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CROCIN ENHANCES THE QUALITY OF FROZEN-THAWED BOAR SPERM BY MODULATING OXIDATIVE STRESS AND GLYCEROPHOSPHOLIPID METABOLISM

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Abstract

BACKGROUND: Cryopreservation is a fundamental tool for conserving endangered species and enhancing reproductive management in livestock; however, post-thaw sperm performance is easily influenced by several stressors. Crocin, a natural carotenoid, has antioxidant properties. However, its effects on frozen-thawed boar sperm remain insufficiently studied. **OBJECTIVE:** This study aimed to assess the role of crocin in improving the quality of frozen boar sperm by focusing on its mechanisms of regulating oxidative stress, mitochondrial membrane potential, and glycerophospholipid metabolism. **MATERIALS AND METHODS:** Boar sperm samples were supplemented with varying crocin concentrations (0, 0.5, 1, and 2 mM). Sperm kinematic parameters and motility were assessed using computer-assisted semen analysis, whereas the DNA fragmentation index and mitochondrial membrane potential were assessed using a fluorescence microscope. **RESULTS:** Treatment with 1 mM crocin significantly enhanced the kinematic parameters ($P < 0.05$) and frozen sperm viability ($P < 0.05$) while enhancing the integrity of the plasma- and acrosomal membranes ($P < 0.05$). The oxidative stress indices, including reactive oxygen species, 8-hydroxy-2-deoxyguanosine, and malondialdehyde content ($P < 0.01$), were effectively lowered by crocin. Furthermore, crocin enhanced the mitochondrial membrane potential and sperm DNA integrity ($P < 0.01$). Metabolomic analysis showed that crocin affected several differential metabolites, including phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and 2-lysophosphatidylcholine ($P < 0.05$), highlighting its role in glycerophospholipid metabolism. **CONCLUSION:** Crocin markedly improved the quality of frozen boar sperm by regulating oxidative stress and lipid metabolism, offering new insights for optimising sperm cryopreservation. This study provides practical insights into the optimisation of boar semen cryopreservation in livestock breeding.

Keywords: crocin; cryopreservation; glyceryl phospholipids; malondialdehyde; natural antioxidant.

INTRODUCTION

The livestock industry plays a critical role globally. Livestock serves as the primary protein source consumed in the human diet, with semen preservation being one of the most economically significant factors in animal breeding (1). Sperm cryopreservation is considered one of the most effective techniques for preserving sperm quality and ensuring long-term fertility (2).

Cryopreservation inflicts substantial damage on sperm, encompassing structural alterations, DNA fragmentation, and disruption of membrane lipid composition including phosphatidylserine (PS) translocation (3, 4, 5, 6, 7). A central mechanism of this cryo-injury is oxidative stress, driven by the accumulation of reactive oxygen species (ROS), which directly induces membrane lipid peroxidation (8). Concurrently, mitochondrial dysfunction arises, characterized by the loss of mitochondrial membrane potential ($\Delta\Psi m$) (9). Together, these interconnected pathways create a vicious cycle that compromises sperm viability and function (10).

The sperm plasma membrane, rich in phospholipids, proteins, and glycolipids, is a critical protective barrier and plays a crucial role in the maintenance of sperm integrity and function (11). Glycerophospholipids are the major phospholipid components. Common glycerophospholipids in the plasma membrane include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and lysophosphatidylcholine (LPC) (12). PC influences the sperm acrosome reaction (13) and prevents ultrastructural damage caused by cold shock (14). PE plays crucial roles in the regulation of sperm maturation (15), as well as mitochondrial function and morphology (16). Thus, glycerophospholipids are indispensable for mammalian sperm function and structural integrity. The addition of cryoprotectants is a widely used method to mitigate sperm damage during freeze-thaw cycles. Antioxidants can effectively decrease the damage caused by cold shock while mitigating the harmful effects of ROS (17).

Crocin is a water-soluble carotenoid with potent antioxidant activity. With regard to male fertility preservation, crocin improves sperm motility and DNA integrity in humans (18), rams (19), chickens (20), and bulls (21) after freezing and thawing, with reduced oxidative stress and lipid peroxidation. However, research on the effects of crocin on boar sperm, especially on glycerophospholipid metabolism, remains limited. This study aimed to elucidate the effect of crocin on the quality of frozen-thawed boar sperm by focusing on kinematics, motility, acrosome integrity, plasma membrane integrity, DNA fragmentation index (DFI), mitochondrial membrane potential (MMP), and 8-hydroxy-2-deoxyguanosine (8-OHdG). Additionally, the effects of crocin on glycerophospholipid metabolism in frozen-thawed boar sperm were assessed using non-targeted metabolomic analysis.

MATERIALS AND METHODS

All experiments were conducted in accordance with the ARRIVE guidelines and approved by the Ethical Committee of Guangxi University, Nanning, China (Approval No. Gxu2022-212). Guangxi Keda Livestock & Poultry Improvement Co., LTD provided the samples and their use complied with the local ethical regulations.

Experimental design

This study employed a preliminary trial design to evaluate the cryoprotective potential of crocin in boar sperm. Semen samples were collected from six Landrace boars and pooled for each experimental replicate. This pooling strategy mimics commercial artificial insemination practices and allows assessment of the average treatment effect on overall semen quality. Four concentrations of crocin (0, 0.5, 1, and 2 mM) were initially screened. Based on the preliminary results (Table 1), 1 mM crocin was selected for all subsequent analyses. Three experimental groups were defined: F (Fresh, untreated semen), O (Frozen control, without crocin), and M (Crocin-treated, frozen with 1 mM crocin). Each group consisted of three independent experimental replicates (n=3), with each replicate derived from a pooled ejaculate collected on separate days.

Semen collection and crocin solution preparation

Semen samples were obtained from six healthy Landrace boars (age: 12-16 months; body weight: 160-200 kg) during March to July 2024,

a period known to minimize seasonal variation in boar semen quality. The boars were maintained under standard husbandry conditions at Guangxi Keda Livestock & Poultry Improvement Co., LTD and were proven fertile. The samples were collected daily at 09:00 h using aseptic disposable gloves, with 100-200 mL of ejaculate collected per boar in sterile containers. All samples from the six boars were immediately pooled to form a composite sample for each experimental replicate. This pooling strategy aligns with standard practices in swine artificial insemination, where pooled semen is commonly used to reduce individual boar variability and ensure consistent quality in commercial doses. While this design does not provide fully independent biological replicates at the individual boar level, the use of 18 ejaculates (six boars across three replicates) effectively mitigates the impact of individual differences, enabling assessment of the population-level effects of crocin treatment. Only samples with total sperm motility $\geqslant$ 80%, microscopically examined, were selected for further experiments. Semen was collected from six healthy adult boars, with each boar providing three ejaculates over three weeks, yielding a total of 18 samples and ensuring three independent experimental replicates representing pooled population samples.

A 10 mM stock solution was prepared by dissolving 1 g of crocin (Sigma-Aldrich, 98% purity) in 100.3 mL of sterile physiological saline (0.9% NaCl). The solution was aliquoted and stored at −80 °C until further use. Before the experimental application, frozen aliquots were thawed at 22 ℃ under aseptic conditions.

Semen freezing and thawing

The method of freezing and thawing semen was modified based on the protocol described by Zheng et al. (22). After centrifugation at 1200 relative centrifugal force (rcf) for 6 min, the supernatant was discarded, and the precipitate was mixed with an equal volume of freezing solution I. Different volumes of 10 mM crocin stock solution were added to obtain 0, 0.5, 1, and 2 mM as the final concentrations. The subsequent steps follow the original procedure (F, fresh, untreated group; O, frozen control group; M, frozen group supplemented with 1 mM crocin). The pooled semen samples were then cooled to 4 ℃ over 2 h, loaded into 0.25 mL straws, and frozen in liquid nitrogen vapor. Thawing was performed at 37 ℃ for 30 s. This entire freeze-thaw procedure was repeated for three independent biological replicates (n=3).

Sperm motility measurement

An aliquot of 10 μL of thawed semen from each group was placed on a pre-warmed glass slide (Sail brand, Guangzhou) and covered with a coverslip (Citotest, Nantong) to create a thin, uniform film for motility analysis. A computer-assisted sperm analysis system (CASA, Beijing Medical) was used to assess the dynamic parameters of the thawed sperm following the procedure outlined by Zheng et al. (22). Each sample was analysed in at least six different fields for each of the three independent experimental replicates (n=3), with over 200 sperm counted per slide. The recorded motion parameters included total motility (TM, %), linearity (LIN, %), average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), and beat cross frequency (BCF, Hz).

Sperm vitality measurement

The sperm survival rate was evaluated using eosin-aniline black staining. Thawed sperm were washed three times with phosphate-buffered saline (PBS) and smeared according to the instructions provided in the Sperm Vitality Staining Kit (Borde Bio, Shenzhen). The prepared smears were subsequently examined under an optical microscope at a magnification of 400 x. In each group, at least 200 spermatozoa were counted in triplicate (n=3). Dead sperm heads appeared red or dark pink, whereas live sperm heads appeared white or light pink.

Sperm vitality = (number of sperm with white or light pink heads/total sperm counted) × 100

Measurement of sperm plasma membrane/acrosome integrity

The sperm plasma membrane's integrity was evaluated using a hypotonic swelling test as described by Zheng et al. (22). In brief, thawed sperm were diluted to a concentration of 1×10^6 SPZ/mL in a fructose-sodium citrate hypotonic solution. The diluted semen was smeared after incubation at 37 ℃ for 30 min. The percentage of sperm with curled tails was determined using an optical microscope at 400 × magnification.

Sperm plasma membrane integrity = (number of sperm with curled tails/total sperm counted) × 100

Sperm acrosome integrity was evaluated using Coomassie Brilliant Blue staining. After

thawing, the sperm were washed three times with PBS (with centrifugation) and fixed with 4% paraformaldehyde for 30 min. After centrifugation and removal of the supernatant, PBS was added to obtain a suspension. Ten microlitres of semen were smeared to prepare a smear. After being air-dried, the smears were subsequently stained in Coomassie Brilliant Blue solution preheated to 37 °C. For each sample, at least 200 sperm were counted, and the process was repeated three times (n=3).

Measurement of the sperm DFI

A nuclear fragmentation detection kit (Xingbo Biological Company) was utilised to determine the sperm DFI, following the method described by Li CY (23). Briefly, sperm samples, after being fixed in 4% paraformaldehyde for 15 min, were permeabilised with 0.1% Triton X-100 for 5 min and subsequently stained with acridine orange (10 μg/mL) for 10 min in the dark. After washing with PBS, at least 200 spermatozoa per sample were analysed using fluorescence microscopy (BDAccuriTMC6, 400 × magnification). The DFI was calculated as the ratio of the red fluorescence intensity to the sum of red and green fluorescence intensities (DFI = red fluorescence intensity / (red fluorescence intensity + green fluorescence intensity)). Three independent replicates (n=3) were conducted for each sample.

Sperm MMP assay

MMP was evaluated using JC-1 fluorescence staining (Beyotime) following the method described by Li et al. (23). The MMP of each sample was expressed as the ratio of red-to-green fluorescence intensity. Three independent replicates (n=3) were conducted for each sample.

Evaluation of intracellular ROS

ROS in boar sperm were detected using an ROS assay kit (Beyotime Biotechnology), following the manufacturer's protocol and the method described by Zheng et al. (22). Briefly, sperm suspensions were incubated with 10 μM DCFH-DA probe at 37 °C for 30 min in the dark before being analysed using a fluorescence microscope(BD AccuriTM C6). Three independent replicates (n=3) were conducted for each sample.

Determination of malondialdehyde (MDA) content and 8-OHdG

The MDA content was measured using a commercial kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Briefly, thawed sperm samples were washed three times with PBS and subjected to ultrasonic disruption (Xinzhi, Ningbo, China) in an appropriate amount of PBS. The supernatant was collected, and absorbance was measured at 636 nm using a microplate reader. Three independent replicates (n=3) were performed for each sample.

The 8-OHdG content was determined using an ELISA kit (Meimian, Jiangsu, China) following the manufacturer's protocol. After thawing, sperm samples were washed three times with PBS and ultrasonically disrupted. The supernatant was collected and used for the assay. All measurements were performed in triplicate (n=3).

Untargeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Nontargeted LC-MS/MS analysis of the boar semen was conducted following a standardised protocol. The samples were thawed at 4 °C, with each tube containing approximately 1×10^7 cells. To each tube, a 6 mm diameter grinding bead and 300 μL of extraction solution (methanol= 4:1, v/v) containing four internal standards, L-2-chlorophenylalanine (0.02 mg/mL), were added. The samples were ground using a frozen tissue grinder for 6 min (−10 °C, 50 Hz) after being thoroughly mixed. The homogenised samples were subsequently subjected to low-temperature ultrasonic extraction for 30 min (5 °C, 40 KHz), followed by incubation at −20 °C for 30 min. Then, the samples were centrifuged for 15 min (13000 ×g, 4 °C), and the supernatant was carefully transferred to vials with inner tubes for analysis. Additionally, 20 μL of the supernatant from each sample was pooled to create a quality control (QC) sample.

Chromatographic conditions: (i) Chromatographic separation was achieved using an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm; Waters, Milford, USA). (ii) The mobile phase consisted of A: 95% water + 5% acetonitrile (containing 0.1% formic acid) and B: 47.5% acetonitrile + 47.5% isopropanol + 5% water (containing 0.1% formic acid). (iii) The flow rate was 0.40 mL/min, with an injection

Table 1. Effects of varying crocin concentrations on sperm kinematic parameters and viability of frozen-thawed boar spermatozoa.

Parameters	Fresh	Crocin concentration (mM)			
		0	0.5	1	2
TM, %	91.30±1.45^{a}	34.78±2.11^{d}	40.50±2.13^{cd}	55.18±2.44^{b}	45.40±2.38^{c}
LIN, %	29.00±0.90^{b}	33.11±2.25^{ab}	34.40±0.57^{ab}	34.39±1.53^{ab}	38.33±1.18^{a}
VAP, um/s	77.92±4.38^{a}	30.75±5.05^{b}	41.68±3.48^{b}	34.45±2.08^{b}	45.64±2.34^{b}
VSL, um/s	46.31±1.72^{a}	29.14±1.93^{b}	35.95±1.85^{b}	34.45±2.08^{b}	36.47±1.22^{b}
VCL, um/s	156.2±7.78^{a}	87.00±7.11^{b}	97.30±3.44^{b}	93.52±3.27^{b}	90.21±2.19^{b}
BCF, Hz	29.96±0.50^{b}	33.05±1.41^{ab}	35.51±1.05^{a}	34.83±1.47^{a}	35.26±0.74^{a}
Viability, %	72.70±11.64^{a}	31.55±1.79^{b}	46.45±2.55^{b}	53.09±2.13^{ab}	45.59±0.89^{b}

Note: Different superscript letters (a, b, c, and d) within the same row indicate statistically significant differences ($P < 0.05$). Abbreviations: TM, total motility; LIN, linearity; VAP, average path velocity; VSL, straight-line velocity; VCL, curvilinear velocity; BCF, beat cross frequency.

volume of 10 µL and a column temperature of 45 °C.

For mass spectrometry, samples were ionised via electrospray, and mass spectrometry signals were acquired in positive and negative ion scanning modes using a Q Exactive HF-X mass spectrometer (Thermo- Fisher Scientific, USA).

QC samples were prepared by pooling equal volumes of extracts from all samples. The volume of each QC was equivalent to that of the analytical samples, and the samples were processed and analysed identically. During instrument analysis, a QC sample was interspersed with every 5–15 analytical samples to monitor the stability of the testing process. Metabolite features with relative standard deviation (RSD) > 30% in QC samples were excluded from downstream analysis.

Bioinformatic analysis of the metabolomic data was conducted using a custom pipeline from Meiji Biotech Co., Ltd., which integrated data preprocessing, multivariate statistical analysis, and pathway enrichment. Multivariate analyses, including PCA and PLS-DA, were performed with the ropls R package (version 1.6.2) using default settings. During preprocessing, missing values were imputed and the data were normalized. Metabolite identification was carried out by matching MS/MS spectra against the KEGG database (release 2017-05-01) and the HMDB database. Differential metabolites (DMs) were defined based on variable importance in projection (VIP) > 1.0 and $P < 0.05$. Pathway enrichment analysis was subsequently performed using the KEGG database.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 8.0.1. Data were tested for normality (Shapiro–Wilk test) and homogeneity of variance (Levene's test). Differences between groups were analysed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. The results were expressed as mean ± standard error of the mean (SEM), and statistical significance was set at $P < 0.05$, <0.01, <0.001 and <0.0001.

The fluorescence intensity of the images was analysed and quantified using ImageJ (National Institutes of Health, Bethesda, Maryland, USA).

RESULTS

Effects of crocin on sperm kinematic parameters and viability of the frozen-thawed boar sperm

The groups are referred to as F (Fresh), O (Frozen control), and M (Crocin-treated) throughout.

The semen diluent was supplemented with varying concentrations (0,0.5,1 and 2 mM) of crocin to investigate its effects on sperm quality. The motion and velocity parameters in the fresh and experimental groups were assessed using the CASA system. Table 1 summarises the results.

Freezing severely damaged the TM in the O group compared to the F group ($P < 0.01$). However, the addition of 1 mM crocin (M group)

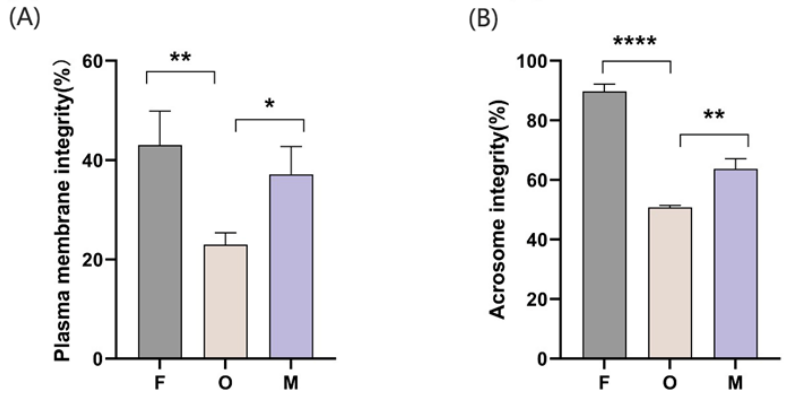

Figure 1. A. Plasma membrane integrity was evaluated via the hypotonic swelling test for Fresh (F), Frozen control (O) and Crocin-treated (M) sperm. B. Acrosome integrity was assessed using Coomassie Brilliant blue staining. Data are presented as mean ± standard error of the mean (n = 3). Significance levels: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

led to a significant recovery of TM compared to the O group ($P < 0.05$). Although the M group did not affect other motility parameters compared to the O group ($P > 0.05$), it showed improved sperm motility overall. These findings suggested a close relationship between crocin and sperm quality, with the addition of 1 mM crocin demonstrating the most beneficial effect on sperm quality maintenance.

Effects of crocin on the plasma membrane and acrosome integrity of frozen-thawed boar sperm

Compared to the O group, sperm plasma membrane integrity was significantly higher in the M group (Figure 1A; $P < 0.05$). Additionally, treatment with 1 mM crocin significantly protected sperm acrosome integrity (Figure 1B; $P < 0.01$).

Effects of crocin on ROS, MDA content and 8-OHdG of frozen-thawed boar sperm

Figures 2A and B show representative ROS images captured using fluorescence microscopy. The images clearly demonstrate that cryoinjury induced a significant increase ($P < 0.0001$) in ROS content in the O group compared to the F group. However, treatment with 1 mM crocin (the M group) led to a considerable reduction in ROS levels compared to the O group ($P < 0.01$). Furthermore, MDA production in the sperm of the O group increased following ultra-rapid freezing compared to the F group ($P < 0.01$). Contrastingly, supplementation with 1 mM crocin reversed these trends and significantly decreased the MDA levels in the spermatozoa of the M group compared to the O group ($P < 0.05$). Additionally, 8-OHdG levels were elevated in the O group after ultrarapid freezing compared to the F group ($P < 0.001$), whereas crocin supplementation (the M group) resulted in lower 8-OHdG levels than those in the O group ($P < 0.05$).

ROS, MDA and 8-OHdG are markers of oxidative stress and DNA damage, and the above results indicate that crocin effectively alleviated the oxidative damage produced during freezing.

Effects of crocin on MMP and DFI of frozen-thawed sperm

Figures 3A and B indicate that ultra-rapid freezing led to a considerable reduction in sperm MMP compared to that in the F group ($P < 0.05$). MMP in sperm treated with 1 mM crocin (the M group) was higher than that of sperm in the O group ($P < 0.01$), indicating that crocin improved the sperm's MMP during ultra-rapid freezing.

Compared to the F group, sperm DNA integrity in the O group was markedly damaged following ultra-rapid freezing (Figures 3C and D). Sperm DFI was significantly lower ($P < 0.0001$) after adding 1 mM crocin than in the O group. Sperm DFI was significantly lower ($P < 0.0001$) after adding 1 mM crocin than in the O group.

Metabolomics analysis and validation of differential metabolites

Principal component analysis (PCA) was performed on the F (Fresh), O (Frozen control), and M (Crocin-treated, 1 mM) groups. Clear separation among groups O, M and F highlighted metabolic differences, despite the fact that a

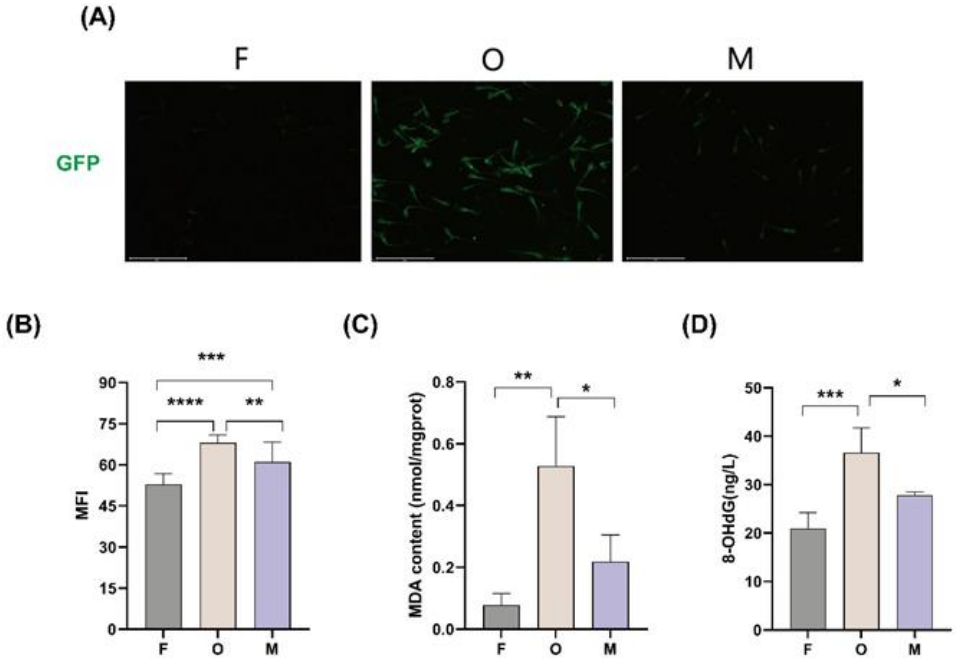

Figure 2. Effects of crocin on the oxidative stress markers in the frozen-thawed boar sperm [Fresh (F), Frozen control (O) and Crocin-treated (M)]. (A) Sperm stained with DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate) exhibited green fluorescence (scale bar = 75 μm). (B) Mean fluorescence intensity (MFI). (C) Malondialdehyde (MDA) content. (D) 8-hydroxy-2-deoxyguanosine (8-OhdG) content. Data are presented as mean ± standard error of the mean (n = 3). Significance levels: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

minor O-M overlap did not affect the downstream analyses (Figure 4A). All samples fell within the 95% confidence interval, and tightly clustered QC samples confirmed high experimental reproducibility and data reliability.

PLS-DA modelling linked metabolite profiles to sample classifications, with score plots (Figure 4B-E) showing distinct separations between O vs. F and M vs. O. Increased R^2/Q^2 values ($Q^2 > 0.5$) indicated robust model stability and predictive power. These findings validate the reliability of the data, and the observed metabolic differences reflect the inherent biological distinctions among the samples.

Figures 5A and B show the volcano plots of the differentially expressed metabolites between the groups. A total of 290 differential metabolites (DMs) were identified between O and F, with 210 upregulated and 80 downregulated metabolites. In the M vs. O group, 134 DMs were identified, 42 of which were upregulated and 92 were downregulated.

A heatmap with hierarchical clustering analysis was generated (Figures 5C and D) to visualise the expression levels of these DMs across groups. The heatmap illustrates the stability of DM expression in each group. Metabolites that clustered together exhibited similar expression patterns, reflecting changes in the relative expression levels and the significance of these differences.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was conducted on the DMs to explore the effects of freezing and crocin treatment on the metabolic pathways of boar sperm. O vs. F involved 290 differential metabolic pathways, predominantly concentrated in glycerophospholipid metabolism, choline metabolism in cancer, sphingolipid metabolism, sphingolipid signalling pathway, and central carbon metabolism in cancer (Figure 5E). Similarly, M vs. O involved 134 differential metabolic pathways, primarily concentrated in glycerophospholipid metabolism; lysosomes; choline metabolism in cancer; neomycin, kanamycin, and gentamicin biosynthesis; and systemic lupus erythematosus (Figure 5F). These findings suggest that the freeze-thaw process mainly influences the membrane lipid composition of the boar sperm and that crocin mitigates post-freezing sperm damage by modulating glycerophospholipid metabolism.

those in the O group, effectively reversing the

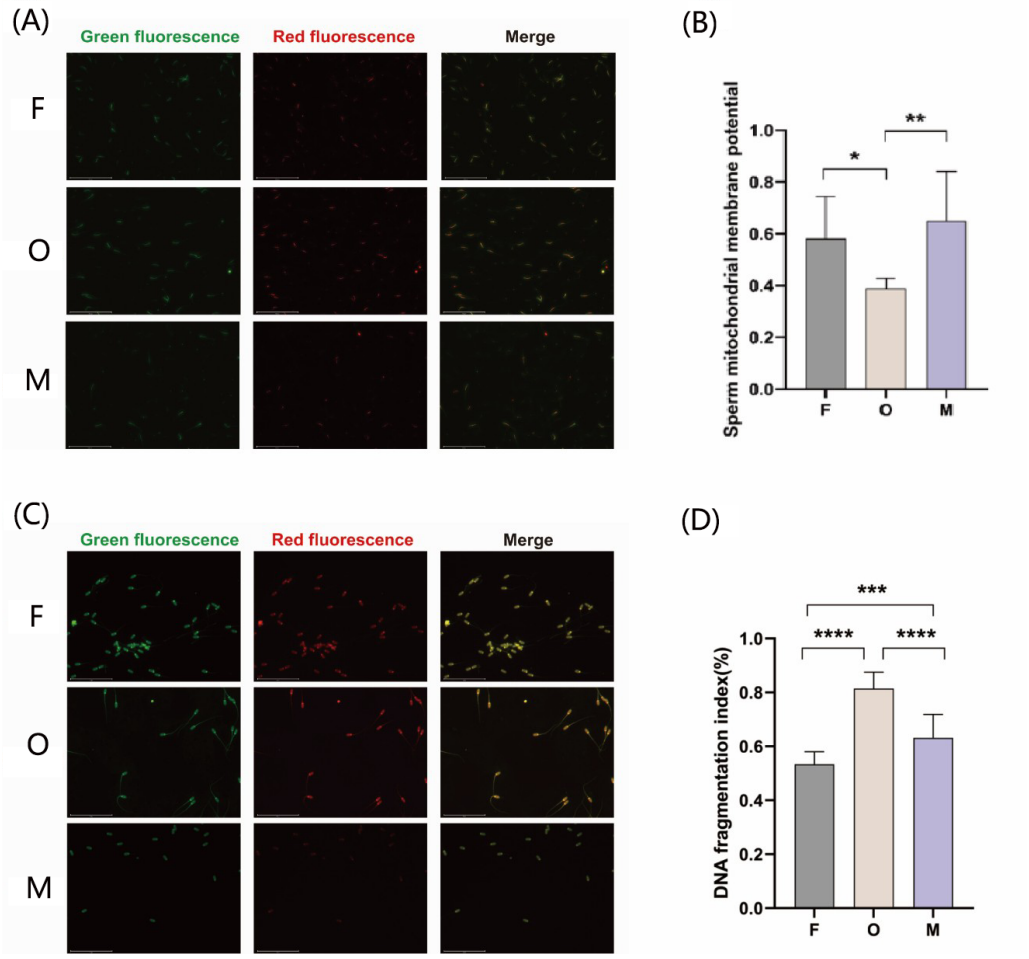

Figure 3. Effects of crocin on the mitochondrial function and DNA integrity in the frozen-thawed boar sperm [Fresh (F), Frozen control (O) and Crocin-treated (M)]. (A) Sperm stained with JC-1 exhibiting red (high Mitochondrial membrane potential [MMP]) and green fluorescence (low MMP) (scale bar = 150 μm). (B) MMP. (C) Sperm DNA fragmentation index (DFI) fluorescence staining (scale bar = 75 μm). (D) DNA fragmentation index (DFI) levels. Data are presented as mean ± standard error of the mean (n=3). Significance levels: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

Identification and analysis of DMs and lipid metabolic pathways

KEGG pathway enrichment analysis of the DMs showed that several metabolic pathways were considerably enriched in the F (Fresh) vs. O (Frozen control) and O vs. M (Crocin-treated, 1 mM) comparisons, with glycerol phospholipid metabolism playing a critical role in maintaining sperm cell membrane integrity. In particular, the DMs within the glycerol phospholipid metabolism pathway included PC, PE, PG, and LPC. In the F vs. O comparison, the O group exhibited significantly reduced PC and LPC levels compared to the F group (Figure 6; $P < 0.05$). Notably, in the M group (crocin-treated), PC and LPC levels were significantly higher than decrease observed after freezing.

DISCUSSION

Crocin improves sperm motility and DNA integrity in humans, rams, chickens, and bulls after freezing and thawing, with reduced oxidative stress and lipid peroxidation. In the present study, we found that crocin also markedly improved the quality of frozen-thawed boar sperm via multiple mechanisms, including decreasing oxidative stress, boosting mitochondrial function, and regulating glycerophospholipid metabolism. These actions increase sperm viability and bolster their

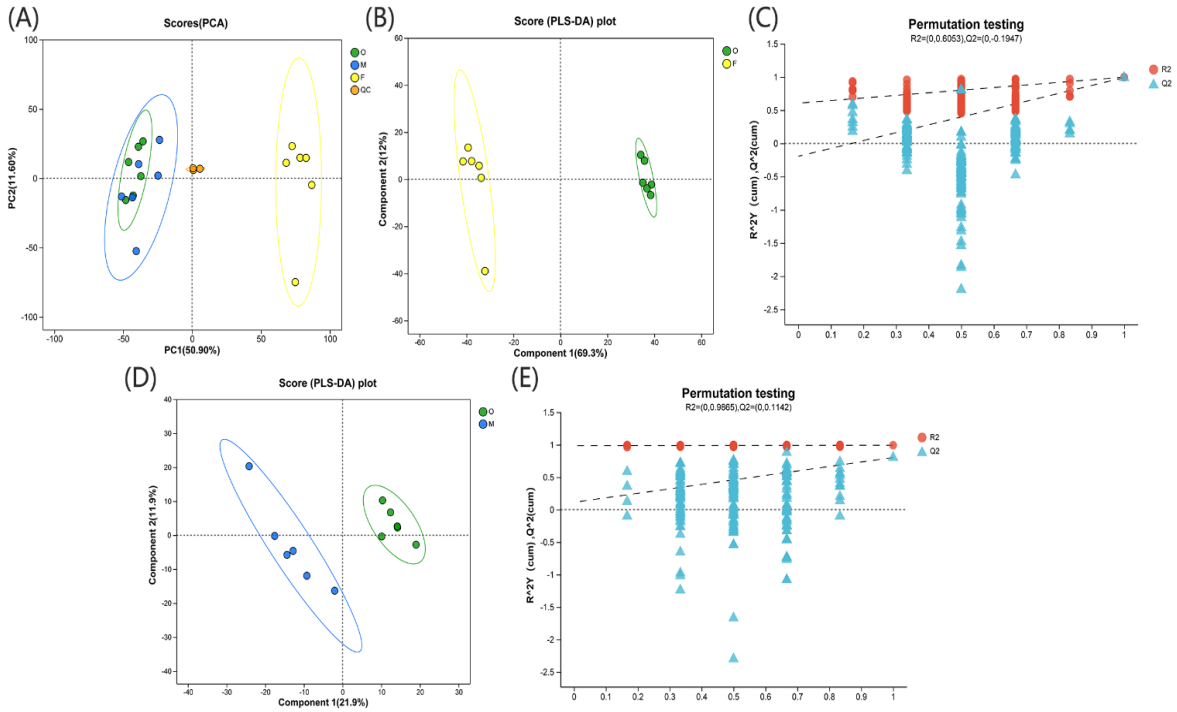

Figure 4. Multivariate analysis of the sperm metabolomic profiles. (A). Principal component analysis (PCA) score plot revealing separation among the F (Fresh), O (Frozen control), and M (Crocin-treated, 1 mM) conditions. (B-C) OPLS-DA score plot (**B**) and permutation test (**C**) for O vs F. (D-E) OPLS-DA score plot (D) and permutation test (E) for O vs M. All analyses represent n=3 biological replicates.

resilience against cryopreservation-associated damage.

Sperm kinematic parameters and viability serve as the primary indicators for evaluating sperm quality following cryopreservation, thus influencing fertilisation ability. Our study did not find a considerable positive effect of crocin on the viability of thawed boar sperm. However, adding 1 mM crocin enhanced the kinematic parameters and motility of thawed boar sperm, implying its efficacy in mitigating freezing-related cell damage. These findings align with previous reports where crocin improved post-thaw motility in rams (19, 20), bulls (21), and chickens (20). The specific improvement in motion characteristics, such as total motility recovery, suggests crocin's role in preserving the functional components necessary for movement.

Mitochondria play a crucial role in male fertility and are involved in critical processes, such as motility, capacitation, acrosome reaction, hyperactivation, and fertilisation. They are essential organelles for maintaining sperm function and energy production (24). Cryopreservation harms mitochondria, causing a decrease in MMP, which adversely affects ATP production and energy metabolism (25). Adding mitochondrial antioxidants to freezing media enhances long-term fertility preservation in males. These antioxidants help protect mitochondrial function during freezing by mitigating oxidative stress and membrane damage, which are critical for maintaining sperm viability and motility. This approach can result in improved outcomes in fertility preservation efforts (26, 27). Our results indicated that crocin markedly enhanced MMP in the boar sperm after freezing and thawing. This suggests a crucial role in preserving mitochondrial function by potentially preventing damage caused by freezing, reducing free radical production, and maintaining mitochondrial integrity. Thus, crocin helps regulate cellular energy metabolism and lowers oxidative stress, thereby supporting sperm vitality and motility after cryopreservation.

Mitochondria are important for providing energy to the sperm and serve as a primary ROS source (28). Although ROS play a crucial role in regulating different physiological processes in sperm, their excessive accumulation can

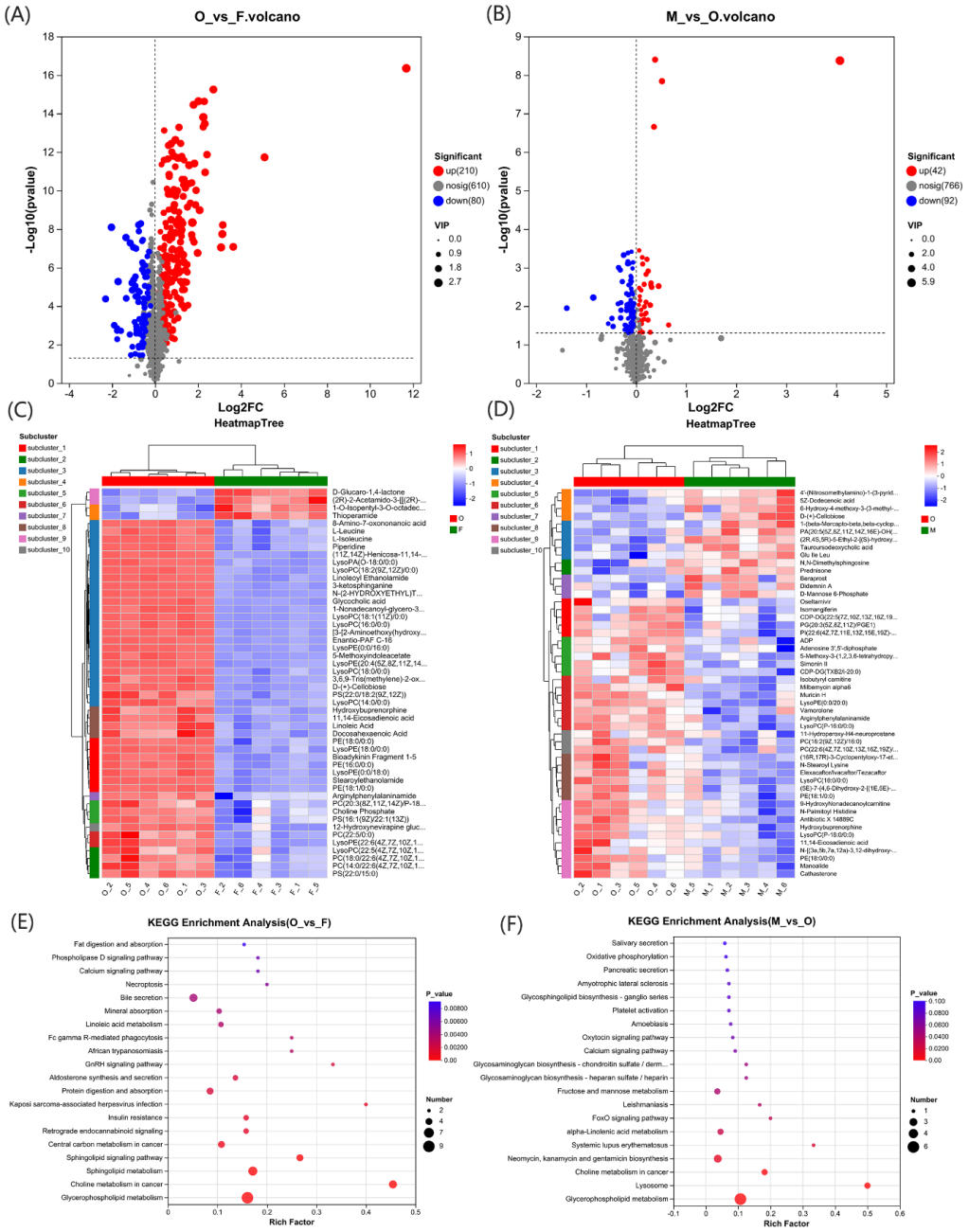

Figure 5. Differential metabolite analysis and pathway enrichment. (A-B) Volcano plots of differently expressed metabolites between O vs F and O vs M. (C-D) Cluster heatmap between O vs F and O vs M. (E-F) Bubble plot of the Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis between O vs F and O vs M.

compromise sperm membrane integrity, impair motility, and hinder sperm-egg fusion. Furthermore, high ROS concentrations can compromise the integrity of sperm nuclear DNA, leading to base modifications, DNA strand breaks and chromatin cross-linking, which negatively impact the fertilisation potential (29).

Previous studies have indicated that crocin improves the quality of bovine sperm and its fertilisation ability after freezing by effectively regulating ROS concentration (30). Furthermore, 8-OHdG serves as a marker of oxidative DNA damage, which is frequently noted during freezing. This highlights the potential impact of oxidative stress on genetic integrity (31). In the present study, crocin markedly decreased ROS levels, 8-OHdG levels, and DNA fragmentation rate after freezing. This suggests that crocin effectively protects the integrity of the sperm genetic material by minimising ROS-induced

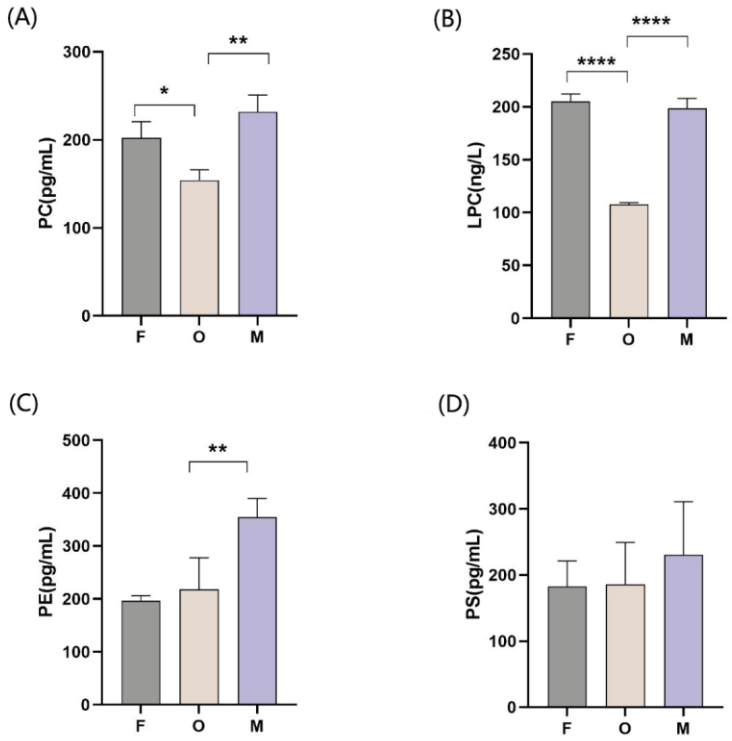

Figure 6. Effects of crocin on sperm glycerophospholipid content following cryopreservation.(A) Sperm phosphatidylcholine (PC); (B) Sperm lysophosphatidylcholine (LPC); (C) Sperm phosphatidylethanolamine (PE) and (D) Sperm phosphatidylserine (PS) contents in the F (Fresh), O (Frozen control), and M (Crocin-treated, 1 mM) groups. Data are presented as mean ± standard error of the mean (n=3). Significance levels: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

DNA damage. Such protection is important for maintaining the sperm fertilisation capability and ensuring the health of potential offspring.

The integrity of the plasma and acrosomal membranes is critical for the functionality of frozen sperm. During freezing, these membranes are especially vulnerable to physical damage caused by ice crystal formation and alterations in osmotic pressure. Our results indicate that crocin markedly improves the integrity of the plasma membrane and acrosome of frozen-thawed boar sperm, thereby stabilising the membrane structure. Phospholipids, necessary components of cell membranes, play a crucial role in maintaining membrane integrity, which largely depends on their balance and metabolic state (32). In addition, LS-MS/MS metabolomics has been extensively used to investigate male reproductive processes. By identifying the metabolites linked to semen freezing, researchers can gain valuable insights into the underlying molecular mechanisms and strategies for fertility preservation. This approach enhances our understanding of sperm cryopreservation (33).

The enriched KEGG pathways in this study mainly focused on glycerophospholipid metabolism, underscoring key DMs, such as PC, PE, PS and LPC. Glycerophospholipid metabolism is vital for cell membrane structure and function and considerably affects membrane fluidity and stability. Each glycerophospholipid type plays a distinct role in maintaining membrane integrity, highlighting its importance in optimising sperm quality and functionality, especially during and after cryopreservation.

The observed preservation of sperm membrane integrity and the concomitant reduction in lipid peroxidation (MDA) can be mechanistically linked to crocin's modulation of glycerophospholipid metabolism. Cryopreservation-induced oxidative stress preferentially targets polyunsaturated fatty acids within membrane phospholipids, initiating peroxidation chain reactions that degrade structural lipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE), while generating cytotoxic aldehydes including MDA (34, 35). This degradation directly undermines

membrane fluidity, stability, and barrier function. Our metabolomic analysis revealed that crocin treatment effectively attenuated the cryopreservation-induced depletion of these key glycerophospholipids. PC is known to play a direct protective role against cold shock and lipid peroxidation (14), and its levels have been negatively correlated with oxidative damage markers like MDA in other sperm pathologies (36, 37). PE is essential for maintaining mitochondrial membrane architecture and function (16). Furthermore, the restoration of lysophosphatidylcholine (LPC), an intermediate involved in membrane remodeling (38), may support membrane repair processes. By restoring the physiological levels of PC, PE, and LPC, crocin likely contributes to the stabilization of the sperm plasma and mitochondrial membranes, as evidenced by the improved membrane integrity and reduced MDA levels. Thus, crocin's cryoprotective action appears to operate through a synergistic mechanism: its direct antioxidant capacity scavenges ROS to limit the initiation of lipid peroxidation, while its regulatory effect on glycerophospholipid metabolism helps to maintain and repair the membrane's structural integrity, thereby enhancing its resilience against oxidative and cryogenic stress.

While this study demonstrates the cryoprotective potential of crocin, several limitations should be acknowledged. The preliminary trial design using pooled semen samples, while appropriate for initial efficacy screening, does not account for potential individual boar variability in response to cryoprotectants, which is a recognized factor in semen cryopreservation studies (19, 24). The in vitro model and limited sample size may not fully capture the complexities of the in vivo reproductive environment. Furthermore, our focus on antioxidant activity and glycerophospholipid metabolism leaves other potential mechanisms unexplored. Future work should prioritize validating these findings using semen from individual boars to assess response heterogeneity. Leveraging multi-omics approaches (e.g., transcriptomics, proteomics) could systematically identify the key signaling pathways and gene networks underlying crocin's effects. Such research will be pivotal in developing novel, natural antioxidant-based formulations to optimize boar semen cryopreservation protocols and enhance reproductive efficiency in the swine industry.

In conclusion, our findings demonstrate that crocin improves the quality of frozen-thawed boar sperm through integrated mechanisms that mitigate oxidative stress, preserve mitochondrial function, and regulate glycerophospholipid metabolism. These actions collectively enhance sperm functional integrity and resilience against cryopreservation damage, supporting the potential of crocin as a natural cryoprotectant for boar semen.

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