Research Article

Title

The effects of crocin on frozen-thawed rooster sperm parameters

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Abstract

This study investigated the effect of crocin on the post-thaw sperm-quality parameters of frozen rooster sperm.

In this study, eight Ross 308 roosters were used to collect semen samples. After the initial evaluation, the semen was diluted using a laboratory-made diluent that closely resembled Beltsville Poultry Semen Extender (BPSE). Crocin was evaluated at 0, 0.5, 1, 2, and 4 mM in Stage 1. Because the 4 mM group showed undesirable sperm morphology and the lowest viability, only this concentration was excluded from the Stage 2 follow-up experiment. Stage 2 evaluated 0, 0.5, 1, and 2 mM using semen from five roosters and six separately prepared pooled-sample replicates per concentration. Samples were pre-cooled to 4°C, equilibrated with glycerol, and cryopreserved by horizontal exposure to liquid nitrogen vapor (4 cm above the liquid phase for 10 min, approximately 15-20°C/min) before immersion into liquid nitrogen (-196°C). Then, after thawing, the samples were evaluated for viability, motility parameters, and membrane integrity.

In the second stage of the experiment, analysis of variance (ANOVA) revealed significant differences among groups for overall motility ($P < 0.001$), progressive motility ($P < 0.001$),

membrane integrity (P = 0.026), and sperm viability (P = 0.044). Subsequent post-hoc analysis using Duncan's multiple range test showed that the 0.5 mM crocin group exhibited significantly higher overall motility compared with the control, 1 mM, and 2 mM groups.

In Stage 2, 0.5 mM crocin produced the highest total motility, progressive motility, and viability, whereas 1 mM produced the highest membrane integrity. These endpoint-specific findings identify 0.5 mM as a promising concentration for preserving the measured post-thaw sperm-quality parameters; confirmation using independent rooster-level replication and fertility outcomes is warranted.

Keywords

Sperm, Crocin, Freezing, Rooster

Introduction

One of the main problems in the poultry industry is the decline in the fertility of parent hen flocks due to genetic selection for production traits that are negatively correlated with fertility. Genetic selection of lines is aimed at producing chicks with fast growth and high slaughter weight, which conflict with reproductive traits, such as fertility rate[1]. Artificial insemination, as one of the techniques used in parent hen flocks, can be a good alternative to natural mating and reduce rearing costs, as well as selecting birds with superior genetic traits from older flocks[2].

Artificial insemination is a valuable tool because it makes optimal use of males that are unable to mate naturally, and also reduces the need for males in the flock[3]. The success of artificial insemination is influenced by various factors, among which the availability of viable and fertile sperm after the freezing and thawing process plays a key role[4, 5].

In the method of storing sperm in liquid form, collected sperm samples are diluted at specific ratios with appropriate diluents. The main purpose of this dilution is to provide conditions for maintaining the normal metabolism, motility, and fertility of sperm[6].

Compared with sperm from other domestic species, cryopreserved poultry spermatozoa exhibit considerably reduced fertilizing capacity, leading to substantially lower fertility rates in inseminated hens relative to those achieved with fresh semen. On the other hand, exposure of sperm to mechanical, chemical, and osmotic stresses during freezing causes extensive changes in their structure, biochemical composition, and function[7].

To reduce or prevent this type of damage during freezing, various antioxidants have been used. It should be noted that the use of antioxidants reduces the damage to sperm cells during freezing[8]. One of the most important challenges is the production of reactive oxygen species and a decrease in antioxidant capacity, which, in turn, reduces sperm quality and efficiency.

Mitochondria are the primary locus of ROS production; nonetheless, the distinctive lipid architecture and selective permeability of mitochondrial membranes restrict the bioavailability and penetration of several non-targeted, conventional antioxidants. Consequently, bioactive compounds capable of partitioning across lipid bilayers or mitigating mitochondrial oxidative stress may provide cryoprotective effects; these proposed mechanisms were not measured in the present study[9].

Crocin (digentiobiosyl ester of crocetin), the principal bioactive constituent of saffron (Crocus sativus L.) [10,25], exhibits potent antioxidant activity primarily by scavenging reactive oxygen species (ROS), notably the superoxide anion [10]. Furthermore, it mitigates oxidative stress by upregulating the expression of endogenous antioxidant enzymes and neutralizing free radicals[11]. Mehdipour et al. reported that crocin improved post-thaw rooster sperm quality and fertility

outcomes in a separate experimental setting and provided evidence of protection against freezing-induced damage by regulating the expression of genes related to apoptosis [12]. Salehi et al. also showed that using crocin in a dog semen extender can improve sperm quality and its indicators after storage in liquid form [13].

The present study aimed to evaluate the effects of crocin on post-thaw total motility, progressive motility, membrane integrity, and viability of frozen rooster sperm. A laboratory-made diluent that closely resembled BPSE was supplemented with crocin. Unlike the previous rooster study, which evaluated 0.5-1.5 mM crocin in a different freezing protocol [12], the present work used a two-stage design with a broader 0-4 mM screening range followed by evaluation of 0-2 mM in Ross 308 rooster semen.

Result

First stage of the experiment (Effect of adding crocin in the diluent on Ross 308 roosters sperm)

In Stage 1, total motility, progressive motility, plasma membrane integrity, and sperm viability were examined across all five concentrations. All Stage 1 results were retained. The 4 mM treatment, which showed the lowest viability and undesirable sperm enlargement and deformation, was excluded only from the Stage 2 follow-up experiment.

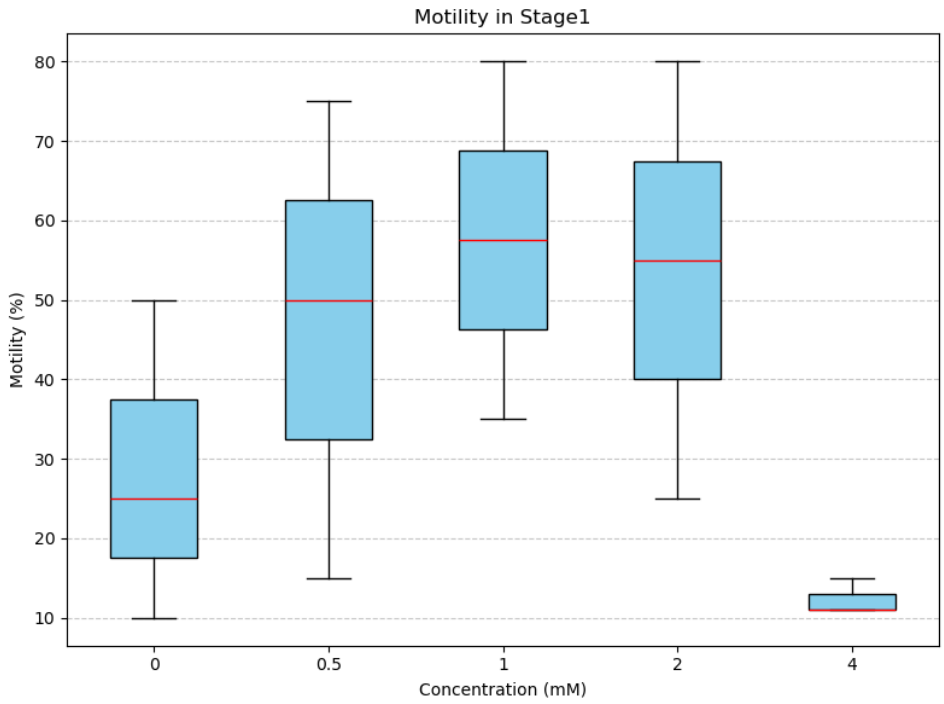

Figure 1. Box plot of total sperm motility after thawing in Stage 1. The centre line shows the median, the box shows the interquartile range, and the whiskers show the plotted range. Semen from three roosters contributed to the pooled Stage 1 screening samples.

In the initial screening stage, overall motility (P = 0.153), progressive motility (P = 0.130), and membrane integrity (P = 0.113) did not differ significantly among groups. Sperm viability was the only parameter that varied significantly across treatments (P = 0.037), primarily driven by the sharp decline in the 4 mM group.

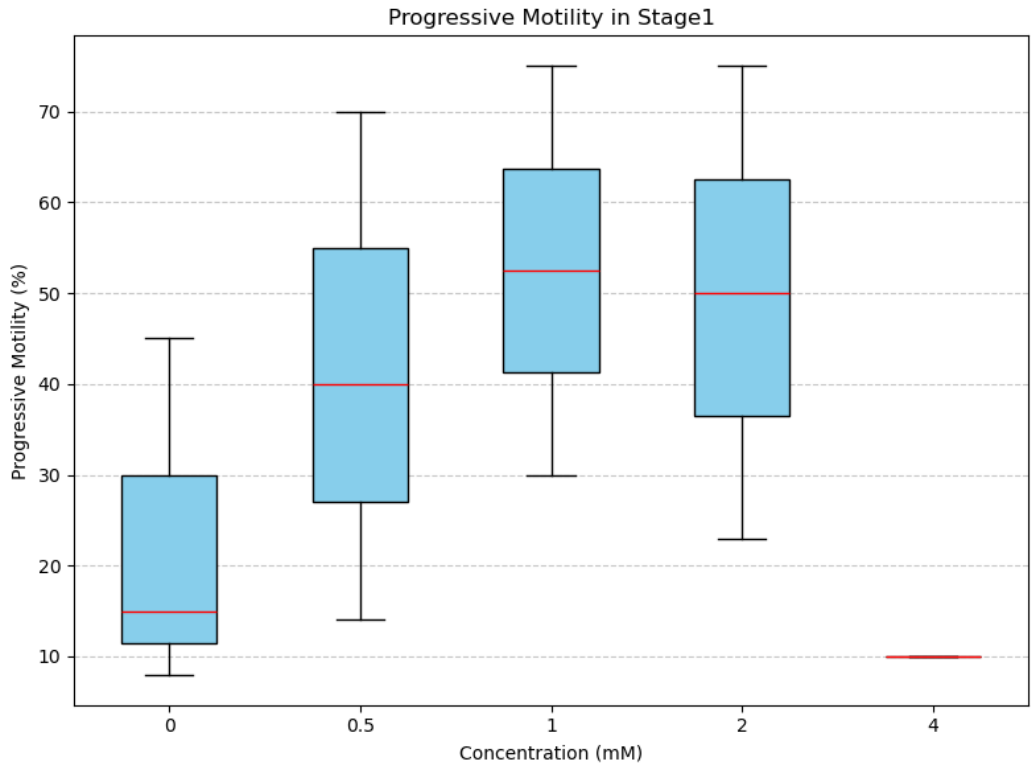

Figure 2. Box plot of progressive sperm motility after thawing in Stage 1. The centre line shows the median, the box shows the interquartile range, and the whiskers show the plotted range. Semen from three roosters contributed to the pooled Stage 1 screening samples.

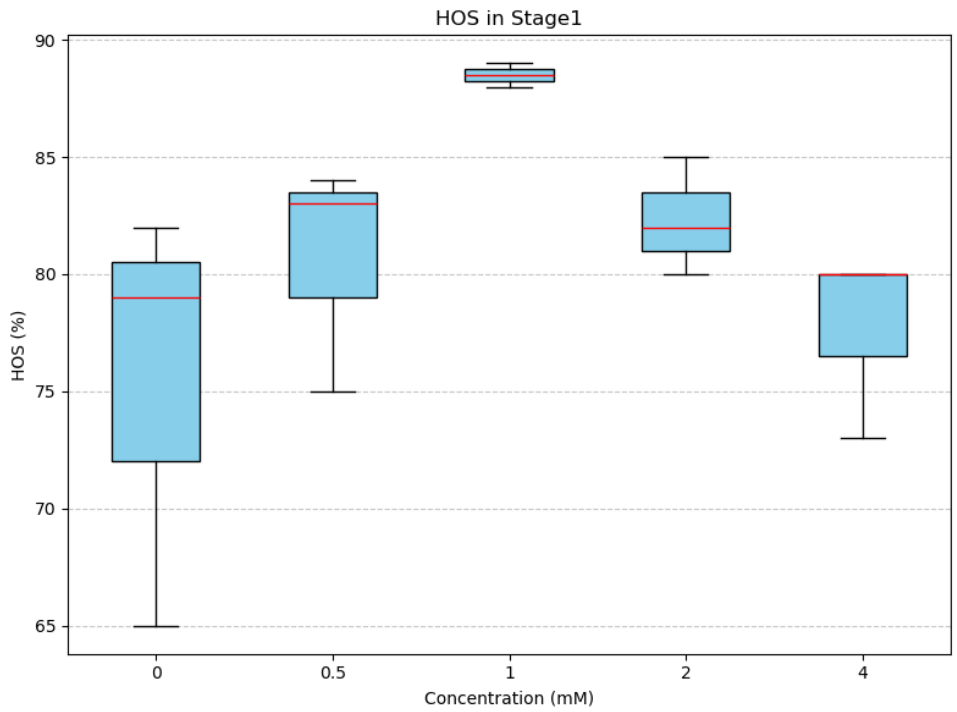

Figure 3. Box plot of sperm membrane integrity after the HOS test in Stage 1. The centre line shows the median, the box shows the interquartile range, and the whiskers show the plotted range. Semen from three roosters contributed to the pooled Stage 1 screening samples.

Membrane integrity did not differ significantly among Stage 1 groups ($P = 0.113$).

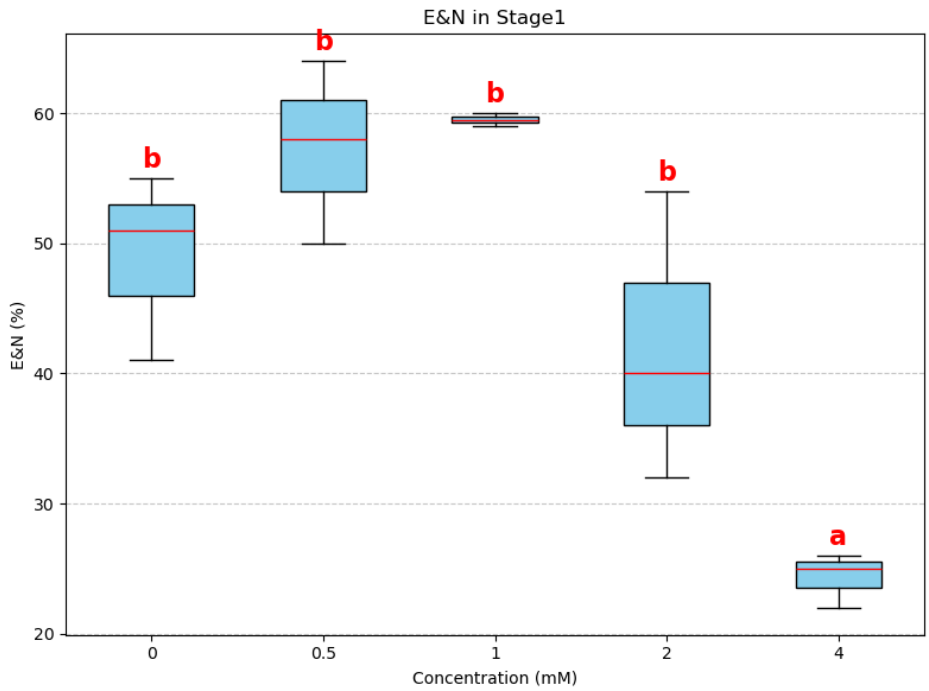

Figure 4. Box plot of sperm viability after thawing in Stage 1. The centre line shows the median, the box shows the interquartile range, and the whiskers show the plotted range. Different letters indicate significant post-hoc differences ($P < 0.05$); groups sharing a letter did not differ significantly.

Sperm viability differed among Stage 1 groups ($P = 0.037$).

Post-hoc grouping showed that viability in the 4 mM group was significantly lower than in the control, 0.5, 1, and 2 mM groups, whereas those four groups did not differ significantly from one another.

Second stage of the experiment (the effect of adding crocin to the diluent on Ross 308 roosters' sperm)

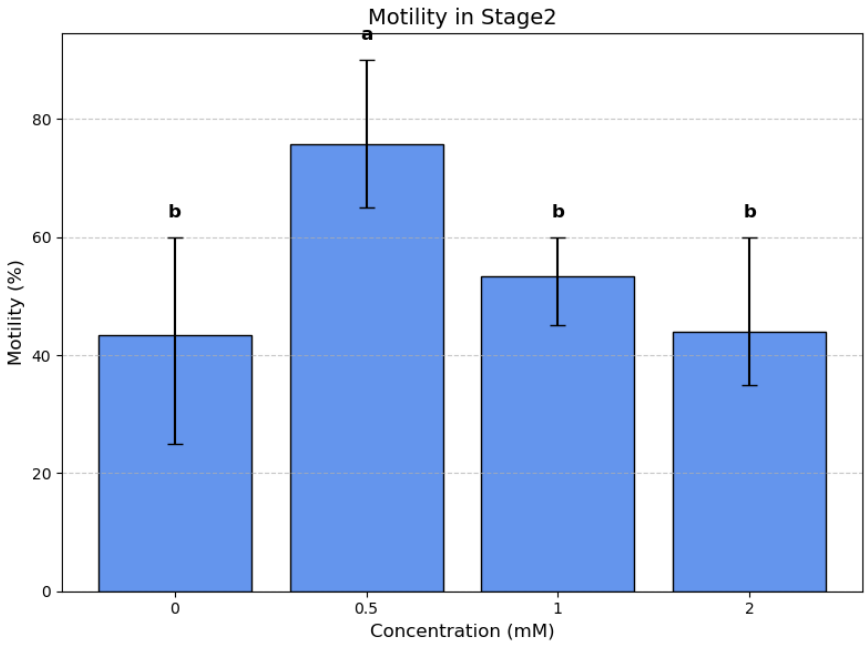

Figure 5. Total sperm motility after thawing in Stage 2. Bars show mean ± SD for six separately prepared pooled-sample replicates per concentration. Different letters indicate significant differences by Duncan's multiple range test (P < 0.05); groups sharing a letter did not differ significantly.

Overall motility differed among groups ($F(3,20) = 552.14$, $P < 0.001$). Duncan's multiple range test showed that 0.5 mM crocin produced the highest post-thaw motility (75.83 ± 14.36%), which was significantly higher than the control (43.33 ± 16.50%), 1 mM (53.33 ± 6.74%), and 2 mM (43.83 ± 16.03%) groups ($P < 0.001$).

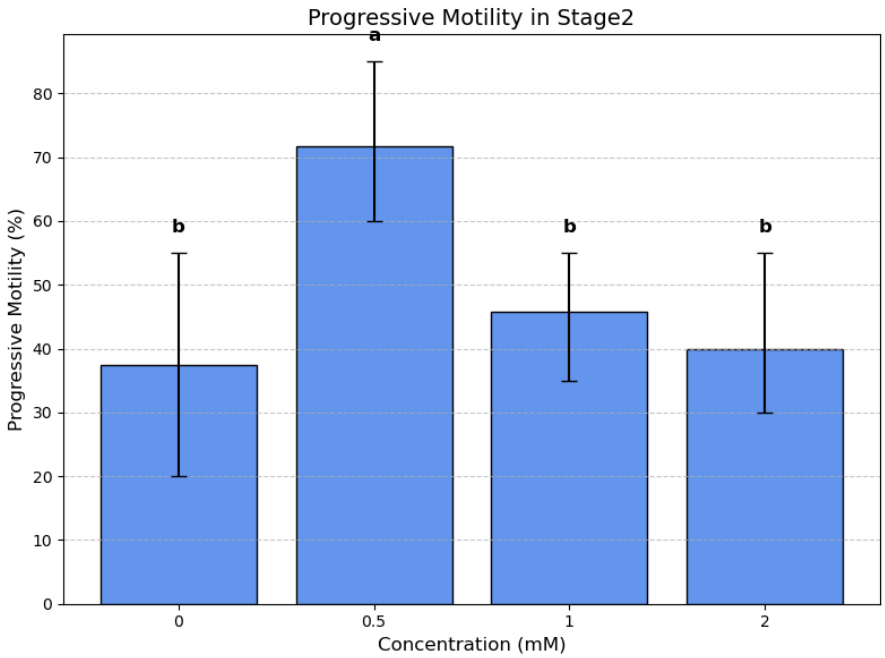

Figure 6. Progressive sperm motility after thawing in Stage 2. Bars show mean ± SD for six separately prepared pooled-sample replicates per concentration. Different letters indicate significant differences by Duncan's multiple range test (P < 0.05); groups sharing a letter did not differ significantly.

Progressive motility differed among groups ($F(3,20) = 15.345$, $P < 0.001$). Duncan's test showed that the 0.5 mM group had significantly higher progressive motility than the control (difference 34.17%, $P < 0.001$), 1 mM (difference 25.83%, $P < 0.001$), and 2 mM (difference 31.60%, $P < 0.001$) groups. The complete statistical grouping is shown by the letters in Figure 6.

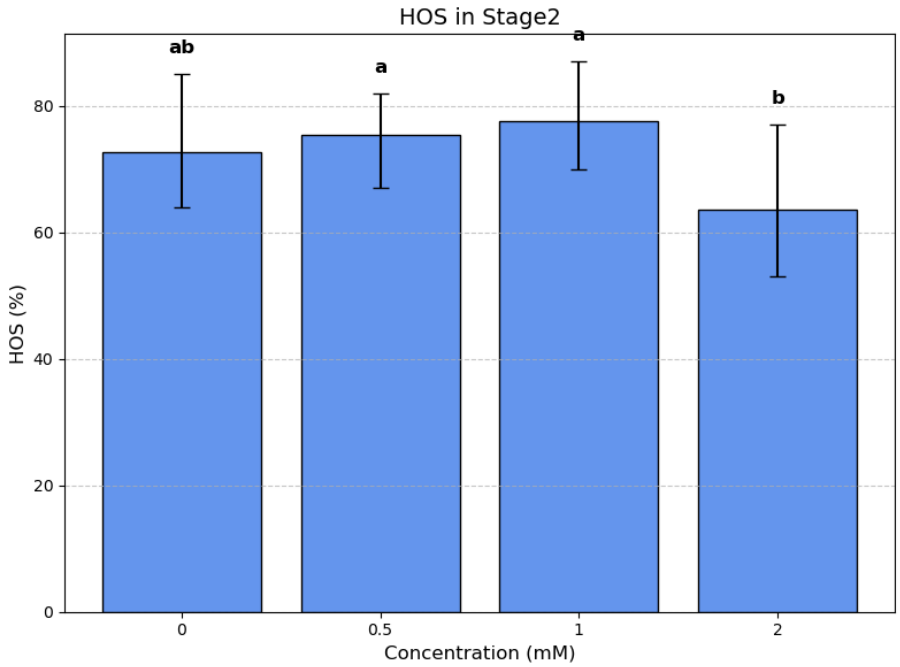

Figure 7. Sperm membrane integrity after the HOS test in Stage 2. Bars show mean ± SD for six separately prepared pooled-sample replicates per concentration. Different letters indicate significant differences by Duncan's multiple range test ($P < 0.05$); groups sharing a letter did not differ significantly.

Membrane integrity differed among groups ($F(3,20) = 3.837$, $P = 0.026$). Duncan's test showed that the 1 mM group had significantly greater membrane integrity than the 2 mM group (difference 14.067%, $P = 0.007$), and the 0.5 mM group had significantly greater membrane integrity than the 2 mM group (difference 11.733%, $P = 0.019$). The 1 mM group had the highest membrane integrity; the complete grouping is shown in Figure 7.

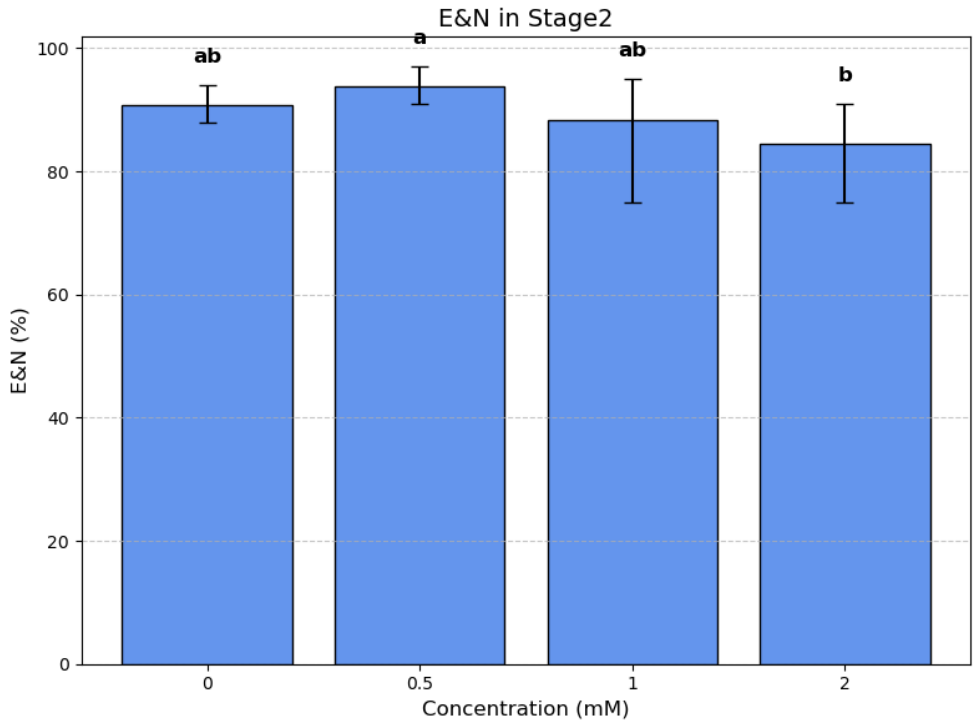

Figure 8. Sperm viability after thawing in Stage 2. Bars show mean ± SD for six separately prepared pooled-sample replicates per concentration. Different letters indicate significant differences by Duncan's multiple range test (P < 0.05); groups sharing a letter did not differ significantly.

Sperm viability differed among groups ($F(3,20) = 3.271$, $P = 0.044$). Duncan's test showed that the 0.5 mM group had significantly higher viability than the 2 mM group (difference 9.433%, $P = 0.011$). The 0.5 mM group had the highest viability; the complete grouping is shown in Figure 8.

Discussion

Sperm freezing is a complex process that causes various forms of cellular damage, known as cold shock[15]. One of the main factors in the reduction of viability rate in frozen sperm is the formation of reactive oxygen species[16]. Destruction of the sperm membrane is known as an important factor in the loss of fertility and reduced motility of male sperm[17]. So far, various antioxidants

have been used to prevent or reduce membrane damage during freezing because adding antioxidant compounds can reduce the severity of damage to sperm cells during freezing[18].

Crocin is a water-soluble carotenoid compound found in saffron and has antioxidant properties [10,11]. Previous literature has also described anti-inflammatory activity in non-sperm experimental systems [21]. Mitochondrial targeting, membrane penetration, intracellular ROS, and lipid peroxidation were not measured in the present study; therefore, any contribution of these mechanisms to the observed sperm-quality results remains proposed rather than demonstrated.

Carotenoids, including crocin, may support sperm structure and function through antioxidant activity [22]. Studies in bovine and other mammalian systems have proposed effects on apoptosis, mitochondrial activity, and sperm energy metabolism [23,24], but the mammalian evidence in reference 24 should not be interpreted as direct evidence for rooster sperm. Previous rooster research has also proposed effects on intracellular detoxifying enzymes and membrane fluidity [12]. These mechanisms were not measured in the present experiment and are discussed only as possible explanations.

In Stage 1, crocin was evaluated at 0, 0.5, 1, 2, and 4 mM. Total motility, progressive motility, and membrane integrity did not differ significantly, although the 1 mM group had the highest numerical values for several endpoints. Viability differed among treatments, with the lowest value at 4 mM. On the basis of these observed Stage 1 findings, the 4 mM treatment was excluded only from Stage 2, which evaluated 0, 0.5, 1, and 2 mM crocin.

Stage 1 was an exploratory dose-screening phase. At each collection session, accepted ejaculates were pooled and divided among treatment concentrations. Stage 2 was a follow-up experiment based on six separately prepared collection-session pools per concentration. In Stage 2, 0.5 mM produced the highest total motility, progressive motility, and viability, whereas 1 mM produced

the highest membrane integrity. Thus, 0.5 mM was the most consistently favourable concentration across three of the four measured endpoints, but it should not be interpreted as a universal optimum. Because individual-rooster variation was not estimated from the pooled samples, further independent rooster-level studies are needed.

In a 2015 study by Sapanidou et al., frozen bovine sperm were incubated with 3 different doses of crocin (0.5, 1, and 2 mM), and a significant reduction in reactive oxygen species, lipid peroxidation, and DNA damage, and improvements in motility, viability, and in vitro fertilization-related parameters were observed at a dose of 1 mM[26].

Another study by Langobardi et al. in 2020 investigated the effect of crocin on goat sperm extenders before freezing. It was found that adding crocin at 1 mM significantly improved sperm motility. Although the species and freezing protocol differed from the present experiment, the results showed that crocin supplementation in extenders reduced oxidative stress and improved sperm motility and DNA integrity in frozen-thawed sperm across different breeds [27].

In Stage 2, 0.5 mM crocin produced the highest total motility, progressive motility, and viability, whereas 1 mM produced the highest membrane integrity. These findings describe post-thaw sperm-quality endpoints only. Fertility, intracellular ROS, and sperm respiration were not measured in the present study.

In a study conducted by Calabria et al. in 2023, 0.5 mM crocin was selected as the most effective concentration compared to other concentrations for assessing intracellular ROS levels in sperm, lipid peroxidation, and DNA fragmentation compared to the control[28]. Because that study used chilled canine semen and different endpoints, it is not directly equivalent to the present rooster cryopreservation experiment; however, it also indicates that crocin effects can be concentration dependent.

In a 2021 study by Langobardi et al., which evaluated the effect of crocin on the quality of frozen-thawed sperm in buffalo, in a different species and protocol, crocin increased the functional integrity of the sperm membrane compared to the control group and reduced sperm DNA fragmentation compared to other groups [29].

A recent review of predominantly mammalian evidence described associations between crocin, sperm motility, oxidative status, and mitochondrial pathways [30]. These findings provide mechanistic context but are not direct evidence for rooster sperm.

While diverse studies consistently confirm the cryoprotective utility of crocin, the optimal effective concentration varies across the literature. When comparing our findings with the rooster trial by Mehdipour et al. (2020), differences in the most effective dosage may reflect methodological variations, including base extender composition, cryoprotectant equilibration protocols, and specific freezing/thawing rates, rather than species divergence. Conversely, discrepancies between avian and mammalian investigations (such as those in bulls, dogs, and buffaloes) may reflect both methodological differences and inter-species variation in sperm plasma membrane lipid architecture, cholesterol-to-phospholipid ratios, and overall vulnerability to osmotic stress during cryopreservation[31].

In the second stage of the present study, the 0.5 mM concentration showed an improvement effect compared to other concentrations. One possible explanation is that higher concentrations increase diluent osmolarity, and sperm cells are sensitive to osmotic changes, especially during freezing/thawing. The 0.5 mM concentration may provide a favourable balance between ROS removal and osmotic tolerance. There may also be a saturation point beyond which additional crocin does not confer increased intracellular protection. These explanations remain hypotheses because osmolarity, intracellular ROS, metabolism, and cell signalling were not measured. Excess

crocin might accumulate around the cell, causing metabolic imbalances or membrane interactions that impair function and interfere with cell signaling pathways necessary for normal sperm function.

Materials and Methods

Ethical Considerations

The protocol, including housing, semen collection, cryopreservation, thawing, and laboratory evaluation of eight roosters, was approved by the Research Ethics Committee of Ferdowsi University of Mashhad (IR.UM.REC.1402.222).

All procedures followed national regulations and the Guide for the Care and Use of Laboratory Animals.

Experimental Compounds

All compounds were laboratory or analytical grade. Tris and D-fructose were obtained from Sigma-Aldrich (USA). Eosin-nigrosin stain, crocin, BPSE-related reagents, potassium citrate tribasic monohydrate, and glycerol were obtained from Merck (Germany). Crocin was used as supplied at analytical grade; no unverified catalogue or batch information has been added.

Animals

This study used eight adult Ross 308 roosters (28 weeks of age) that were clinically healthy at enrolment and weighed approximately 5 kg. The experiment was conducted in two stages:

- **Stage 1 (exploratory dose screening):** Semen was collected from three roosters and tested with five concentrations of crocin (0, 0.5, 1, 2, and 4 mM).
- **Stage 2 (follow-up experiment): Semen was collected from five roosters. Four crocin concentrations (0, 0.5, 1, and 2 mM) were evaluated in six separately prepared pooled-sample replicates per concentration.**

The roosters were housed on litter under a 15:9 h light:dark cycle at 21-25°C in the Poultry Department, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad. Each bird received 150 g/day of a commercial pelleted rooster diet prepared by Gohar Daneh Company and had free access to water. Roosters were trained for semen collection for 2-3 weeks before the study.

Semen was collected from each rooster two to three times per week by the dorso-abdominal massage method. At each collection session, ejaculates that met the inclusion criteria were pooled, mixed, and divided among the treatment concentrations. Each separately prepared collection-session pool was treated as one experimental replicate; aliquots from the same pool were not counted as separate replicates. Samples were examined microscopically for motility, clarity, and absence of urate or red-blood-cell contamination. They were maintained at 35-37°C during transfer and processed promptly after arrival at the laboratory.

Preparation steps of test compounds

The diluent used throughout the study was laboratory-made and closely resembled, but was not identical to, BPSE (Beltsville Poultry Semen Extender), containing 5 mM fructose, 2.71 mM Tris, and 0.64 mM potassium citrate, with different concentrations of crocin added [14]. The egg yolk was separated using the standard method. The yolk and diluent were mixed in equal proportions (1:1), placed in a 50 cc Falcon tube, and homogenized for 30 minutes in a Rotomix to ensure thorough mixing. The resulting mixture was poured into two separate 10 cc tubes and centrifuged for 30 minutes at 5500 rpm. From the supernatant, the clear middle layer (representing the clarified egg yolk plasma, devoid of dense sedimented yolk granules) was transferred to a 1.5 mL microtube and centrifuged again for 45 minutes at 13,000 rpm. The recovered clarified egg-yolk plasma was added at 20% (v/v), calculated relative to the final extender volume.

Crocin (molecular weight approximately 977 g/mol) was dissolved in physiological saline to prepare a 200 mM stock solution (195.5 mg in 1.0 mL). For each 1.0 mL of final extender, 2.5, 5.0, 10.0, or 20.0 µL of stock was used to obtain 0.5, 1, 2, or 4 mM crocin, respectively. Physiological saline was added as needed so that every preparation, including the control, contained a total vehicle volume of 20 µL/mL.

Only ejaculates with a volume of 0.2-0.7 mL, motility >75%, viability >80%, and no visible contamination were included. Sperm concentration was measured before treatment allocation, and aliquots from each pooled replicate were standardized by equal division among its treatment groups.

Evaluation of sperm quality parameters

Sperm count

A 10 µL semen sample was mixed with 90 µL physiological saline containing 4% formalin (1:10 dilution) to immobilize sperm. The diluted sample was loaded into a haemocytometer and examined by light microscopy. At least five counting-grid squares were assessed, with cells touching the upper and left borders included and those touching the lower and right borders excluded. Sperm concentration per millilitre was calculated from the counted chamber volume and the dilution factor. Concentration was recorded before equal aliquots from each pooled sample were assigned to treatments.

Evaluation of sperm motility parameters

Total and progressive sperm motility were assessed as percentages by subjective light-microscopic evaluation; no 1-5 or 1-10 scoring scale was used. These motility percentages were treated as sperm-quality endpoints and not as direct measures of fertility.

A 10 µL undiluted semen sample was placed on an evaluation slide at room temperature. At least five fields were examined by the same trained evaluator, and the percentages of total motile and progressively motile sperm were recorded.

Evaluation of sperm membrane integrity

The hypo-osmotic swelling test (HOST) was used to assess sperm membrane integrity. Diluted semen (30 µL) was mixed with 300 µL hypo-osmotic solution (100 mOsm/L) and incubated for 30 min at 37°C. Two hundred sperm were examined by phase-contrast microscopy. Sperm showing the characteristic swollen or curled tail response were classified as membrane intact, whereas sperm without tail swelling were classified as membrane damaged. Membrane integrity (%) was calculated as: membrane-intact sperm/200 × 100.

Evaluation of sperm viability rate

The semen sample was thoroughly mixed with eosin-nigrosin stain, spread on a slide, and air-dried. Two hundred sperm were examined using a 100× objective. Unstained sperm were classified as viable because their plasma membranes excluded eosin, whereas pink or red-stained sperm were classified as non-viable. Viability (%) was calculated as: unstained sperm/200 × 100.

First stage of the experiment

Because the effective crocin range under the present conditions was uncertain, Stage 1 was conducted as an exploratory dose-screening experiment using semen from three adult Ross 308 roosters (28 weeks old). Selection of 0, 0.5, 1, and 2 mM for Stage 2 was made after examination of the Stage 1 results; it was not a prespecified confirmatory selection rule.

Cryopreservation followed a static-vapour cooling regimen. Extended semen was placed in cryovials and cooled to 4°C over 60 min. Glycerol-containing diluent was added at 4°C, followed by a 10 min equilibration. Cryovials were mounted horizontally on a floating rack 4 cm above

liquid nitrogen in a closed Styrofoam chamber for 10 min, corresponding to an approximate cooling rate of 15-20°C/min, and were then immersed in liquid nitrogen (-196°C). For thawing, each cryovial was placed in a 60°C water bath for 3-5 s and removed immediately when the contents were fully thawed. The same container type and thawing endpoint were used for every group before sperm evaluation.

Stage 1 showed that 4 mM crocin was associated with undesirable sperm morphology and the lowest viability. Accordingly, only the 4 mM treatment was excluded from Stage 2. Stage 2 was conducted as a follow-up experiment using 0, 0.5, 1, and 2 mM crocin, semen from five roosters, and six separately prepared pooled-sample replicates per concentration to further evaluate the observed concentration-response pattern.

Second stage of the experiment

Stage 2 used semen from five adult Ross 308 roosters (28 weeks old). At each of six collection sessions, accepted ejaculates were pooled and divided among the four crocin concentrations (0, 0.5, 1, and 2 mM), yielding six pooled-sample replicates per concentration. The 4 mM treatment was not included because of the adverse findings observed in Stage 1.

After completing this stage, all the steps performed in the previous experiment, such as sperm cooling, freezing and thawing, and evaluation of sperm quality parameters, were repeated in this experiment.

Statistical Analysis

Data were analysed using SigmaPlot 14. Each separately prepared collection-session pool was treated as the experimental unit. Normality and homogeneity of variance were assessed using the Shapiro-Wilk and Brown-Forsythe tests, respectively. Stage 2 groups were compared by one-way ANOVA; when the overall ANOVA was significant, Duncan's multiple range test was used to

compare treatment means. Stage 1 was treated as exploratory. Non-normal Stage 1 data were analysed using the Kruskal-Wallis test followed by pairwise Mann-Whitney tests with Bonferroni adjustment. Statistical significance was set at $P < 0.05$, with adjusted thresholds applied to the Stage 1 pairwise tests.

Data availability

The summary data supporting the findings are presented in the Results and figures. Replicate-level data are available from the corresponding author upon reasonable request.

Authors' Contributions:

All authors contributed to the study conception and design. Soroush Entezari performed material preparation, data collection, and investigation. Pezhman Mirshokraei carried out project administration, supervision, methodology, conceptualization, review and editing of the manuscript. Vahid Hamedianasl carried out the formal analysis, software processing, validation, and visualization. Soroush Entezari wrote the first draft of the manuscript, and all authors commented on previous versions. All authors read and approved the final manuscript.

Competing Interests:
The authors declare no conflict of interest.

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