 per treatment). The pooled seminal plasma (FT and NFT) was the treatment, not the experimental unit. Three linked experiments were carried out sequentially: screening of seminal plasma concentrations via sperm quality detection; functional component verification and molecular mechanism exploration using ultracentrifugal supernatant and DIA-based proteomics; and field artificial insemination (AI) trials to verify practical application value. All experimental operations were conducted consistently from May 2023 to May 2024 to minimize systematic error. Boars were classified as freeze-tolerant (FT) or non-freeze-tolerant (NFT) based on post-thaw progressive motility (PM) after repeated standardized semen cryopreservation procedures [24]. Boars with consistently higher post-thaw PM were categorized as FT, whereas those with poorer post-thaw PM were categorized as NFT.

2.2. Equipment and Reagents

The following instruments were purchased from Beijing Tianyuan Aorui Biotechnology Co., Ltd. (Beijing, China): computer-assisted sperm analysis (CASA) system (Model TYCASA.V1.0), electronic balance (Model TYPHY10), automatic straw filling and sealing machine (Model TYGZJ-1-50), programmable freezer (Model TYLDY-900), large-capacity centrifuge (Model TYLXJ-0-4500), −150 °C low-temperature refrigerator (Model TYDWCZG-150), and 100 L self-pressurized liquid nitrogen tank (Model BGHN-ZZYG-100). Additional equipment included a 35 L liquid nitrogen tank (Jinfeng, Chengdu, China), Mini-TB4 protein electrophoresis transfer tank and DYY-8C electrophoresis apparatus (Beijing Liuyi Biotechnology Co., Ltd.) (Beijing, China), Tanon 5200 chemiluminescence imaging system (Tanon Science & Technology Co., Ltd.) (Shanghai, China), Eppendorf pipettes of all specifications, and an ultra-clean workstation (Suzhou Purification, Suzhou, China). A commercial boar semen cryopreservation kit (containing Reagents A–E) was supplied by Beijing Tianyuan Aorui Biotechnology Co., Ltd. The detailed formulation of this kit is proprietary commercial information and is not publicly disclosed by the manufacturer. Ultrapure water was obtained from Merck Millipore (Darmstadt, Germany). Primary and secondary antibodies were purchased from Proteintech (Rosemont, IL, USA): mouse anti-human CLU (Cat. MK18016), mouse anti-LGMN monoclonal antibody (Cat. 67017-1-Ig), mouse anti-SERPINA1 monoclonal antibody (Cat. 66135-1-Ig), rabbit anti-PLS1 polyclonal antibody (Cat. 13143-1-AP), rabbit anti-β-actin antibody (Cat. 20536-1-AP), mouse anti-rabbit secondary antibody (Cat. SA00001-19), and goat anti-mouse secondary antibody (Cat. SA00013-1).

2.3. Methods for Semen Cryopreservation, Thawing, and Quality Detection

2.3.1. Semen Cryopreservation Method

Fresh semen was obtained from a commercial boar stud (Beijing Sifanghong Breeding Pig Co., Ltd., Beijing, China) and immediately diluted with pre-warmed 37 °C pre-diluent at a 1:2 (v/v) semen:diluent ratio upon arrival. A 1 mL aliquot was sampled to test sperm concentration, total motility, and morphological abnormality rate. Only qualified semen (motility ≥ 80%, abnormality rate ≤ 10%) was equilibrated at 17 °C for 1 h. Qualified samples were centrifuged at 850× g for 13 min at 17 °C, and the supernatant was discarded. The sperm pellet was resuspended in Equilibrated Cryoprotectant Extender I at 17 °C to reach a final concentration of 2×10^9 sperm/mL. Samples were slowly cooled from 17 °C to 4 °C over 90 min using a programmable freezer. After cooling, semen was transferred to a 4 °C operation cabinet and mixed equally with pre-chilled Cryoprotectant Extender II (1:1, v/v), resulting in a final concentration of 1×10^9 sperm/mL in 0.5 mL straws. The mixture was loaded into 0.5 mL straws and sealed automatically. The freezing curve was set as follows: cool from 4 °C to 1 °C within 2 min, then drop to −140 °C over 4.7 min, hold at −140 °C for 5 min, and finally store straws long-term in liquid nitrogen (−196 °C).

2.3.2. Semen Thawing and Quality Detection Method

Frozen straws were retrieved from liquid nitrogen and thawed in a 60 °C water bath for 15 s, following the procedure described in our previous study [24]. The basal thawing medium was Reagent E of the commercial boar semen cryopreservation kit (Beijing Tianyuan Aorui Biotechnology Co., Ltd., Beijing, China), which mainly contains glucose, citric acid, sodium citrate, sodium bicarbonate, and EDTA-Na2. Thawed semen was diluted with pre-warmed 37 °C basal thawing medium at a 1:8 (v/v) ratio, according to the manufacturer's instructions and our previously published protocol [24], and incubated at 37 °C for 10 min to restore sperm motility before testing. Sperm motility parameters were quantified via CASA. The microscope stage and counting chambers were maintained at 37–38 °C throughout detection. Samples were pre-incubated at 37 °C and gently inverted 30 times to ensure homogeneous sperm distribution; 10 μL of sample was loaded into a pre-warmed 10 μm-deep counting chamber and settled for 10 s before observation. Four random microscopic fields were captured per sample using a 10× phase-contrast objective. System parameters: frame interval 20 ms, exposure time 10 ms, total frames captured = 25. Motility criteria: motile sperm = curvilinear velocity (VCL) ≥ 35 μm/s; progressively motile sperm = straight-line velocity (VSL) ≥ 25 μm/s, or 10 μm/s ≤ VSL < 25 μm/s with linearity (LIN) ≥ 0.4. Additional kinematic parameters included average path velocity (VAP), amplitude of lateral head displacement (ALH), straightness (STR), and wobble (WOB). Each sample was analyzed in triplicate (technical replicates), and the mean value was recorded as the final result.

2.4. Experimental Design for Seminal Plasma Treatment and Grouping

2.4.1. Seminal Plasma Concentration Screening Experiment

FT seminal plasma was obtained from 11 freeze-tolerant boars, and NFT seminal plasma from 11 non-freeze-tolerant boars. Ejaculates were collected from these boars at the same commercial boar stud according to its routine schedule, at intervals of approximately 2–3 days, and more than 30 ejaculate aliquots were obtained for each phenotype. All aliquots within the same phenotype were pooled at equal volumes. Therefore, each phenotype corresponded to a single mixed seminal plasma pool (n = 1 pool per phenotype), and the pooled seminal plasma served as the treatment, not as the experimental unit. The pooled samples were sequentially filtered through 3 μm, 1 μm and 0.22 μm membranes to reduce bacterial and fungal microbial loads. The processed seminal plasma was aliquoted and

stored at −80 °C. Thawing media with final seminal plasma ratios of 0%, 10%, 20% and 30% (v/v) were prepared based on the basal medium. For each biological replicate (individual boar, n ≥ 15 per treatment), four frozen straws from the same boar were thawed and pooled, then diluted with each test medium at a 1:8 (v/v) ratio and incubated at 37 °C. Sperm motility and kinematic indices were measured at 10 min and 2 h post-thawing.

2.4.2. Seminal Plasma Ultracentrifugation Supernatant Verification Experiment

Ultracentrifugal supernatants were harvested from FT and NFT seminal plasma separately and added to thawing medium at the selected concentration screened above. Frozen semen was thawed, mixed with supernatant-containing medium at 1:8 (v/v), and incubated at 37 °C. Sperm quality detection at 10 min and 2 h post-thawing followed identical protocols to the concentration screening test.

2.4.3. Proteomic Analysis Experiment

For proteomic analysis, 15 frozen straws from 15 independent boars were used. The straws were thawed and pooled in groups of five, yielding three pooled semen samples. Each pooled sample was divided into three equal aliquots and diluted separately with FT-supplemented, NFT-supplemented, or control thawing medium at 1:8 (v/v) and incubated at 37 °C for 10 min. The three pooled samples served as biological replicates (n = 3 per treatment). These pooled proteomic samples were distinct from the individual boar samples used for subsequent Western blot validation (n = 9 per group). Samples were washed by centrifugation at 800× g for 10 min at 4 °C, and sperm pellets were lysed with radioimmunoprecipitation assay (RIPA) buffer containing 1% protease inhibitor cocktail for data-independent acquisition (DIA)-based quantitative proteomics to screen differentially expressed proteins (DEPs).

2.5. Methods for Seminal Plasma and Its Ultracentrifugation Supernatant Treatment in Thawing Medium

2.5.1. Treatment Method of Seminal Plasma Addition in Thawing Medium

Thawing media supplemented with 0%, 10%, 20%, or 30% FT/NFT seminal plasma were aliquoted into 80 mL bottles and stored at −80 °C to avoid activity loss from repeated freeze–thaw cycles. For each biological replicate (n ≥ 15 independent boars), four frozen straws from the same boar were thawed, mixed, and diluted with seminal plasma-supplemented thawing medium at 1:8 (v/v) at 37 °C. CASA was used to test total motility (TM), progressive motility (PM), and all kinematic parameters at 10 min and 2 h after thawing. The supplementation ratio was selected based on these measurements.

2.5.2. Treatment Method of Seminal Plasma Ultracentrifugation Supernatant Addition in Thawing Medium

Ultracentrifugal supernatants from FT and NFT seminal plasma were separately incorporated into basal thawing medium at the screened selected concentration, aliquoted, and stored at −80 °C. Using the same number of biological replicates (n ≥ 15 independent boars), four frozen straws originating from each boar were thawed and mixed, and diluted with supernatant-supplemented thawing medium at 1:8 (v/v) at 37 °C. CASA was used to compare sperm motility indices between intact seminal plasma and supernatant-only groups at 10 min and 2 h post-thawing.

2.6. Proteomic Analysis Methods for Frozen–Thawed Sperm

2.6.1. Sample Preparation and Protein Extraction

Semen samples from each group were diluted with the corresponding thawing medium at a ratio of 1:8 (v/v) and incubated at 37 °C for 10 min. The samples were then washed twice by centrifugation at 12,000× g for 1 min to remove seminal plasma impurities. The final sperm pellet was resuspended in 200 μL RIPA lysis buffer containing 1% protease inhibitor cocktail, followed by lysis on ice for 20 min with 1 min vortexing every 5 min. After lysis, samples were centrifuged at 12,000× g for 20 min at 4 °C, and the supernatant containing total sperm protein was collected, preserved at −80 °C, and shipped on dry ice for subsequent proteomic detection.

2.6.2. Protein Enzymatic Digestion Method

Frozen protein extracts were thawed slowly on ice and concentrated with 10 kDa molecular weight cutoff ultrafiltration tubes at 5000× g, and the buffer was replaced five times with 4 M urea to eliminate interference from lysis buffer components. Silver staining with bovine serum albumin (BSA) as the standard was applied to quantify protein concentration. The digestion procedure followed standard protocols: proteins were denatured with 5 mM dithiothreitol (DTT) at 56 °C for 30 min, then alkylated with 11 mM iodoacetamide (IAA) in darkness at room temperature for 15 min. Triethylammonium bicarbonate (TEAB) buffer was added to dilute urea to below 2 M. Trypsin was added at an enzyme-to-protein mass ratio of 1:50 for overnight digestion at 37 °C, and extra trypsin (1:100, w/w) was supplemented for another 4 h to achieve complete peptide hydrolysis.

2.6.3. Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS) Analysis Method

Enzymatically digested peptides were separated using an Easy-nLC 1200 (Thermo Fisher Scientific, Waltham, MA, USA) ultra-high performance liquid chromatography system. Mobile phase A consisted of 0.1% formic acid in 2% acetonitrile, and mobile phase B consisted of 0.1% formic acid in 90% acetonitrile. The linear separation gradient was configured as follows: 6–22% mobile phase B over 22.5 min, 22–34% mobile phase B over 4 min, 34–80% mobile phase B over 2 min, and 80% mobile phase B for 1.5 min, with a constant flow rate of 700 nL/min. After chromatographic separation, peptides were ionized by electrospray ionization and analyzed on an Orbitrap Exploris 480 (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer in data-independent acquisition (DIA) mode.

2.7. Western Blotting

Western blotting was performed with minor modifications to the protocol described by Taylor and Posch (2014) [25]. Briefly, extracted total sperm proteins were separated on 5% stacking/12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) separating gels, then wet-transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk for 1 h, incubated with primary antibodies overnight at 4 °C, and washed three times (5 min each) with Tris-buffered saline with Tween (TBST). Corresponding secondary antibodies were applied for 1 h at room temperature, followed by three additional TBST washes. Protein bands were visualized via chemiluminescence using a Tanon 5200 imaging system, and gray values were quantified with ImageJ software (version 1.8.0). Standard pre-stained protein molecular weight markers were used for SDS-PAGE. For statistical comparisons of WB relative quantification data, one-way ANOVA was applied. All WB experiments were performed under identical experimental conditions across all blots. Due to the limited volume of each protein sample, each target protein was analyzed on a separate gel, with β-actin run in parallel on the same

gel as the loading control. For relative quantification, the ratio of target protein to β-actin band intensity within each sample served as the relative abundance of the target protein, correcting for variations in sample loading; the relative expression level of each treatment group was then calculated relative to the control group, which was set as 100%.

2.8. Artificial Insemination

Thawing media were prepared in advance, dispensed into 40 mL bottles, and stored at −20 °C to avoid repeated freeze–thaw damage. For each insemination dose, four 0.5 mL frozen straws were thawed in a 60 °C water bath for 15 s, rapidly pooled and added to one pre-warmed 40 mL bottle of thawing medium, and used immediately. The total insemination volume was 40 mL per dose. The sperm concentration before freezing was 2×10^9 sperm/mL. After 1:1 dilution with Cryoprotectant Extender II, the final concentration in 0.5 mL straws was 1×10^9 sperm/mL, corresponding to 5×10^8 sperm per straw. Four straws per insemination dose yielded a total of 2×10^9 sperm per dose. Two inseminations were performed per sow 12 h apart, resulting in a total of 4×10^9 sperm administered per sow. Farm technicians controlled insemination timing strictly: sows with morning estrus were inseminated in the afternoon, and afternoon-estrus sows received insemination the next morning, adjusted according to parity and estrus intensity. Deep intrauterine insemination was performed using a disposable deep intrauterine insemination catheter by trained farm technicians following the farm's standard operating procedures. All sows used in this trial were commercial Landrace sows with parity 2 to 4 and moderate body condition. Sows were randomly allocated to the frozen semen group and the fresh semen group at the time of breeding. No additional inclusion or exclusion criteria were applied, and all sows presented for breeding during the trial period were included in the analysis. Pregnancy was diagnosed by trans-abdominal ultrasonography (B-mode) according to the farm's routine procedure. The control group used fresh semen collected within 3 days (motility ≥ 85%), with identical insemination volume, operation and timing as the frozen semen group.

2.9. Data Processing and Analysis

Raw experimental data were cleaned and outliers removed in Microsoft Excel, followed by statistical analysis using SPSS 23.0. All quantitative results were expressed as mean ± standard error of the mean (Mean ± SEM). For sperm quality data, a two-way ANOVA was performed with boar as a random effect and thawing medium concentration as a fixed effect. The 10 min and 2 h post-thawing time points were analyzed separately. Dunnett's multiple comparison test was used as the post-hoc procedure to compare each treatment group with the control. Outliers were defined as values beyond mean ± 2 standard deviations; no outliers were identified, and no missing observations occurred in this study. The experimental unit was the semen sample from an individual boar (n ≥ 15 per treatment), and technical replicates (three CASA measurements per sample) were averaged prior to analysis. All statistical inferences regarding treatment effects were based on these independent biological replicates, not on the seminal plasma pools. Direct pairwise comparisons among the 10%, 20%, and 30% groups were not performed. Sperm motility and kinematic parameter graphs displayed baseline-subtracted values relative to the within-boar control for each group to eliminate inter-individual boar variability; absolute control means were subsequently overlaid to ensure that group means reflect their actual absolute values. Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Differentially expressed proteins (DEPs) were screened at fold change (FC) > 1.5 and $p < 0.05$. For the proteomic experiment, three pooled semen samples per treatment were used as biological replicates ($n = 3$ per treatment). Gene Ontology (GO) annotation covered biological process (BP), cellular component (CC),

and molecular function (MF). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment was conducted with $p < 0.05$ as the significance cutoff.

3. Results

3.1. Effects of Freeze-Tolerant (FT) Seminal Plasma on Post-Thaw Sperm Motility and Kinematics

At 10 min post-thawing, supplementation with 10%, 20% and 30% FT seminal plasma significantly improved total motility (TM) and progressive motility (PM) compared with the control group ($p < 0.05$) (Figure 1). No differences in VCL, VAP or ALH were observed ($p > 0.05$). The 10% group only elevated STR and LIN ($p < 0.05$), while 20% and 30% groups produced far higher STR, LIN and WOB ($p < 0.01$, $p < 0.001$). All three treatment groups showed significantly increased WOB ($p < 0.01$ or $p < 0.001$).

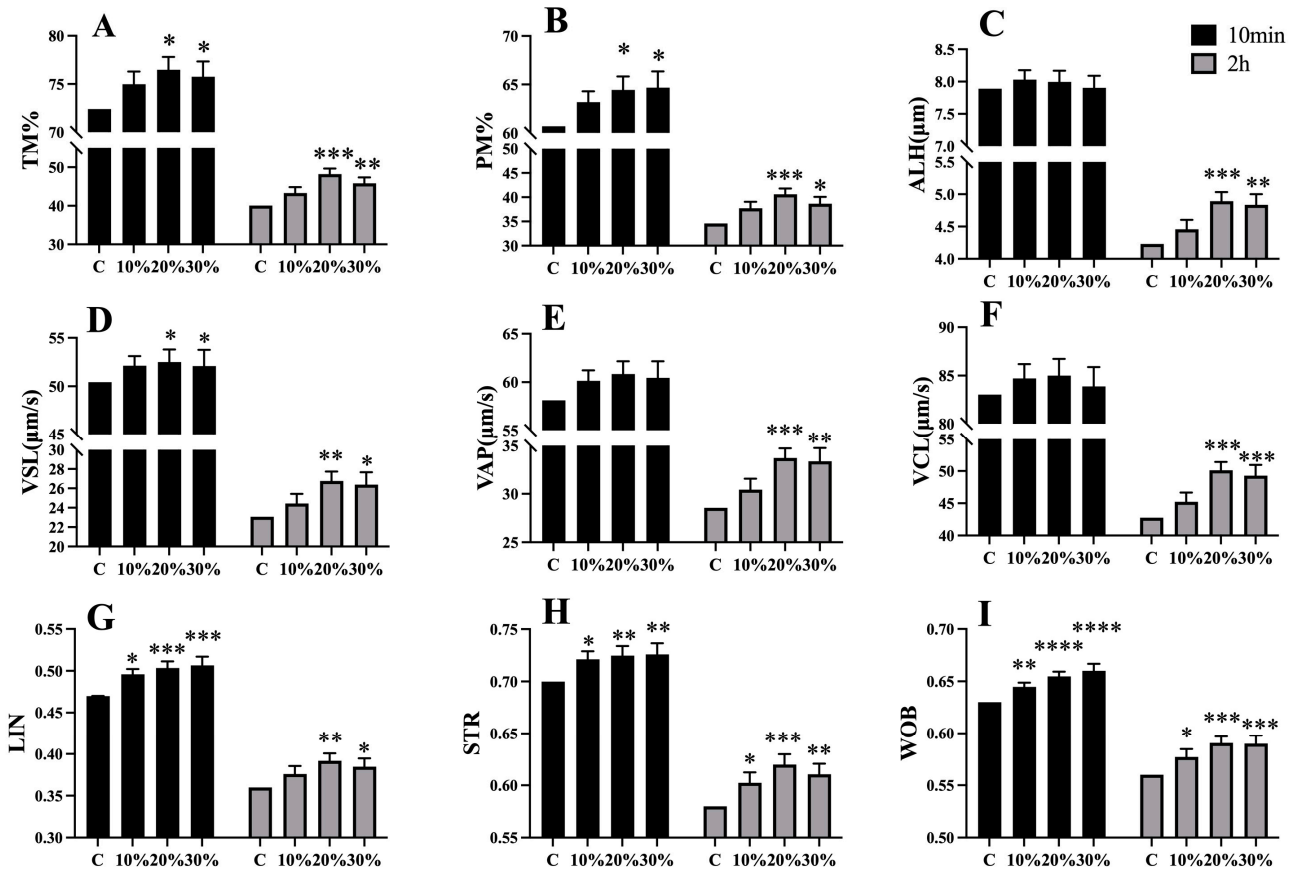


Figure 1. Effects of freeze-tolerant (FT) boar seminal plasma supplementation in thawing medium on motility and kinematic traits of frozen–thawed boar sperm. Sperm were treated with 0% (control, C), 10%, 20%, and 30% (*v*/*v*) freeze-tolerant seminal plasma; black bars indicate detection at 10 min post-thawing, and gray bars represent measurements at 2 h post-thawing. (**A**) Total motility (TM); (**B**) progressive motility (PM); (**C**) amplitude of lateral head displacement (ALH); (**D**) straight-line velocity (VSL); (**E**) average path velocity (VAP); (**F**) curvilinear velocity (VCL); (**G**) linearity (LIN); (**H**) straightness (STR); (**I**) wobble (WOB). Asterisks denote statistical significance relative to the control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Data are presented as mean ± SEM ($n \geq 15$ independent boars per treatment).

At 2 h post-thawing, all treatment groups showed higher values than controls, with the 20% group showing the highest numerical values for most parameters. Only STR and WOB rose significantly in the 10% group ($p < 0.05$), whereas all measured parameters were significantly increased at 20% ($p < 0.001$). The 30% group displayed significant improvements across indices ($p < 0.05$, $p < 0.01$) but remained inferior to the 20% treatment.

3.2. Effects of Non-Freeze-Tolerant (NFT) Seminal Plasma on Post-Thaw Sperm Motility and Kinematics

At 10 min post-thawing, supplementation with NFT seminal plasma at three concentrations elevated all measured sperm motility and kinematic parameters relative to the control group (C) (Figure 2). VCL and ALH exhibited significant increases only in the 10% NFT group ($p < 0.05$). VSL and VAP were significantly improved across all three concentrations; significance was $p < 0.01$ for the 10% group, and $p < 0.05$ for the 20% and 30% groups. The maximum TM value was recorded in the 20% group, with significant differences in TM detected at 10% ($p < 0.05$), 20% ($p < 0.001$) and 30% ($p < 0.05$). PM reached its highest numerical value at 20% supplementation ($p < 0.001$). STR, LIN and WOB were significantly higher in all NFT treatment groups ($p < 0.001$).

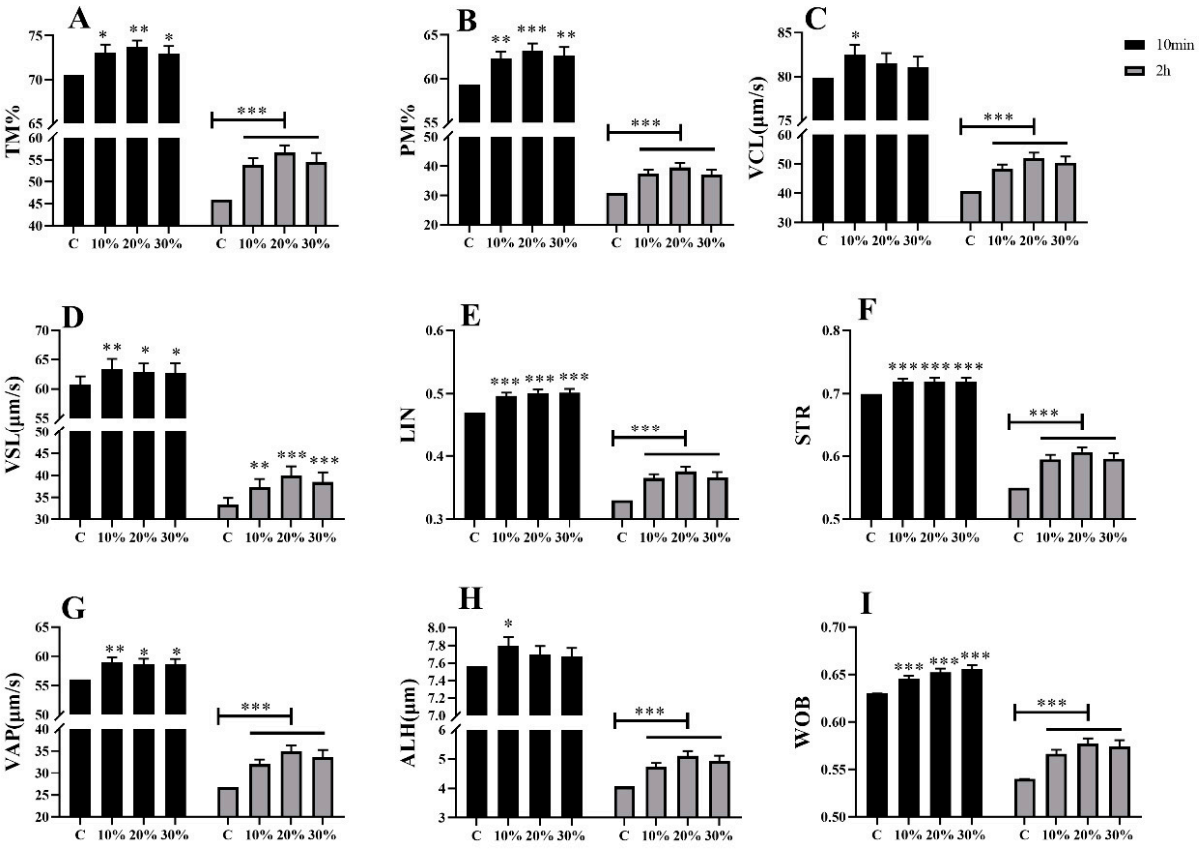


Figure 2. Effects of non-freeze-tolerant (NFT) boar seminal plasma supplementation in thawing medium on motility and kinematic traits of frozen–thawed boar sperm. Sperm were treated with 0% (control, C), 10%, 20%, and 30% (v/v) non-freeze-tolerant seminal plasma; black bars indicate detection at 10 min post-thawing, and gray bars represent measurements at 2 h post-thawing. (**A**) TM; (**B**) PM; (**C**) VCL; (**D**) VSL; (**E**) LIN; (**F**) STR; (**G**) VAP; (**H**) ALH; (**I**) WOB. Asterisks denote statistical significance relative to the control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are presented as mean ± SEM ($n \geq 15$ independent boars per treatment).

At 2 h post-thawing, all NFT groups retained higher values of the above kinematic indicators than the control, and the 20% group showed the highest values for most indicators. LIN was significantly increased in the 10% group ($p < 0.01$); significant improvements in LIN and all other kinematic indicators were also found in the 20% and 30% groups ($p < 0.001$).

3.3. Motility Changes After Supplementation with Seminal Plasma Supernatant (Initial PM < 65%)

For semen samples with low initial PM below 65% (Figure 3), all measured sperm motility and kinematic parameters were significantly elevated in the 20% FT and NFT seminal plasma supernatant groups at 10 min post-thawing. TM, ALH and VCL were significantly higher in the FT and NFT groups ($p < 0.05$), while PM, VSL, VAP, STR, LIN and WOB showed more pronounced improvements ($p < 0.01$ or $p < 0.001$). No statistically significant differences were observed between the FT supernatant group and NFT supernatant group at this time point.

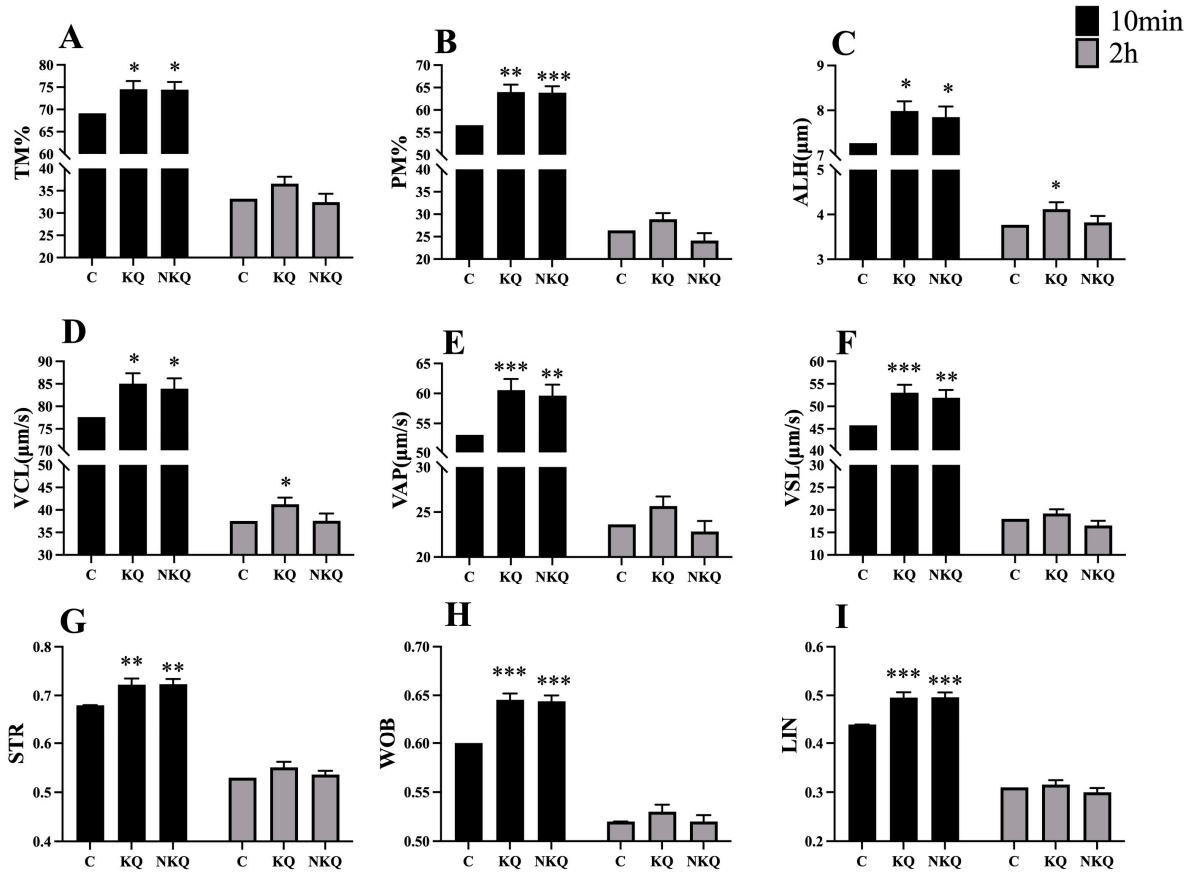


Figure 3. Effects of freeze-tolerant (FT) and non-freeze-tolerant (NFT) boar seminal plasma supernatant supplementation in thawing medium on motility and kinematic traits of frozen–thawed boar sperm (Initial PM < 65%). Sperm were treated with 20% (v/v) supernatant derived from FT and NFT seminal plasma, and the control group (C) received no supernatant addition; black bars indicate detection at 10 min post-thawing, and gray bars represent measurements at 2 h post-thawing. (**A**) TM; (**B**) PM; (**C**) ALH; (**D**) VCL; (**E**) VAP; (**F**) VSL; (**G**) STR; (**H**) WOB; (**I**) LIN. Asterisks denote statistical significance relative to the control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are presented as mean ± SEM ($n \geq 15$ independent boars per treatment).

At 2 h post-thawing, only ALH and VCL remained significantly higher in the FT supernatant group relative to the control (C) ($p < 0.05$). No other kinematic indicators exhibited significant differences between the FT group and control. No sperm motility or kinematic parameters differed significantly between the NFT supernatant group and the control group at 2 h post-thawing.

3.4. Motility Changes After Supplementation with Seminal Plasma Supernatant (Initial PM > 65%)

For high-quality frozen semen with initial PM above 65% (Figure 4), FT supernatant significantly increased WOB ($p < 0.001$) and LIN ($p < 0.01$) at 10 min post-thawing. NFT supernatant only produced a significant rise in WOB ($p < 0.05$), and all remaining kinematic indicators did not differ significantly from the control.

At 2 h post-thawing, the FT supernatant group displayed significant gains in VAP ($p < 0.05$), as well as elevated ALH and VCL ($p < 0.01$). The NFT supernatant group only showed an increased ALH value ($p < 0.05$), with no other significant differences from the control group.

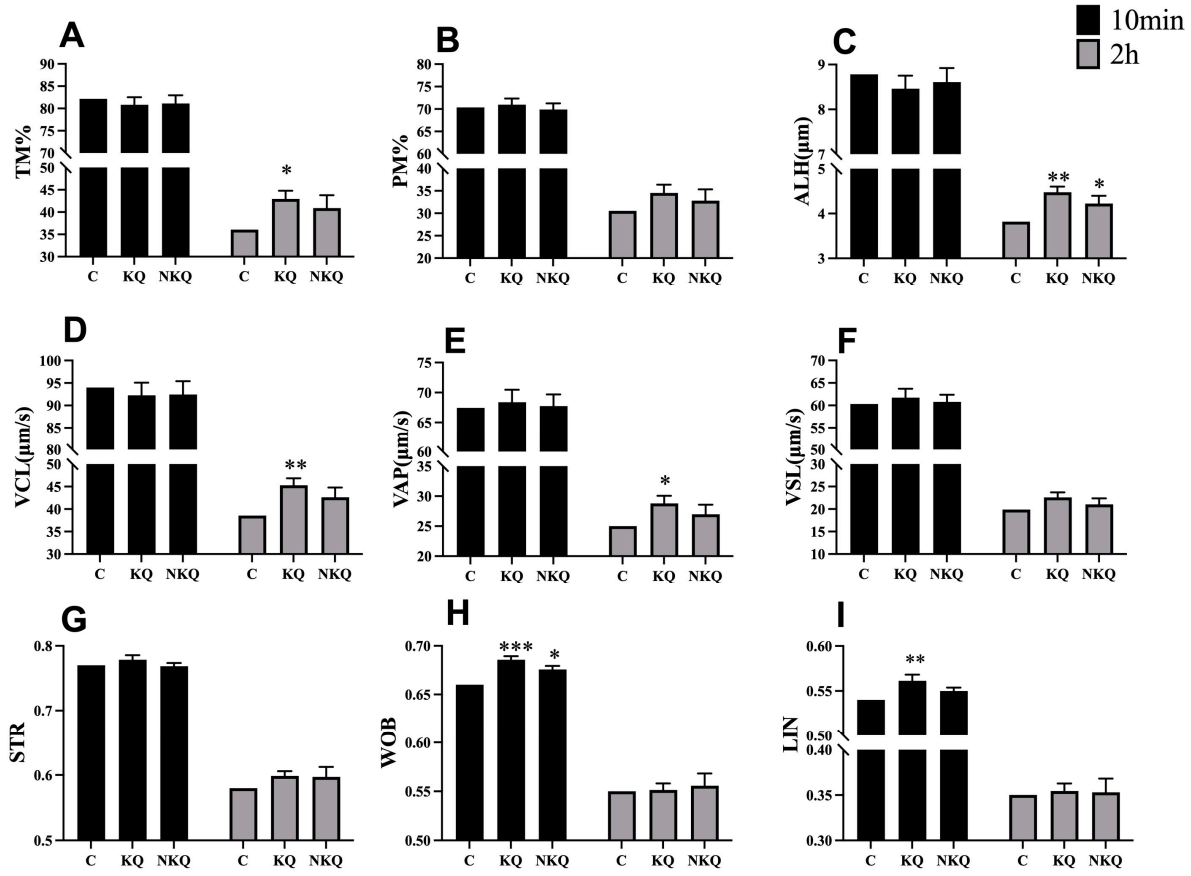


Figure 4. Effects of freeze-tolerant (FT) and non-freeze-tolerant (NFT) boar seminal plasma supernatant supplementation in thawing medium on motility and kinematic traits of frozen–thawed boar sperm (Initial PM > 65%). Sperm were treated with 20% (v/v) supernatant derived from FT and NFT seminal plasma, and the control group (C) received no supernatant addition; black bars indicate detection at 10 min post-thawing, and gray bars represent measurements at 2 h post-thawing. (**A**) TM; (**B**) PM; (**C**) ALH; (**D**) VCL; (**E**) VAP; (**F**) VSL; (**G**) STR; (**H**) WOB; (**I**) LIN. Asterisks denote statistical significance relative to the control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are presented as mean ± SEM ($n \geq 15$ independent boars per treatment).

3.5. Artificial Insemination Reproductive Outcomes

This field trial was conducted under identical commercial farm conditions and comprised two groups: sows inseminated with frozen–thawed semen supplemented with 20% seminal plasma, and sows inseminated with fresh semen as the reference group. No contemporaneous conventional frozen-semen control was included in this trial, and the comparison with conventional frozen semen therefore relies on historical breeding records rather than a

concurrent experimental control. For sow artificial insemination (AI), all sows inseminated with frozen–thawed semen using this selected 20% seminal-plasma-supplemented thawing medium achieved successful conception. A total of 26 sows were assigned to the frozen semen treatment group, and 27 sows inseminated with fresh semen served as controls (Figure 5). The average litter size of the frozen semen group was 12.46 ± 0.43 piglets, which was not significantly different from the fresh semen group (12.56 piglets; $p > 0.05$). Similarly, the number of live piglets did not differ significantly between fresh and frozen semen groups.

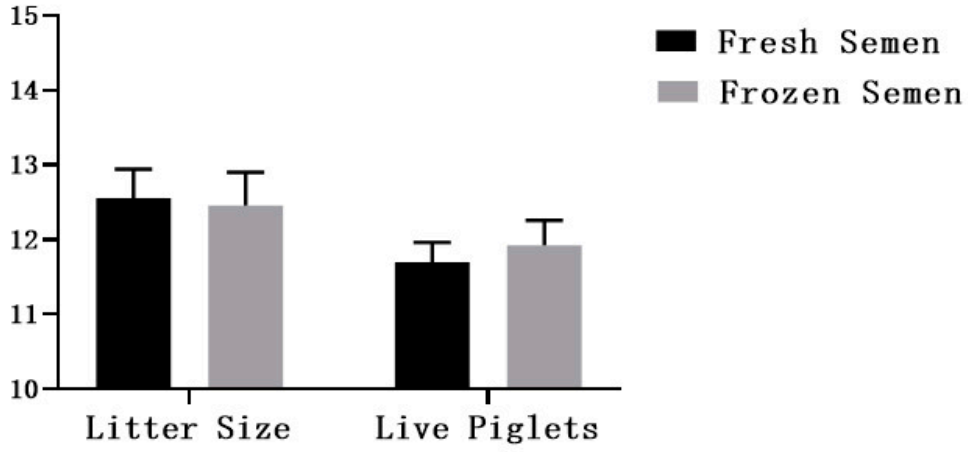


Figure 5. Reproductive performance of sows subjected to artificial insemination with fresh boar semen and frozen–thawed semen supplemented with 20% seminal plasma in thawing medium. Sows were inseminated with either fresh semen (control, black bars) or frozen semen thawed with 20% seminal plasma (gray bars); 26 sows were assigned to the frozen semen group, and 27 sows received fresh semen. Litter Size, total piglets per litter; Live Piglets, viable piglets per litter. Asterisks denote statistical significance relative to the control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; no asterisk indicates $p > 0.05$.

3.6. Proteomic Analysis of Sperm Treated with Different-Freezability Seminal Plasma

3.6.1. Screening of Differentially Expressed Proteins (DEPs)

Principal component analysis (PCA) and Pearson correlation heatmaps confirmed tight intra-group clustering for control (C), freeze-tolerant (FT) and non-freeze-tolerant (NFT) samples (Figure 6A,B). Samples from the FT and NFT groups partially overlapped, while both treatment groups were clearly separated from the control, with all intra-group correlation coefficients above 0.98 (n = 3 pooled samples per group). Supplementary quality results including SDS-PAGE of sperm proteins, relative standard deviation (RSD) distribution, peptide length distribution and protein abundance distribution further validated reliable proteomic detection (Supplementary Figures S1–S4). A total of 14,141 peptides, 12,557 unique peptides and 2965 quantified sperm proteins were identified (Figure 6C). Using fold change > 1.5 and $p < 0.05$ as screening thresholds, 513 DEPs were detected between the FT and control (C) groups, with 356 DEPs upregulated and 157 DEPs downregulated in FT relative to C. Similarly, 366 DEPs were detected between the NFT and C groups, with 273 DEPs upregulated and 93 DEPs downregulated in NFT relative to C. Only 11 DEPs were identified between the FT and NFT groups (5 upregulated, 6 downregulated) (Figure 6D). Volcano plots presented distinct expression shifts between the control and each seminal plasma group, whereas minimal protein abundance differences existed between FT and NFT (Figure 6E–G). The DEP clustering heatmap displayed consistent intra-group expression patterns and obvious inter-group separation (Figure 6H).

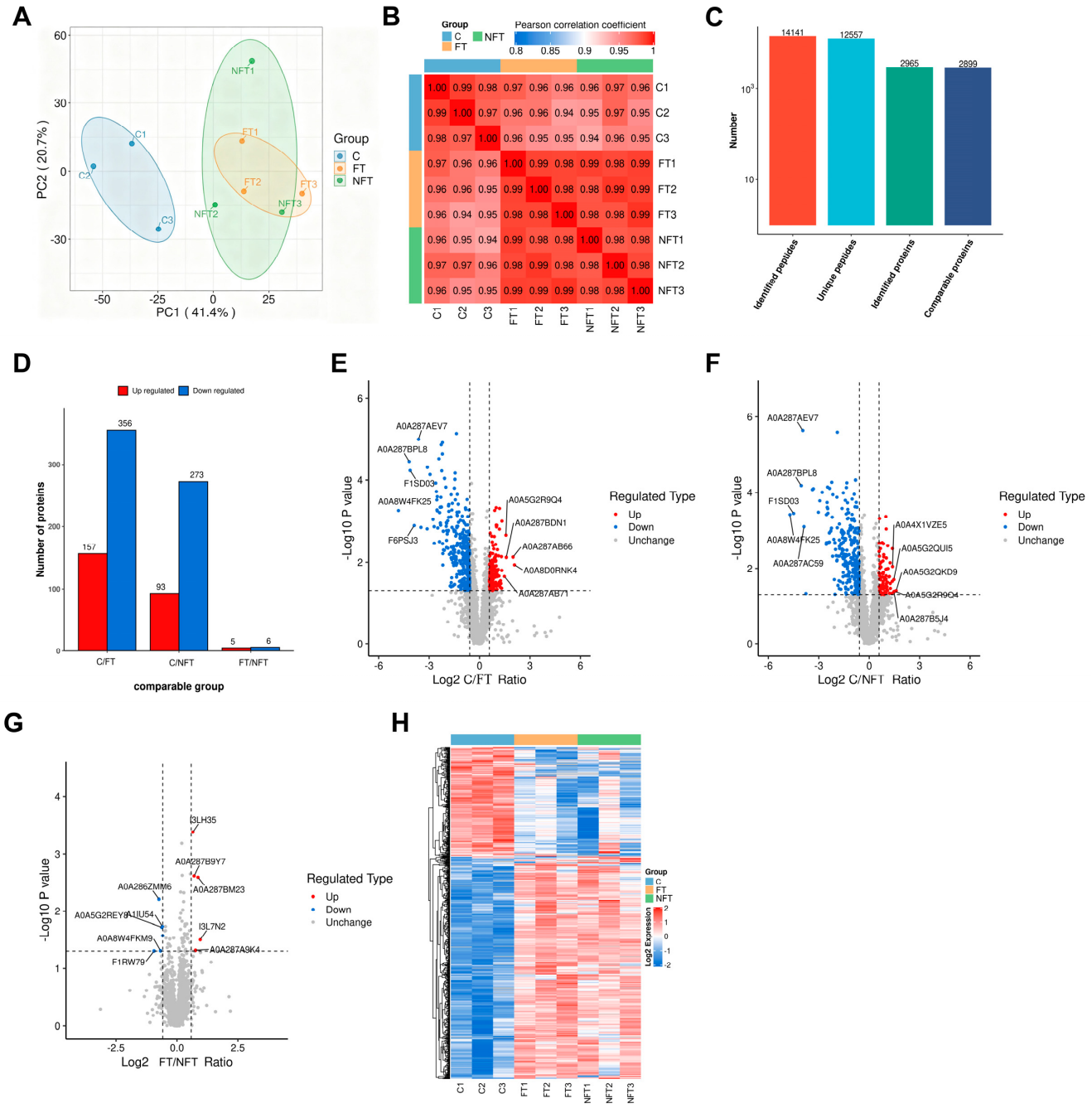


Figure 6. Screening and repeatability analysis of differentially expressed proteins (DEPs) in frozen–thawed boar sperm treated with control (C) FT and NFT seminal plasma. (**A**) Principal component analysis (PCA) plot of biological replicates; (**B**) sample Pearson correlation heatmap; (**C**) bar chart of total peptides and quantified proteins; (**D**) statistics of upregulated and downregulated DEPs; (**E**–**G**) volcano plots for C vs. FT, C vs. NFT, FT vs. NFT groups; (**H**) DEP clustering heatmap. DEPs were filtered by fold change (FC) > 1.5 and $p < 0.05$. Red = high protein expression; blue = low protein expression. $n = 3$ pooled semen samples per group; each pooled sample was prepared from five independent boars.

3.6.2. GO and KEGG Enrichment Analysis

GO and KEGG enrichment and subcellular localization analyses were performed on DEPs from C vs. FT and C vs. NFT groups to characterize their functional features and protein origins (Figure 7, Table 1).

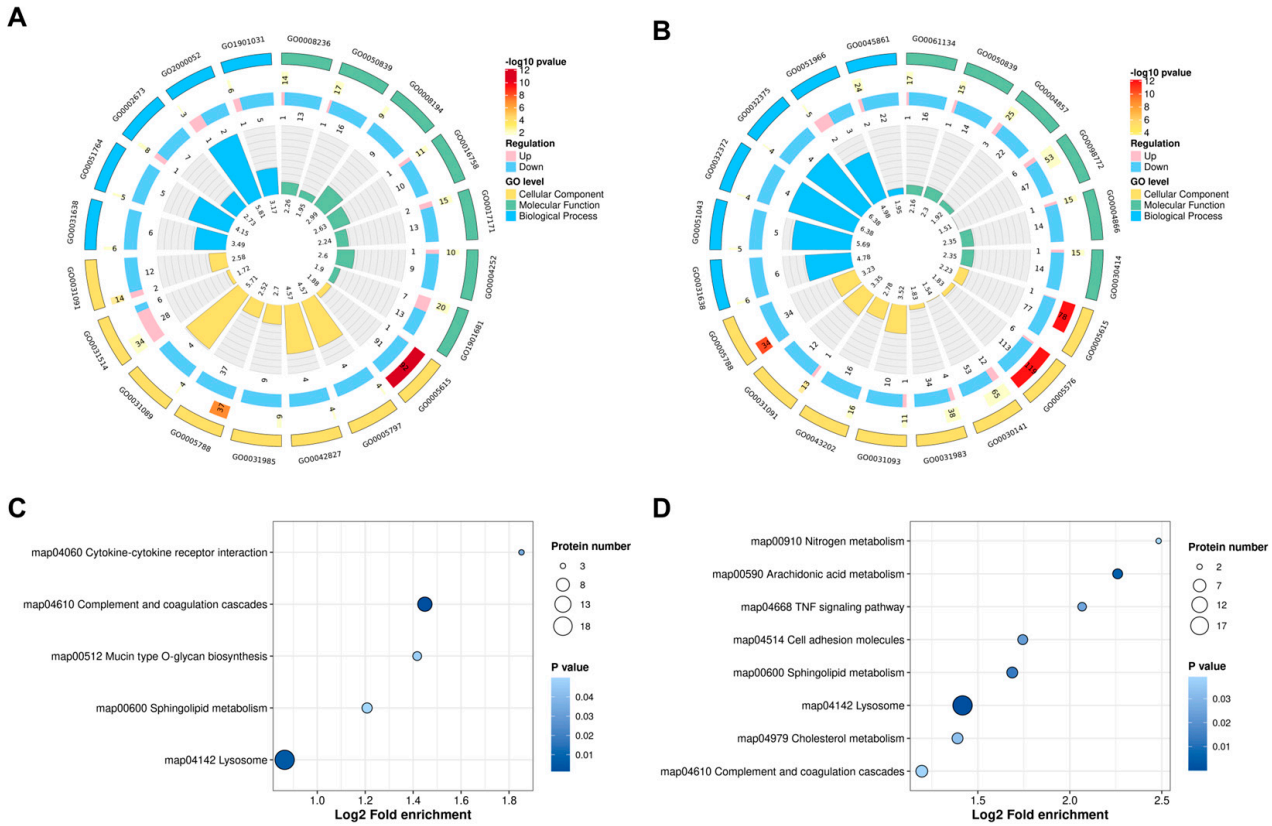


Figure 7. GO and KEGG enrichment of differentially expressed proteins (DEPs) in boar sperm (C vs. FT, C vs. NFT). (**A**) C vs. FT GO enrichment; (**B**) C vs. NFT GO enrichment; (**C**) C vs. FT KEGG enrichment; (**D**) C vs. NFT KEGG enrichment. Circular GO plot color represents statistical significance, with terms grouped into biological process, cellular component and molecular function. For KEGG bubble diagrams, the horizontal axis displays fold enrichment; bubble color denotes *p*-value, and bubble size indicates the total number of enriched DEPs. C, control; FT, 20% freeze-tolerant seminal plasma; NFT, 20% non-freeze-tolerant seminal plasma; DEPs, differentially expressed proteins. $n = 3$ pooled semen samples per group; each pooled sample was prepared from five independent boars.

Table 1. Overlap proportions of upregulated sperm differentially expressed proteins (DEPs) shared with seminal plasma proteins in the FT/C and NFT/C comparisons, separated by extracellular and non-extracellular subcellular localization.

Localization Type	Overlap with Seminal Plasma (∩)	Total Number (n)	Ratio
Total sperm proteins	556	2965	0.188 ^{Aa}
FT/C up-regulated	173	356	0.486 ^{Bb}
FT/C extracellular up-regulated	85	124	0.685 ^{Cc}
FT/C non-extracellular, up-regulated	88	232	0.379 ^{Bb}
Total sperm proteins	556	2965	0.188 ^{Aa}
NFT/C up-regulated	139	273	0.509 ^{Bbc}
NFT/C extracellular up-regulated	64	104	0.615 ^{Bc}
NFT/C non-extracellular, up-regulated	75	169	0.444 ^{Bb}

Note: FT, freeze-tolerant seminal plasma treatment group; NFT, non-freeze-tolerant seminal plasma group; C, untreated control group. Ω = number of overlapping proteins between sperm and seminal plasma proteomes; n = total quantified proteins in each subset. Different lowercase superscripts denote differences at $p < 0.05$; distinct uppercase superscripts denote highly significant differences at $p < 0.01$. n = 3 pooled semen samples per group; each pooled sample was prepared from five independent boars.

Top 20 enriched GO entries were categorized into biological process (BP), cellular component (CC), and molecular function (MF) categories for both comparisons (Figure 7A,B). For C vs. FT, BP terms were enriched for actin assembly and zymogen activation; major CC terms were extracellular and endoplasmic reticulum compartments; MF terms centered on glycosyltransferase and cell adhesion activity (Figure 7A). For C vs. NFT, prominent BP functions included zymogen activation and cholesterol transport inhibition, while CC enrichment concentrated on extracellular and lysosomal regions, with peptidase inhibitor activity dominating MF terms (Figure 7B).

KEGG bubble plots showed shared and unique enriched pathways (Figure 7C,D). Lysosome, complement and coagulation cascades, and sphingolipid metabolism were enriched in both groups. Five pathways were detected for C vs. FT, and eight for C vs. NFT; the latter additionally contained arachidonic acid metabolism, TNF signaling, and cholesterol metabolism. The lysosome pathway involved 20 DEPs in C vs. FT and 21 DEPs in C vs. NFT.

Subcellular localization profiles of DEPs are shown in Supplementary Figures S5 and S6, with quantitative overlap ratios listed in Table 1. The baseline overlap ratio between all sperm and seminal plasma proteins was 0.188 (556/2965). For DEPs upregulated in FT relative to C, the overlap rate reached 0.486 (173/356): extracellular proteins hit 0.685 (85/124), and non-extracellular proteins reached 0.379 (88/232). For DEPs upregulated in NFT relative to C, the total overlap was 0.509 (139/273), including 0.615 (64/104) extracellular and 0.444 (75/169) non-extracellular proteins. All overlap proportions of upregulated proteins were significantly higher than the baseline value, and extracellular DEPs showed markedly higher overlap rates than intracellular counterparts in both comparisons.

Enrichment clustering of GO and KEGG categories (Supplementary Figures S7–S11) demonstrated highly consistent functional profiles between the FT and NFT groups, while both treatment groups differed distinctly from the control. Combined with subcellular localization data, the two types of seminal plasma exerted broadly consistent regulatory effects on the sperm proteome.

3.6.3. Western Blot Analysis of Differentially Expressed Proteins

Four key DEPs linked to lysosome and complement–coagulation cascades were selected for Western blot (WB) analysis to assess consistency with the proteomic data: LGMN, PLS1, SERPINA1, CLU, with β-actin as the internal reference. Nine independent boars (biological replicates, n = 9) were included for each group (Figure 8A,B).

WB expression trends were consistent with proteomic data. Compared with control (C), all four proteins were highly upregulated in the FT group ($p < 0.01$): LGMN (2.13 ± 0.46), PLS1 (1.49 ± 0.14), SERPINA1 (1.86 ± 0.25), CLU (1.40 ± 0.11). In the NFT group, LGMN (1.87 ± 0.29) and SERPINA1 (1.56 ± 0.22) rose significantly ($p < 0.05$), CLU increased markedly ($p < 0.01$), while PLS1 showed no significant change relative to control ($p > 0.05$). Direct pairwise comparison between the FT and NFT groups showed no significant differences for any of the four target proteins.

These WB results were consistent with the proteomic DEP data, supporting substantial similarity in sperm proteome regulation between FT and NFT seminal plasma and corroborating the preceding GO/KEGG enrichment analyses.

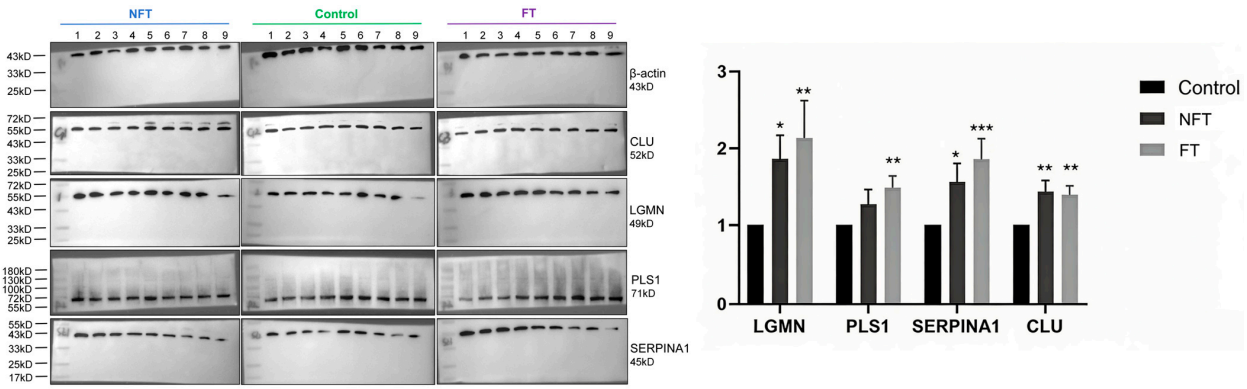


Figure 8. Western blot band profiles and quantitative relative expression of target proteins in boar sperm from control (C), FT and NFT groups. (**A**) Raw WB bands of β-actin, LGMN, PLS1, SERPINA1, and CLU; lanes 1–9 represent nine biological replicates per row, and rows correspond to C, FT and NFT groups respectively. (**B**) Quantitative relative expression of the four target proteins normalized to the control. Data are presented as mean ± SEM (n = 9). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control; ns denotes no significant difference between FT and NFT groups. LGMN, asparaginyl endopeptidase; PLS1, fimbrin 1; SERPINA1, serine protease inhibitor A1; CLU, clusterin; β-actin, internal reference protein. n = 9 independent boars per group.

4. Discussion

4.1. Concentration Screening and Protective Effects of Two Types of Seminal Plasma

Supplementation of seminal plasma into thawing medium markedly improved the overall quality of post-thaw boar semen, with an effective supplementary concentration clearly identified in this work. Consistent with the data measured at 10 min and 2 h post-thawing, 10–30% (v/v) addition of either FT or NFT seminal plasma significantly elevated TM, PM, VCL, VSL and other kinematic indices, and the protective efficacy persisted for at least 2 h after thawing. The 20% supplementation level showed the highest numerical values for most measured sperm performance metrics among the tested concentrations. Based on these numerical trends and the consistency of its protective effect across time points, 20% was selected as the recommended supplementation concentration, and this dosage is more practical for large-scale pig breeding compared with the 50% seminal addition reported previously [26], as lower raw material consumption may reduce production costs.

Notably, no statistically significant difference in protective outcomes was detected between the FT and NFT groups, indicating that the core cryoprotective bioactive substances contained in seminal plasma are not exclusive to freeze-tolerant boars. There is no necessity to screen boars based on semen freeze resistance to collect usable seminal plasma, which broadens the available raw material sources. Seminal plasma harvested from boars producing low-quality frozen semen (NFT phenotype) also exerted beneficial effects on thawed sperm, further expanding its industrial application value.

4.2. Protective Effects of Multiple Components in Intact Seminal Plasma

Comparisons between intact seminal plasma and its ultracentrifugal supernatant showed that multiple components in seminal plasma contribute jointly to its protective effects. After ultracentrifugation, exosomes and particulate vesicles were removed from seminal plasma supernatant. Although the supernatant slightly ameliorated sperm motility, its protective capacity was weaker and shorter-lived relative to complete seminal plasma. For batches with low baseline PM (<65%), supernatant only generated transient improvements at 10 min post-thawing with no sustained effects at 2 h. For high-quality semen with

initial PM > 65%, the supernatant produced negligible short-term benefits and only mild protective effects at 2 h, a trend consistent with the independent function of exosomes alone. These observations are consistent with soluble metabolites and vesicular components both contributing to counteract cold-induced sperm damage, and suggest that exosomes, despite their low abundance, may be important for long-term sperm protection. Combined soluble and vesicle fractions achieve similar protective effects at merely 0.1–0.3 × the semen volume required for exosome single treatment.

4.3. Field Artificial Insemination Verification of Industrial Application Potential

Given the parallel protective phenotypes of FT and NFT seminal plasma observed in in vitro tests, we mixed the two types indiscriminately without separate sorting to simplify farm operation workflows for subsequent AI trials. Consistent with the reproductive performance data presented in Section 3.5, thawing medium supplemented with 20% mixed seminal plasma generated fertility outcomes that did not differ significantly from fresh semen under actual production conditions. The average total litter size of sows receiving treated frozen semen (n = 26) reached 12.46 ± 0.43 piglets, while the fresh semen group recorded 12.56 piglets; the number of viable piglets was 11.92 ± 0.33 for the frozen group, slightly higher than the 11.70 piglets seen in fresh semen inseminations, with no statistically significant inter-group differences.

This study is subject to an experimental limitation arising from on-farm operational constraints: no concurrent control group using conventional unsupplemented frozen semen was included under identical feeding and breeding management. Establishing a contemporaneous conventional frozen-semen control group in a commercial production setting is logistically challenging, because sows allocated to such a group would be expected to show reduced reproductive performance relative to the farm's standard breeding output, which would compromise farm productivity. Notably, the conventional frozen-semen data cited herein are derived from long-term historical field breeding records accumulated by our research team and the collaborating farm, and serve as an observational benchmark for contextual comparison rather than a contemporaneous experimental control. Additionally, all seminal plasma samples in this work were pooled from multiple donor boars to reduce individual-boar-specific variation for practical farm-oriented application. Accordingly, our dataset cannot directly differentiate protective effects between autologous and heterologous seminal plasma, which would require follow-up experiments with separate treatment groups. Notably, all sows in both groups achieved conception in this trial with identical 100% conception outcomes, so further comparative analysis focused on litter-size-related productive traits. Based on these historical records, standard frozen semen without seminal plasma additives requires a total dosage of approximately 5 billion sperm per insemination to achieve litter performance similar to fresh semen when post-thaw PM exceeds 60% [27]. In contrast, our selected protocol only needs approximately 2 billion total sperm per insemination dose (roughly 1.2 billion motile sperm) for similar prolificacy, which substantially reduces elite boar germplasm consumption and may lower breeding costs for large-scale pig operations. Minor natural fluctuations in average litter size inevitably occur across farms due to disparities in sow parity, nutrition and routine insemination proficiency; the favorable reproductive indicators recorded at our test farm represented auxiliary influencing factors rather than the core driver of balanced fertility. The key factor underlying the similar litter outcomes was that 20% seminal plasma sustained stable high sperm motility for at least 2 h post-thaw, extending the fertilizable lifespan of sperm within the sow reproductive tract [28].

From a broader industrial research perspective, conventional frozen semen consistently yields lower farrowing rates and smaller litters than liquid fresh semen even when deep

intrauterine insemination is adopted [29,30]. Most existing strategies to boost frozen semen fertility rely on costly purified antioxidant additives in thawing extenders, which limits widespread on-farm uptake [31]. Earlier seminal plasma supplementation research mostly adopted a 50% volumetric addition ratio to gain measurable fertility improvements, a dosage that wastes large volumes of seminal plasma and cannot sustain continuous factory-scale use [26]. One prior in vitro study confirmed the motility benefits of 10–20% seminal plasma supplementation, yet lacked supporting on-field AI validation data [32]. Our field breeding trials supply complementary reproductive evidence to support the practical value of low-dose seminal plasma treatment. In line with our in vitro motility findings, seminal plasma from individuals with different cryotolerance delivers similar protective effects, with no statistically significant difference detected, supporting that mixing freeze-tolerant and non-freeze-tolerant seminal plasma is feasible for conventional breeding work [33]. Collectively, these artificial insemination data strongly support the industrial applicability of this practical, easy-to-implement thawing optimization protocol, whose economic benefits remain to be validated. The absence of a contemporaneous conventional frozen-semen control is a limitation of this study. The comparison with conventional frozen semen is based on historical records and should therefore be considered observational rather than experimental.

4.4. Global Proteomic Characteristics of Sperm Regulated by Two Seminal Plasma Types

Quantitative proteomics further uncovered the molecular mechanisms through which seminal plasma modulates thawed sperm function. All sample groups exhibited RSD values below 0.2, reflecting reliable proteomic detection and low intra-sample variation. PCA and Pearson correlation analyses showed tight clustering of FT and NFT samples, with clear separation from the control group, consistent with the phenotypic sperm quality data. Using FC $>$ 1.5 and $p < 0.05$ as screening thresholds, 513 DEPs were identified between C and FT, with 356 DEPs upregulated and 157 DEPs downregulated in FT relative to C. Similarly, 366 DEPs were identified between C and NFT, with 273 DEPs upregulated and 93 DEPs downregulated in NFT relative to C. In stark contrast, merely 11 DEPs (5 upregulated, 6 downregulated) were differentially expressed between FT and NFT groups, accounting for only 0.4% of all quantified proteins. This minor inter-group molecular divergence is consistent with the largely similar functional phenotypes of the two seminal plasma types. Our total quantified protein count (2965) aligns with previous mammalian sperm proteomic research; 1893 proteins were identified in buffalo sperm in a prior comparative proteomic investigation of seminal plasma exosomes from buffaloes with divergent fertility [23], corroborating the robustness of our mass spectrometry data. When compared with existing porcine sperm proteome studies, most prior work only detected hundreds of sperm proteins after cryopreservation [24], while the deeper proteome coverage achieved here allows more comprehensive profiling of cryoprotection-associated molecular changes. Notably, consistent with our finding that divergent freezability phenotypes are associated with minimal sperm proteome differences under the applied analysis, another pig proteomic study also reported limited inter-group DEPs between high- and low-freezability boar seminal plasma treatments [34], further supporting our core molecular observation.

The WNT pathway inhibitor DKK1 participates in early embryonic development and pregnancy establishment [35]. Its homologous protein DKKL1 localizes to the sperm acrosome during spermatogenesis, implying a potential regulatory role in acrosomal function [36–38]. The WNT signaling cascade governs sperm release [39] and epididymal maturation [40], while the WNT/GSK3 axis stabilizes structural proteins to maintain normal sperm motility [41]. As a canonical WNT antagonist, DKK1 shapes sperm maturation and motility by modulating this signaling network.

Multiple DEPs modulated by seminal plasma are functionally tied to sperm physiological activity. Lipocalin 5 (LCN5), significantly upregulated in both treatment groups, participates in lipid transport and spermatogenic processes; knockout of lipocalin family members including LCN5 induces male subfertility or complete sterility in mice [42,43]. Matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) family proteins were also enriched. MMP2 localizes to the inner acrosomal membrane and mediates sperm–egg binding as well as polyspermy blockage, and its expression positively correlates with sperm DNA integrity [44,45]. Clinically, higher MMP2 activity is detected in sperm from patients achieving pregnancy via intrauterine insemination [46]. In stallion semen, MMP2 and MMP9 concentrations correlate with sperm density but not routine motility parameters [47]. ADAM3A, ADAM9 and ADAM10 were significantly elevated in treated sperm; ADAM7 deletion impairs mouse epididymal and sperm structural integrity and reduces fertility, highlighting the essential role of ADAM proteins in gamete recognition and spermatogenesis [48,49].

4.5. Core Functional Pathways and Analysis of Target Proteins

GO and KEGG enrichment analyses clarified core functional cascades modulated by seminal plasma. For the C vs. FT comparison, DEPs were enriched in actin assembly and zymogen activation (BP), extracellular and endoplasmic reticulum compartments (CC), and glycosyltransferase/cell adhesion activities (MF). For C vs. NFT, prominent BP terms included zymogen activation and cholesterol transport inhibition, with CC enrichment concentrated in extracellular and lysosomal compartments, and peptidase inhibitor activity dominating MF annotations. KEGG analysis identified three shared core pathways: lysosome, complement and coagulation cascades, and sphingolipid metabolism. The lysosomal pathway contained 20–21 DEPs including LGMN, a lysosomal cysteine protease responsible for intracellular protein turnover and organelle clearance during sperm maturation. The acrosome is recognized as a specialized secretory lysosome, and intact lysosomal function is critical for successful acrosome reactions and fertilization [50,51]. The complement and coagulation cascade pathway contains six shared DEPs including SERPINA1, and CLU. SERPINA1 deficiency is associated with human asthenospermia [52,53], while vitronectin (VTN) localizes to capacitated sperm heads and facilitates gamete adhesion [54,55]. Sphingolipid metabolism proteins such as NEU1 and PSAP preserve sperm membrane structural stability [56], and PSAP enhances sperm–egg binding efficiency [57]. Arachidonic acid metabolism, TNF signaling and cholesterol metabolism were uniquely enriched in the NFT group, yet these secondary pathways did not alter the shared core regulatory machinery.

Subcellular localization statistics and WB analysis jointly support the reliability of our proteomic data. Extracellular proteins constituted 25.34% and 29.78% of all DEPs in the C vs. FT and C vs. NFT groups, respectively, higher than the proportion of intracellular DEPs. Approximately half of all upregulated DEPs overlapped with seminal plasma proteins in both treatments, with extracellular DEPs showing overlap rates exceeding 60%, far above the baseline 0.188 overlap ratio for total sperm proteins. These data are consistent with a model whereby seminal plasma proteins may bind the sperm surface or could enter cells via endocytosis and exosome fusion to remodel the sperm proteome, which may account for the weaker protective effects of vesicle-free supernatant. Four representative proteins (LGMN, PLS1, SERPINA1, CLU) from core pathways were quantified via WB. All four target proteins were significantly elevated in the FT group ($p < 0.01$) relative to the control group. In the NFT group, LGMN and SERPINA1 were moderately upregulated ($p < 0.05$), CLU showed highly significant elevation ($p < 0.01$), while PLS1 exhibited no statistical difference relative to controls. No inter-group differences were detected between FT and NFT for any target

protein. WB expression patterns were consistent with proteomic quantification, providing experimental support for our pathway analysis.

It should be noted that mechanistic inferences derived from our proteomic data are preliminary. We attempted immunoblotting for additional candidate proteins; however, unsatisfactory antibody specificity precluded reliable, publication-grade data. Since comprehensive functional assays were not performed, further work is required to confirm the causal roles of these proteins in boar sperm cryoprotection. Additionally, due to the limited volume of available protein extract, each target protein was analyzed on independent gels rather than a common reference gel. Although all WB experiments were performed under identical conditions, and the ratio of target protein band intensity to the corresponding β-actin intensity within each sample was calculated to correct for variations in sample loading, potential inter-gel variability cannot be completely excluded. Therefore, the WB data should be interpreted as supportive evidence consistent with the proteomic findings rather than as absolute quantitative comparisons.

5. Conclusions

Among the tested concentrations (10%, 20%, and 30%), 20% (v/v) FT or NFT boar seminal plasma produced the highest numerical values for post-thaw sperm total motility and maintained stable kinematic performance over a 2 h incubation period. No significant difference was observed in cryoprotective efficiency between the two seminal plasma phenotypes, which eliminates the need to screen boars with high freeze tolerance and expands available raw material sources for on-farm use. In field AI trials, frozen–thawed semen supplemented with 20% seminal plasma achieved litter sizes and live piglet numbers that did not differ significantly from fresh semen using approximately 2 billion sperm per insemination dose. However, because no contemporaneous conventional frozen-semen control was included, the comparison with conventional frozen semen relies on historical data and should be interpreted with caution. Further comparison showed that the ultracentrifugal supernatant of seminal plasma only exerted weak, transient protective effects, consistent with joint contributions from soluble metabolites and extracellular vesicle components to the protective activity of intact seminal plasma.

Quantitative proteomic analysis identified only 11 differentially expressed proteins between FT and NFT treatment groups, indicating substantial similarity in their molecular profiles under the applied analysis. Lysosome, complement and coagulation cascades, and sphingolipid metabolism were identified as three shared core functional pathways, with LGMN, SERPINA1, and CLU supported as key functional proteins by WB analysis. Subcellular protein overlap analysis is consistent with a model whereby seminal plasma components may attach to sperm surfaces or could enter sperm through endocytosis and exosome fusion to remodel the sperm proteome, which may underlie its sustained capacity to alleviate cryodamage. This work clarifies the effective supplementary dosage and core molecular regulatory network of boar seminal plasma for thawing optimization, delivering a practical, easy-to-implement technical workflow for industrial boar frozen semen production, with potential cost benefits that require further economic validation, and providing candidate protein markers for subsequent cryoprotection mechanism research. Collectively, this study identified 20% as the recommended seminal plasma concentration for thawing media based on overall numerical trends, demonstrated similar cryoprotective effects of FT and NFT seminal plasma, with no statistically significant difference detected between the two phenotypes, identified three core molecular pathways by quantitative proteomics, and validated the recommended protocol in field artificial insemination trials.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/vetsci13111182/s1, Figure S1: SDS-PAGE profiling of total sperm proteins; Figure S2: RSD distribution of proteomic data across three groups; Figure S3: Peptide length distribution; Figure S4: Global protein abundance distribution; Figures S5 and S6: Subcellular localization pie charts of DEPs for C vs. FT and C vs. NFT; Figures S7–S11: Enrichment clustering heatmaps for BP, CC, MF, KEGG pathways and protein domains.

Author Contributions: Conceptualization, A.A., B.W. and G.L.; Methodology, A.A. and K.Z.; Validation, A.N.; Formal Analysis, A.A. and A.N.; Investigation, A.A., P.W. and K.Z.; Resources, K.Z.; Data curation, A.A., P.W. and A.N.; Writing—Original Draft, A.A. and P.W.; Visualization, K.Z.; Supervision, B.W. and G.L.; Project Administration, B.W. and G.L.; Funding Acquisition, B.W. and G.L. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Key Program of the National Natural Science Foundation of China (Grant No. 32430101) and the General Program of the National Natural Science Foundation of China (Grant No. 32172738).

Institutional Review Board Statement: Formal ethical committee approval was not required for this study in accordance with national livestock welfare regulations and standardized commercial farming guidelines. All procedures of boar semen collection, cryopreservation and sow artificial insemination are routine breeding practices on certified pig farms. No invasive surgery, painful administration or terminal experimental treatments were performed on animals, and all animal handling followed standard animal welfare norms.

Informed Consent Statement: All boar semen samples used in this study were standard commercial breeding products purchased from Beijing Sifanghong Breeding Pig Co., Ltd. All sows used for artificial insemination were commercial breeding animals provided by Guangxi Nanning Yawei Agriculture and Animal Husbandry Co., Ltd. (Nanning, Guangxi, China). Artificial insemination was performed by the farm's technical staff as part of routine breeding management. No informed consent was required because all animals were commercially owned and the procedures did not involve any privately owned animals.

Data Availability Statement: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (https://proteomecentral.proteomexchange.org) via the iProX partner repository [58,59] with the dataset identifier PXD086373.

Acknowledgments: This study acknowledges the help and support provided by Beijing Tianyuanaorui Biotechnology, Ltd.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

CASA	Computer-assisted sperm analysis
TM	Total motility
PM	Progressive motility
VAP	Average path velocity
VCL	Curvilinear velocity
STR	Straightness
VSL	Straight line velocity
ALH	Amplitude of lateral head displacement
WOB	Wobble
AI	Artificial insemination
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
PCA	Principal component analysis
FC	Fold change
DEPs	Differentially expressed proteins

RSD	Relative standard deviation
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
WB	Western blot

References

1. Toledo-Guardiola, S.M.; Luongo, C.; Martinez-Pastor, F.; Soriano-Úbeda, C.; Matás, C. The Individual Variations in Sperm Quality of High-Fertility Boars Impact the Offspring Production and Early Physiological Functions. *Vet. Sci.* **2025**, *12*, 582. [CrossRef] [PubMed]
2. Feng, Q.; Yang, Y.; Zhang, B.; Shi, W.; Fang, Y.; Xu, C.; Deng, Z.; Feng, W.; Shi, D. The Effects of Limonin, Myo-Inositol, and L-Proline on the Cryopreservation of Debao Boar Semen. *Animals* **2025**, *15*, 2204. [CrossRef] [PubMed]
3. Estudillo, E.; Jiménez, A.; Bustamante-Nieves, P.E.; Palacios-Reyes, C.; Velasco, I.; López-Ornelas, A. Cryopreservation of Gametes and Embryos and Their Molecular Changes. *Int. J. Mol. Sci.* **2021**, *22*, 10864. [CrossRef] [PubMed]
4. Hai, E.; Li, B.; Zhang, J.; Zhang, J. Sperm freezing damage: The role of regulated cell death. *Cell Death Discov.* **2024**, *10*, 239. [CrossRef] [PubMed]
5. Almubarak, A.; Kim, E.; Yu, I.-J.; Park, H.; Jeon, Y. The Effect of kappa-Carrageenan on Porcine Sperm Cryo-Survival. *Animals* **2024**, *14*, 1387. [CrossRef] [PubMed]
6. Narida, A.; Tsai, S.; Huang, C.-Y.; Wen, Z.-H.; Lin, C. The Effects of Cryopreservation on the Cell Ultrastructure in Aquatic Organisms. *Biopreserv. Biobank.* **2023**, *21*, 23–30. [CrossRef] [PubMed]
7. Shi, L.; Zhang, Y.; Huang, X.; Shi, M.; Sun, D.; Zhang, Y.; Li, W.; Jin, T.; Feng, J.; Xing, J.; et al. Effects of mitoquinone (MitoQ) supplementation during boar semen cryopreservation on sperm quality, antioxidant status and mitochondrial proteomics. *Anim. Reprod. Sci.* **2022**, *247*, 107099. [CrossRef] [PubMed]
8. Ramírez-López, C.J.; Barros, E.; Vidigal, P.M.P.; Silva Okano, D.; Duarte Rodrigues, J.N.; Lopes Gomes, L.; Montes-Vergara, J.C.; Petro Hernandez, V.G.; Baracat-Pereira, M.C.; Guimarães, S.E.F.; et al. Relative Abundance of Spermadhesin-1 in the Seminal Plasma of Young Nellore Bulls Is in Agreement with Reproductive Parameters. *Vet. Sci.* **2023**, *10*, 610. [CrossRef] [PubMed]
9. Bolarin, A.; Berndtson, J.; Tejerina, F.; Cobos, S.; Pomarino, C.; D'Alessio, F.; Blackburn, H.; Kaeoket, K. Boar semen cryopreservation: State of the art, and international trade vision. *Anim. Reprod. Sci.* **2024**, *269*, 107496. [CrossRef] [PubMed]
10. Lin, H.; Hu, Z.; Li, Y.; Li, Y.; Ma, W.; Zheng, S.; Zhou, J.; Zhao, Z.; Gan, S.; Chen, Z.; et al. Impact of Curcumin on Frozen Bovine Sperm Quality and In Vitro Bovine Oocyte Maturation. *Vet. Sci.* **2025**, *12*, 441. [CrossRef] [PubMed]
11. Liu, Y.; Zhang, H.; Meng, Q.; Min, L.; Zhang, M.; Adetunji, A.O.; Li, W.; Zhu, Z. Nicotinamide Mononucleotide Enhances Boar Sperm Quality via Maintaining Mitochondrial Function During Liquid Storage. *Animals* **2025**, *15*, 3383. [CrossRef] [PubMed]
12. Kujoana, T.C.; Sehlabela, L.D.; Mabelebele, M.; Sebola, N.A. The potential significance of antioxidants in livestock reproduction: Sperm viability and cryopreservation. *Anim. Reprod. Sci.* **2024**, *267*, 107512. [CrossRef] [PubMed]
13. Ausejo-Marcos, R.; Tejedor, M.T.; Miguel-Jiménez, S.; Gómez-Giménez, B.; Soriano-Úbeda, C.; Mendoza, N.; Vicente-Carrillo, A.; Hurtado, W.F.; Ávila Holguín, C.; Moreno, B.; et al. Spermiogram, Kinetics, Flow Cytometric Characteristics and DNA Damage Degree in Boar Ejaculates: Summarization and Clustering. *Vet. Sci.* **2024**, *11*, 420. [CrossRef] [PubMed]
14. Xu, Y.; Sun, S.; Fu, Y.; Wang, L.; Ren, C.; Ling, Y.; Zhang, Z.; Cao, H. Positive In Vitro Effect of ROCK Pathway Inhibitor Y-27632 on Qualitative Characteristics of Goat Sperm Stored at Low Temperatures. *Animals* **2024**, *14*, 1441. [CrossRef] [PubMed]
15. Yeste, M.; Rodríguez-Gil, J.E.; Bonet, S. Artificial insemination with frozen-thawed boar sperm. *Mol. Reprod. Dev.* **2017**, *84*, 802–813. [CrossRef] [PubMed]
16. Reckinger, F.; Luther, A.-M.; Riesenbeck, A.; Sitzenstock, F.; Wassmuth, R.; Waberski, D. Fertility with cervical insemination and boar effects using hypothermic stored semen. *Theriogenology* **2025**, *236*, 45–51. [CrossRef] [PubMed]
17. Bromfield, J.J. A role for seminal plasma in modulating pregnancy outcomes in domestic species. *Reproduction* **2016**, *152*, R223–R232. [CrossRef] [PubMed]
18. Kumar, D.; Naqvi, S.M.K. Effect of time and depth of insemination on fertility of Bharat Merino sheep inseminated trans-cervical with frozen-thawed semen. *J. Anim. Sci. Technol.* **2014**, *56*, 8. [CrossRef] [PubMed]
19. Meeker, J.D.; Godfrey-Bailey, L.; Hauser, R. Relationships between serum hormone levels and semen quality among men from an infertility clinic. *J. Androl.* **2007**, *28*, 397–406. [CrossRef] [PubMed]
20. Zhu, J.; Xu, X.; Cosgrove, J.R.; Foxeroft, G.R. Effects of semen plasma from different fractions of individual ejaculates on IVF in pigs. *Theriogenology* **2000**, *54*, 1443–1452. [CrossRef] [PubMed]
21. Fang, Y.; Zhong, R.; Zhang, X.; Zhang, J.; Zhou, D. Boar seminal plasma inhibits cryo-capacitation of frozen-thawed ram sperm and improves fertility following intracervical insemination. *Theriogenology* **2018**, *105*, 84–89. [CrossRef] [PubMed]
22. Okazaki, T.; Akiyoshi, T.; Kan, M.; Mori, M.; Teshima, H.; Shimada, M. Artificial insemination with seminal plasma improves the reproductive performance of frozen-thawed boar epididymal spermatozoa. *J. Androl.* **2012**, *33*, 990–998. [CrossRef] [PubMed]

23. Yu, K.; Xiao, K.; Sun, Q.-Q.; Liu, R.-F.; Huang, L.-F.; Zhang, P.-F.; Xu, H.-Y.; Lu, Y.-Q.; Fu, Q. Comparative proteomic analysis of seminal plasma exosomes in buffalo with high and low sperm motility. *BMC Genom.* **2023**, *24*, 8. [CrossRef] [PubMed]
24. Zhu, K.; Song, Y.; He, Z.; Wang, P.; Wang, X.; Liu, G. Effect of Seminal Plasma on the Freezability of Boar Sperm. *Animals* **2024**, *14*, 3656. [CrossRef] [PubMed]
25. Taylor, S.C.; Posch, A. The design of a quantitative western blot experiment. *BioMed Res. Int.* **2014**, *2014*, 361590. [CrossRef] [PubMed]
26. Garcia, J.C.; Dominguez, J.C.; Pena, F.J.; Alegre, B.; Gonzalez, R.; Castro, M.J.; Habing, G.G.; Kirkwood, R.N. Thawing boar semen in the presence of seminal plasma: Effects on sperm quality and fertility. *Anim. Reprod. Sci.* **2010**, *119*, 160–165. [CrossRef] [PubMed]
27. Roca, J.; Carvajal, G.; Lucas, X.; Vazquez, J.M.; Martinez, E.A. Fertility of weaned sows after deep intrauterine insemination with a reduced number of frozen-thawed spermatozoa. *Theriogenology* **2003**, *60*, 77–87. [CrossRef] [PubMed]
28. Yeste, M. Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs. *Theriogenology* **2016**, *85*, 47–64. [CrossRef] [PubMed]
29. Estrada, E.; Rodríguez-Gil, J.E.; Rocha, L.G.; Balasch, S.; Bonet, S.; Yeste, M. Supplementing cryopreservation media with reduced glutathione increases fertility and prolificacy of sows inseminated with frozen-thawed boar semen. *Andrology* **2014**, *2*, 88–99. [CrossRef] [PubMed]
30. Alkmin, D.V.; Martinez-Alborcia, M.J.; Parrilla, I.; Vazquez, J.M.; Martinez, E.A.; Roca, J. The nuclear DNA longevity in cryopreserved boar spermatozoa assessed using the Sperm-Sus-Halomax. *Theriogenology* **2013**, *79*, 1294–1300. [CrossRef] [PubMed]
31. Martinez-Alborcia, M.J.; Morrell, J.M.; Gil, M.A.; Barranco, I.; Maside, C.; Alkmin, D.V.; Parrilla, I.; Martinez, E.A.; Roca, J. Suitability and effectiveness of single layer centrifugation using Androcoll-P in the cryopreservation protocol for boar spermatozoa. *Anim. Reprod. Sci.* **2013**, *140*, 173–179. [CrossRef] [PubMed]
32. Fernández-Gago, R.; Domínguez, J.C.; Martínez-Pastor, F. Seminal plasma applied post-thawing affects boar sperm physiology: A flow cytometry study. *Theriogenology* **2013**, *80*, 400–410. [CrossRef] [PubMed]
33. Catalán, J.; Yánez-Ortiz, I.; Tvarijonaviciute, A.; González-Arostegui, L.G.; Rubio, C.P.; Yeste, M.; Miró, J.; Barranco, I. Impact of Seminal Plasma Antioxidants on Donkey Sperm Cryotolerance. *Antioxidants* **2022**, *11*, 417. [CrossRef] [PubMed]
34. Perez-Patiño, C.; Barranco, I.; Parrilla, I.; Valero, M.L.; Martinez, E.A.; Rodriguez-Martinez, H.; Roca, J. Characterization of the porcine seminal plasma proteome comparing ejaculate portions. *J. Proteom.* **2016**, *142*, 15–23. [CrossRef] [PubMed]
35. Amaral, T.F.; de Grazia, J.G.V.; Martinhao, L.A.G.; De Col, F.; Siqueira, L.G.B.; Viana, J.H.M.; Hansen, P.J. Actions of CSF2 and DKK1 on bovine embryo development and pregnancy outcomes are affected by composition of embryo culture medium. *Sci. Rep.* **2022**, *12*, 7503. [CrossRef] [PubMed]
36. Zhu, J.; Wang, Y.; Lei, L.; Chen, C.; Ji, L.; Li, J.; Wu, C.; Yu, W.; Luo, L.; Chen, W.; et al. Comparative genomic survey and functional analysis of DKKL1 during spermatogenesis in the Chinese soft-shelled turtle (*Pelodiscus sinensis*). *Int. J. Biol. Macromol.* **2024**, *254*, 127696. [CrossRef] [PubMed]
37. Zhuang, X.-J.; Hou, X.-J.; Liao, S.-Y.; Wang, X.-X.; Cooke, H.J.; Zhang, M.; Han, C. SLXL1, a novel acrosomal protein, interacts with DKKL1 and is involved in fertilization in mice. *PLoS ONE* **2011**, *6*, e20866. [CrossRef] [PubMed]
38. Kohn, M.J.; Sztein, J.; Yagi, R.; DePamphilis, M.L.; Kaneko, K.J. The acrosomal protein Dickkopf-like 1 (DKKL1) facilitates sperm penetration of the zona pellucida. *Fertil. Steril.* **2010**, *93*, 1533–1537. [CrossRef] [PubMed]
39. Liu, D.-X.; Li, Z.-F.; Zhao, Y.-S.; Wang, L.-M.; Qi, H.-Y.; Zhao, Z.; Tan, F.-Q.; Yang, W.-X. Es-β-CATENIN affects the hemolymph-testes barrier in Eriocheir sinensis by disrupting cell junctions and cytoskeleton. *Int. J. Biol. Macromol.* **2023**, *242*, 124867. [CrossRef] [PubMed]
40. Koch, S.; Acebron, S.P.; Herbst, J.; Hatiboglu, G.; Niehrs, C. Post-transcriptional Wnt Signaling Governs Epididymal Sperm Maturation. *Cell* **2015**, *163*, 1225–1236. [CrossRef] [PubMed]
41. Hu, S.; Song, Y.; Chen, J.; He, X.; Li, G.; Liu, T.; He, Z.; Li, X.; Wang, Z.; Hu, J.; et al. Transcriptome analysis of testis and epididymis identifies key genes and pathways regulating gander sperm motility. *Poult. Sci.* **2025**, *104*, 105012. [CrossRef] [PubMed]
42. Sakurai, N.; Fujihara, Y.; Kobayashi, K.; Ikawa, M. CRISPR/Cas9-mediated disruption of lipocalins, Ly6g5b, and Ly6g5c causes male subfertility in mice. *Andrology* **2024**, *12*, 981–990. [CrossRef] [PubMed]
43. Otčenášková, T.; Macíčková, E.; Vondráková, J.; Frolíková, M.; Komrskova, K.; Stopková, R.; Stopka, P. Proteomic analysis of the mouse sperm acrosome—Towards an understanding of an organelle with diverse functionality. *Eur. J. Cell Biol.* **2023**, *102*, 151296. [CrossRef] [PubMed]
44. Belardin, L.B.; Antoniassi, M.P.; Camargo, M.; Intasqui, P.; Fraietta, R.; Bertolla, R.P. Semen levels of matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinases (TIMP) protein families members in men with high and low sperm DNA fragmentation. *Sci. Rep.* **2019**, *9*, 903. Correction in *Sci. Rep.* **2019**, *9*, 10234. [CrossRef] [PubMed]
45. Watabe, M.; Hiraiwa, A.; Sakai, M.; Ueno, T.; Ueno, S.; Nakajima, K.; Yaoita, Y.; Iwao, Y. Sperm MMP-2 is indispensable for fast electrical block to polyspermy at fertilization in *Xenopus tropicalis*. *Mol. Reprod. Dev.* **2021**, *88*, 744–757. [CrossRef] [PubMed]

46. Sharifi, S.; Mohseni, R.; Amiri, I.; Tavilani, H. Sperm matrix metalloproteinase-2 activity increased in pregnant couples treated with intrauterine insemination: A prospective case control study. *J. Obstet. Gynaecol.* **2019**, *39*, 675–680. [CrossRef] [PubMed]
47. Kareskoski, M.; Vakkamäki, J.; Laukkanen, K.; Palviainen, M.; Johannisson, A.; Katila, T. Matrix metalloproteinase (MMP)-2, MMP-9, semen quality and sperm longevity in fractionated stallion semen. *Theriogenology* **2021**, *164*, 93–99. [CrossRef] [PubMed]
48. Choi, H.; Han, C.; Jin, S.; Kwon, J.T.; Kim, J.; Jeong, J.; Kim, J.; Ham, S.; Jeon, S.; Yoo, Y.J.; et al. Reduced Fertility and Altered Epididymal and Sperm Integrity in Mice Lacking ADAM7. *Biol. Reprod.* **2015**, *93*, 70. [CrossRef] [PubMed]
49. Chen, Z.; Xie, S.; Zou, G.; Zhu, H.; Wang, L.; Sun, X.; Mu, J.; Sang, Q. Garin3 regulates sperm-oocyte fusion and male fertility. *Cell. Mol. Life Sci.* **2026**, *83*, 277. [CrossRef] [PubMed]
50. Litscher, E.S.; Wassarman, P.M. The mammalian egg's zona pellucida, fertilization, and fertility. In *Current Topics in Developmental Biology*; Elsevier: Amsterdam, The Netherlands, 2025; Volume 162, pp. 207–258. [CrossRef] [PubMed]
51. Chávez, J.C.; Carrasquel-Martínez, G.; Hernández-Garduño, S.; Matamoros Volante, A.; Treviño, C.L.; Nishigaki, T.; Darszon, A. Cytosolic and Acrosomal pH Regulation in Mammalian Sperm. *Cells* **2024**, *13*, 865. [CrossRef] [PubMed]
52. Liu, X.; Liu, G.; Zhu, P.; Wang, Y.; Wang, J.; Zhang, W.; Wang, W.; Li, N.; Wang, X.; Zhang, C.; et al. Characterization of seminal plasma proteomic alterations associated with the IVF and rescue-ICSI pregnancy in assisted reproduction. *Andrology* **2020**, *8*, 407–420. [CrossRef] [PubMed]
53. Liu, X.; Wang, W.; Zhu, P.; Wang, J.; Wang, Y.; Wang, X.; Liu, J.; Li, N.; Wang, X.; Lin, C.; et al. In-depth quantitative proteome analysis of seminal plasma from men with oligoasthenozoospermia and normozoospermia. *Reprod. Biomed. Online* **2018**, *37*, 467–479. [CrossRef] [PubMed]
54. Fusi, F.M.; Bernocchi, N.; Ferrari, A.; Bronson, R.A. Integrins and adhesion molecules: Is vitronectin the velcro that binds the gametes together? *Mol. Hum. Reprod.* **1996**, *2*, 859–866. [CrossRef] [PubMed]
55. Boissonnas, C.C.; Montjean, D.; Lesaffre, C.; Auer, J.; Vaiman, D.; Wolf, J.-P.; Ziyyat, A. Role of sperm αvβ3 integrin in mouse fertilization. *Dev. Dyn.* **2010**, *239*, 773–783. [CrossRef] [PubMed]
56. Mostafa, S.; Nader, N.; Machaca, K. Lipid Signaling During Gamete Maturation. *Front. Cell Dev. Biol.* **2022**, *10*, 814876. [CrossRef] [PubMed]
57. Hammerstedt, R.H.; Cramer, P.G.; Barbato, G.F.; Amann, R.P.; O'Brien, J.S.; Griswold, M.D. A fragment of prosaposin (SGP-1) from rooster sperm promotes sperm-egg binding and improves fertility in chickens. *J. Androl.* **2001**, *22*, 361–375. [CrossRef]
58. Ma, J.; Chen, T.; Wu, S.; Yang, C.; Bai, M.; Shu, K.; Li, K.; Zhang, G.; Jin, Z.; He, F.; et al. iProX: An integrated proteome resource. *Nucleic Acids Res.* **2019**, *47*, D1211–D1217. [CrossRef] [PubMed]
59. Chen, T.; Ma, J.; Liu, Y.; Chen, Z.; Xiao, N.; Lu, Y.; Fu, Y.; Yang, C.; Li, M.; Wu, S.; et al. iProX in 2021: Connecting proteomics data sharing with big data. *Nucleic Acids Res.* **2022**, *50*, D1522–D1527. [CrossRef] [PubMed]

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