

Article

Crocin Supplementation of Semen Extender Preserves Chilled Ram Semen Quality During Extended Storage

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Simple Summary

Ram semen is particularly vulnerable to damage from temperature fluctuations, as chilling or freezing semen during storage induces the production of reactive oxygen species (ROS). ROS are highly reactive molecules that induce oxidative stress and thus can inflict damage to cell molecules and structures, like membranes, all of which culminate in reduced sperm function. This has rather important implications for artificial insemination (AI), a practice that is applied for the genetic improvement of herds. In the present study, we investigated the molecular mechanisms implicated in cold shock of ram spermatozoa and the potential of crocin, a potent antioxidant, to inhibit the ROS-mediated damage and modulate the implicated pathways. We have identified that crocin protects spermatozoa from cold shock by inhibiting the induction of regulated sperm death (apoptosis) and fortifying cell survival.

Abstract

Ram semen is highly susceptible to cold shock, which induces irreversible damage to the integrity and fluidity of membranes. Chilled semen is commonly used within 24 h of collection. However, while its storage at 5 °C extends semen lifespan, it is often accompanied by quality deterioration due to accumulation of reactive oxygen species (ROS). This study evaluated the potential of crocin, a carotenoid with antioxidant properties, to improve the quality of chilled ram semen stored at 5 °C for up to three days in a soybean lecithin-based extender supplemented with two crocin concentrations (0.5 and 1 mM). Sperm motility, viability, glutathione levels, the expression of proteins involved in the heat stress response (HSR), and apoptosis were assessed at 24 h intervals. Crocin preserved motility (up to Day 1), viability (up to Day 2), and kinematic parameters (up to Day 3). In addition, crocin enhanced intracellular glutathione and Hsp70 levels and inhibited apoptotic levels dose-dependently, indicating the antioxidant and cytoprotective role of crocin. Despite 0.5 mM being effective up to Day 1, 1 mM crocin augmented antioxidant capacity, modulated stress response mechanisms, and preserved sperm quality during chilled storage up to Day 3, highlighting its potential as a valuable additive of ram semen extenders.



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conditions of the [Creative Commons](https://creativecommons.org/licenses/by/4.0/)[Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.**Keywords:** chilled semen; ram; crocin; glutathione; heat shock protein 70; apoptosis; Bax; Bcl-2; mitochondria; oxidative stress

1. Introduction

The application of advanced reproductive techniques plays a pivotal role in improving fertility rates and livestock production [1]. Artificial insemination (AI) and in vitro fertilization (IVF) are among the most well-established techniques utilized in farm animal reproduction. In sheep, however, application of these technologies is still limited due to two notable drawbacks. First, the complex cervix anatomy of ewe predisposes that semen deposit directly into the uterus is inapplicable, while cervical insemination results in low pregnancy success rates (20–30%) [2,3]. Conception could be achieved by laparoscopic intrauterine insemination (LAI), a sophisticated technique which is only addressed by trained personnel, and that also raises important considerations regarding sheep welfare due to its invasive nature and handling requirements [4]. The second reason is the susceptibility of ram spermatozoa to cold shock [5,6].

Different storage temperatures ranging from 4 to 22 °C have been employed to preserve extended ram spermatozoa. Although high temperatures (15 °C) guarantee higher conception rates [7], storage at this temperature is accompanied by increased nutrient consumption and elevated metabolism rates, compared to 5 °C [8], which eventually lead to a shortening of the shelf life of extended ram semen to 9–10 h [7]. Typically, liquid-chilled semen preservation at 5 °C is widely considered a good alternative method for extended storage of ram spermatozoa, administered in ewes via vaginal insemination [9–11]. Decreasing temperature from 15 °C to 5 °C extends semen storage lifespan up to 3 days [10], while preserving motility, viability, and fertilizing capacity [10,11]. However, this process presents significant challenges for ram spermatozoa, as it inflicts thermal, structural, and biochemical damage [6]. Ram spermatozoa contain higher amounts of polyunsaturated fatty acids (PUFAs) with a low cholesterol/phospholipid ratio compared to other species [5]. Therefore, they are highly susceptible to cold, shock which produces reactive oxygen species (ROS), responsible for irreversible damage to membrane integrity and fluidity [12]. As the storage period is prolonged, ROS continue to accumulate, thus leading to oxidative stress, which ultimately leads to increased pro-apoptotic proteins, such as Bax, and decreased anti-apoptotic proteins, such as Bcl-2 [13]. On the other hand, the cells have protective mechanisms against cold stress; they respond to low temperatures with increased synthesis of heat shock proteins (Hsps), a group of specialized proteins that act as molecular chaperones [14]. Specifically in bovine spermatozoa, Hsp70 plays a central role in critical cellular processes, such as energy metabolism and protein folding [15]. Moreover, Hsp70 abundance is closely associated with fertilizing capacity in water buffalo spermatozoa [16]. Deletion of the *hsp70-2* gene in male knockout mice induces extensive apoptosis in spermatocytes, resulting in infertility [17]. The antiapoptotic effect of Hsp70 has been attributed to its ability to stabilize Bcl-2 and inhibit Bax [18,19]. Although the role of Hsps in ram spermatozoa has not yet been elucidated, we hypothesize that a comparable protective response operates in this species.

Crocin, the main water-soluble antioxidant constituent of saffron (*Crocus sativus* L.), plays a critical role in physiological responses in ruminant species, including bovine, ram, and buffalo [20–24]. Crocin, as a potent radical scavenger (particularly against superoxide anion) [25], ensures optimal ROS levels in bovine spermatozoa and preserves normal sperm function [22–24]. In addition, crocin may indirectly enhance the antioxidant capacity of spermatozoa by upregulating antioxidant enzyme activity and preventing glutathione depletion [24,26]. In bovine spermatozoa, crocin demonstrates anti-apoptotic properties through heat shock response (HSR) modulation [24]. Upregulation of Hsp70 enhances the resistance of spermatozoa to the damaging effects of stressors such as cryopreservation, thereby preserving sperm motility and post-thaw fertility in bovine and buffalo species [16,24]. This observation has also been verified in other cell types, such as rat

cardiomyocytes and myocardium [27,28], suggesting that crocin may exert a broader cytoprotective role in all cells. Although published data support that the addition of crocin (0.5 mM) improves to a certain extent liquid-chilled canine semen quality [29], to the best of our knowledge, no similar data exist for other species. In the present study, the effect of crocin on ram semen physiology during prolonged chilled storage was investigated, in terms of sperm motility and viability. Emphasis was given on the modulation of the HSR and the mitochondrial apoptotic mechanism in spermatozoa.

2. Materials and Methods

2.1. Animals

Five Chios rams with an average age of 6–8 years and body weight of 75 ± 5 kg, trained to donate semen with an artificial vagina, were used in the present study. The animals were housed at the facilities of the Department of Animal Reproduction and Artificial Insemination (EL54303115), Directorate of Veterinary Center of Thessaloniki, Ministry of Rural Development and Food, Ionia, Thessaloniki, Greece (Longitude: $22^{\circ}86'$ E, Latitude: $40^{\circ}68'$ N). The animals were acclimated to the environment of the sheepfold and exposed to the natural photoperiod. They were fed mixed grass hay and concentrate, as needed to maintain a healthy body condition score, and had access to fresh water, ad libitum. The general health management practices were followed as per the health calendar of the Center. During the experimental period, no pharmaceutical or dietary interventions were administered. All animal procedures were performed in accordance with the European Union Regulation 2010/63. Because the animals were trained semen donors and semen collection was performed during routine semen collection sessions, with no additional handling, approval from an Ethics Committee was not required. All analyses were conducted exclusively on the collected spermatozoa, without any intervention or manipulation of the animals. This study was conducted during May and June 2025, which is considered a breeding period for Chios sheep [30].

2.2. Semen Collection and Processing

Semen was collected with an artificial vagina. Soon after collection of each ejaculate, collection cups were placed in a 37°C water bath and transferred to the laboratory to be evaluated. Only semen samples that met standard criteria (volume: >1 mL, concentration: $>2 \times 10^9$ spermatozoa/mL, mass motility: >4 , individual motility: $>70\%$, morphological abnormalities: $<10\%$) were included in the study. Equal volume from each ejaculate was then mixed to eliminate individual variation, and the pooled sample was further divided into three equal parts, following determination of sperm concentration. The aliquots were diluted with pre-warmed (37°C) soy lecithin-based semen extender (OviXcell, IVM Technologies, Saint Uuen sur Iton, L'Aigle, France), to reach a final concentration of approximately 400×10^6 sperm/mL. The dilution rate (ejaculate: extender) was 1:4. Subsequently, the aliquots were equilibrated for 2 h at room temperature (25°C) before the assessment.

Semen collections were performed every 48 h throughout the experimental period. All collections were carried out under standardized and controlled conditions to ensure consistency and minimize potential variability affecting semen quality. Each semen collection was considered an independent replicate in the statistical analysis and was repeated 5 times ($n = 5$).

2.3. Crocin Preparation

A fresh stock solution of crocin (200 mM) (17304, Merck KGaA, Darmstadt, Germany) in water for embryo transfer (W1503, Sigma-Aldrich, Darmstadt, Germany) was prepared before the experiment.

2.4. Experimental Design

In each sampling, the semen pool was divided into 3 groups: one group served as a control, while the two others were supplemented with 10 μ L and 20 μ L of crocin's stock solution to reach a final concentration of 0.5 mM and 1mM crocin (grouped as CR 0.5 and CR 1), respectively. To maintain equal volumes across treatments, an equivalent volume of extender corresponding to the added crocin concentrations was removed from each crocin group prior to supplementation. The day of sampling was considered as Day 0. All tubes were kept at 5 °C for 72 h (Day 3) and aliquots were taken at 24 h intervals, warmed at 37 °C in a water bath for 15 min before evaluation.

2.4.1. Motility

Sperm motility and kinematic parameters were assessed using a computer-assisted sperm analyzer (CASA) system. The CASA system consisted of an optical phase-contrast microscopy system (Nikon Eclipse C1; Nikon, Tokyo, Japan), a warming plate (Tokai, Tokyo, Japan) at 37 °C, a Basler Scout CCD digital camera (Basler Vision Technologies, Ahrensburg, Germany), and a computer (Dell, Austin, TX, USA) using Integrated Semen Analysis System Software version 1 (ISAS MvCo, Valencia, Spain). The samples were analyzed in terms of motility, following the dilution of the sample with the extender to reach a concentration of 50×10^6 spermatozoa/mL [31], to minimize the occurrence of collisions between the sperm cells, as well as the inclusion of debris [32]. A pre-warmed slide was loaded with 10 μ L of the diluted semen sample, and 5 non-consecutive, randomly selected microscopic fields per sample were recorded.

The following sperm characteristics were analyzed: spermatozoa (%) with rapid, total (sum of rapid, medium, and slow), and progressive motility, curvilinear velocity (VCL, $\mu\text{m s}^{-1}$), straight linear velocity (VSL, $\mu\text{m s}^{-1}$), average path velocity (VAP, $\mu\text{m s}^{-1}$), and average lateral head displacement (ALH, μm). The CASA settings were as follows: 25 frames were captured with a rate of 50 frames per second. The medium cell size was 5 pixels and the minimum contrast was 80. Spermatozoa with progressive motility have a cutoff value of 50 $\mu\text{m/s}$ for VAP and 80% for progressive straightness. Spermatozoa were recorded as static with a VAP cutoff < 5 $\mu\text{m/s}$, slow with a VAP cutoff of 10 $\mu\text{m/s}$, and medium with a VAP cutoff of 50 $\mu\text{m/s}$.

2.4.2. Viability

Sperm viability was assessed using a one-step eosin-nigrosin staining technique [33]. In brief, equal volumes (10 μ L) of semen samples and pre-warmed stain were mixed in an Eppendorf tube and incubated for 5 min in a water bath at 37 °C. Ending incubation, 10 μ L of the mixture was smeared on a preheated microscope slide. The smears were dried on a heating plate. A total of 200 spermatozoa per slide were examined microscopically ($\times 1000$) to evaluate viability (NikonEclipse C1, Nikon, Tokyo, Japan). Alive spermatozoa appeared white, whereas dead spermatozoa had a pink-to-purple color, due to the penetration of the stain because of compromised cell membrane integrity.

2.4.3. Determination of Intracellular Glutathione Levels

Spermatozoa (20×10^6) were washed with PBS (phosphate-buffered saline, pH 7.4) and centrifuged at $300 \times g$ for 10 min (25 °C) to remove the antioxidant compounds. The

pellet was resuspended in 670 μL phosphate buffer (67 mM KH_2PO_4 , 67 mM Na_2HPO_4 , pH 8) and subjected to two cycles of sonication at 28 kHz for 60 s, each. The lysates were incubated with 0.33 mM DTNB [5,5'-dithiobis (2-nitrobenzoic acid)] for 60 min at RT, in the dark. Following centrifugation, the absorbance was measured at 412 nm with a spectrophotometer (Pharmacia LKB-Novaspec II, Northwich, Cheshire, UK) [24].

2.4.4. SDS-PAGE/Immunoblot and Dot Blot Analysis

Following washing, spermatozoa (25×10^6) were homogenized in 50 μL of ice-cold lysis buffer [20 mM β -glycerophosphate, 50 mM NaF, 2 mM EDTA, 20 mM Hepes, 0.2 mM Na_3VO_4 , 10 mM benzamidine, pH 7, 200 μM leupeptin, 10 μM trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane, 5 mM dithiothreitol, 300 μM PMSF, 50 $\mu\text{g mL}^{-1}$ pepstatin, and 1% *v/v* Triton X-100]. After extraction on ice for 30 min, the samples were centrifuged at $10,000 \times g$ for 10 min at 4 °C. Protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Supernatants were combined with sample buffer (330 mM Tris-HCl, 13% *v/v* glycerol, 133 mM DTT, 10% *w/v* SDS, 0.2% *w/v* bromophenol blue) at a 3:1 ratio (*v/v*) and fully denatured by heating at 100 °C.

Hsp70, Bax, Bcl-2, and β -actin levels were assessed following standard SDS-PAGE and immunoblotting procedures. Equal protein amounts (50 μg) were loaded onto 10% (*w/v*) acrylamide/0.275% (*w/v*) bisacrylamide gels, electrophoresed, and transferred to 0.45 μm nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Membranes were blocked for 30 min at room temperature with 5% (*w/v*) non-fat milk in TBST [20 mM Tris-HCl pH 7.5, 137 mM NaCl, 0.1% (*v/v*) Tween 20]. Primary antibodies were applied at the manufacturer-recommended dilutions: anti-Hsp70 (H5147, Sigma, Darmstadt, Germany), anti-Bcl-2 (2872, Cell Signaling, Beverly, MA, USA), anti-Bax (B-9) (2772, Cell Signaling, Beverly, MA, USA), and anti- β -actin (3700, Cell Signaling, Beverly, MA, USA), used as a loading and transfer control.

Following three 5 min washes in TBST, membranes were incubated with HRP-conjugated secondary antibodies, washed again (3×5 min), and visualized by enhanced chemiluminescence (Chemicon, Buena Park, CA, USA). Signal intensities were quantified by laser scanning densitometry using GelPro Analyzer Software version 3.0.00.00 (GraphPad, San Diego, CA, USA).

2.5. Statistical Analysis

Data were analyzed using a linear mixed-effects model (LMM) to account for repeated measures and group-specific variability with crocin concentration, exposure time (days), and their interaction as fixed effects. Estimated marginal means (EMMs) with corresponding 95% confidence interval (CI) were calculated, and a Tukey-adjusted post hoc test was used for all pairwise contrasts. Statistical analyses were performed using Python 3.10 via the Anaconda platform and the Jupyter Notebook 6.4.5 (referred to as Python). Statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Motility

Figure 1 depicts the percentages of spermatozoa with rapid (RM), total (TM), and progressive motility (PM). RM progressively decreased during storage at 5 °C from Day 0 to Day 3. Crocin supplementation significantly ($p \leq 0.05$) increased RM levels compared to its respective control of the same day. Nevertheless, these levels were lower to the initial control of Day 0 on Day 2 and Day 3 at both crocin concentrations [0.5 mM (CR 0.5) or 1 mM (CR 1)]. No differences ($p > 0.05$) were observed between the two examined concentrations on Day 1 and Day 3, while on Day 2, CR 0.5 (EMM: 68.56, 95% CI: 63.94–73.62) exhibited

significantly (diff: 9.8, $p = 0.0027$) higher rapid motility levels compared to the CR 1 group (EMM: 58.68, 95% CI: 53.61–63.47). The LMM revealed a significant main effect of exposure time ($p < 0.001$), concentration $\times$ exposure time interaction ($p < 0.001$), whereas no main effect was observed for concentration ($p > 0.05$).

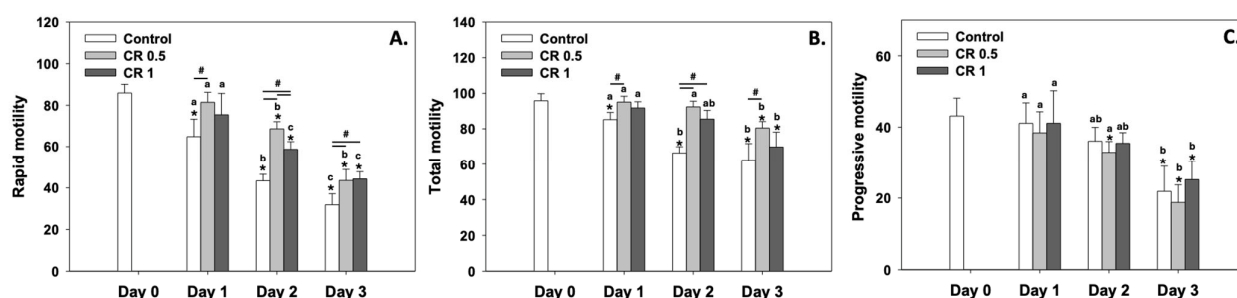


Figure 1. Effect of crocin on rapid (A), total (B), and progressive (C) motility in ram spermatozoa stored for 1, 2, or 3 days at 5 °C. Data are presented as mean % values of spermatozoa with rapid motility (A) and % of spermatozoa with motility (B) or progressive motility (C). Error bars correspond to S.D. ($n = 5$). Statistically significant differences compared to control (day 0) are denoted by asterisk (*), whereas differences between experimental groups of the same day are denoted by hashtag (#). Lower case letters denote statistically significant changes of each parameter between different days for each group ($p \leq 0.05$).

A similar pattern was observed regarding TM. However, on Day 1 and Day 2, CR 1 group exhibited no significant ($p > 0.05$) differences compared to the respective control group of the same day, while on Day 3, both concentrations of crocin exhibited significantly higher TM levels compared to the respective control group (for control EMM: 60.13, 95% CI: 55.36–64.69; for CR 0.5 EMM: 80.48, 95% CI: 75.81–85.14; for CR 1 EMM: 69.80, 95% CI: 65.13–74.46; control vs. CR 0.5 diff: -7.5 , $p = 0.024$; control vs. CR 1 diff: 10.68 , $p = 0.0028$) (Figure 1B). The LMM revealed a significant main effect of exposure time ($p < 0.001$), concentration $\times$ exposure time interaction ($p < 0.001$), whereas no main effect was observed for concentration ($p > 0.05$).

A different pattern was observed regarding PM, which gradually decreased towards Day 3 in all experimental groups (control, CR 0.5 and CR 1) being significantly ($p \leq 0.05$) lower on Day 3 compared to Day 0 (for control day 0 EMM: 43.12, 95% CI: 37.79–48.44; for CR 0.5 EMM: 18.78, 95% CI: 13.15–24.185; for CR 1 EMM: 69.80, 95% CI: 65.13–74.46). The LMM revealed a significant main effect of exposure time ($p < 0.001$), concentration $\times$ exposure time interaction ($p < 0.001$), whereas no main effect was observed for concentration ($p > 0.05$).

The effect of crocin supplementation on CASA kinematic parameters is presented in Table 1. All kinematic parameters progressively decreased as the time in storage at 5 °C increased, with the most pronounced reduction observed during Day 3 of storage. Statistically significant ($p \leq 0.05$) differences were detected on Day 3 between the control group and the crocin-treated groups for the parameters.

VCL: for control EMM: 66.90, 95% CI: 58.79–75.01; for CR 0.5 EMM: 91.92, 95% CI: 79.01–104.82; for CR 1 EMM: 86.90, 95% CI: 76.09–96.81; control vs. CR 0.5 diff: -20.00 , $p = 0.011$; control vs. CR 1 diff: 5.02 , $p = 0.039$; the LMM revealed a significant main effect of exposure time ($p < 0.001$), concentration $\times$ exposure time interaction ($p < 0.001$), whereas no main effect was observed for concentration ($p > 0.05$).

Table 1. CASA kinematic parameters* of ram spermatozoa stored for 1, 2, or 3 days at 5 °C, in the presence of 0.5 mM (CR 0.5) or 1 mM (CR 1) crocin. Values constitute means $\pm$ S.D. ($n = 5$). Statistically significant differences compared to control (Day 0) are denoted by asterisk (*), whereas differences between experimental groups of the same day are denoted by hashtag (#). Lowercase letters denote statistically significant changes of each parameter between different days for each group ($p \leq 0.05$).

Day	Group	VCL ($\mu\text{m s}^{-1}$)	VSL ($\mu\text{m s}^{-1}$)	VAP ($\mu\text{m s}^{-1}$)	ALH (μm)
0	Control	143.60 $\pm$ 16.44	33.66 $\pm$ 6.27	70.33 $\pm$ 10.48	6.58 $\pm$ 0.93
1	Control	121.9 $\pm$ 13.10 ^a	29.48 $\pm$ 5.52 ^a	59.78 $\pm$ 7.62 ^a	5.24 $\pm$ 0.34 ^a
	CR 0.5	124.82 $\pm$ 16.57 _a	32.82 $\pm$ 3.96 ^a	67.28 $\pm$ 8.74 ^a	5.88 $\pm$ 0.75 ^a
	CR 1	115.54 $\pm$ 13.10 _{*a}	28.56 $\pm$ 6.74 ^a	61.21 $\pm$ 16.52 ^a	5.52 $\pm$ 1.05 ^a
2	Control	104.72 $\pm$ 25.27 _{*a}	27.96 $\pm$ 5.72 ^a	52.84 $\pm$ 12.24 ^a	5.6 $\pm$ 0.95 ^a
	CR 0.5	106.94 $\pm$ 8.15 _{*ab}	30.52 $\pm$ 5.07 ^{ab}	56.38 $\pm$ 9.11 ^{ab}	5.44 $\pm$ 0.32 ^a
	CR 1	105.12 $\pm$ 12.63 _{*ab}	27.2 $\pm$ 6.5 ^a	57.68 $\pm$ 6.47 ^a	5.52 $\pm$ 0.45 ^a
3	Control	66.9 $\pm$ 2.20 ^{*b#}	17.42 $\pm$ 4.63 ^{*b}	32.42 $\pm$ 5.79 ^{*b#}	3.72 $\pm$ 0.66 ^{*b#}
	CR 0.5	89.92 $\pm$ 7.09 ^{*b}	24.68 $\pm$ 2.23 ^b	47.3 $\pm$ 4.70 ^{*b}	5.3 $\pm$ 0.37 ^a
	CR 1	86.9 $\pm$ 9.58 ^{*b}	22.4 $\pm$ 3.62 ^a	43.73 $\pm$ 5.11 ^{*b}	5.16 $\pm$ 0.36 ^a

Curvilinear velocity (VCL), straight linear velocity (VSL), average path velocity (VAP), and average lateral head displacement (ALH).

VAP: for control EMM: 32.40, 95% CI: 25.98–38.81; for CR 0.5 EMM: 47.30, 95% CI: 39.88–54.71; for CR 1: EMM: 43.70, 95% CI: 39.28–48.11; control vs. CR 0.5 diff: -14.90 , $p = 0.011$; control vs. CR 1 diff: -11.30 , $p = 0.039$; the LMM revealed a significant main effect of exposure time ($p < 0.001$), concentration $\times$ exposure time interaction ($p < 0.001$), whereas no main effect was observed for concentration ($p > 0.05$).

ALH: for control EMM: 3.72, 95% CI: 3.02–4.42; for CR 0.5 EMM: 5.30, 95% CI: 4.59–6.01; for CR 1 EMM: 5.16, 95% CI: 4.46–5.86; control vs. CR 0.5 diff: -1.58 , $p = 0.0001$; control vs. CR 1 diff: -1.44 , $p = 0.00033$; the LMM revealed a significant main effect of exposure time ($p < 0.001$), concentration $\times$ exposure time interaction ($p < 0.001$), whereas no main effect was observed for concentration ($p > 0.05$).

3.2. Viability

The percentage of alive spermatozoa gradually decreased, reaching the lowest levels on Day 3. On Days 1 and 3, no statistically significant ($p > 0.05$) differences were observed among the groups (Figure 2). However, on Day 2, both concentrations of crocin exhibited statistically significant ($p \leq 0.05$) higher viability rates compared to the respective control [for control EMM: 53.80, 95% CI: 51.08–56.51; for CR 0.5 EMM: 79.4, 95% CI: 76.68–82.11; for CR 1 EMM: 73.20% CI: 70.48–75.91; control vs. CR 0.5 diff: -25.6 , $p = 0.0001$; control vs. CR 1 diff: -19.4 , $p = 0.0001$; the LMM revealed a significant main effect of exposure time ($p < 0.001$), concentration $\times$ exposure time interaction ($p < 0.001$), whereas no main effect was observed for concentration ($p > 0.05$)].

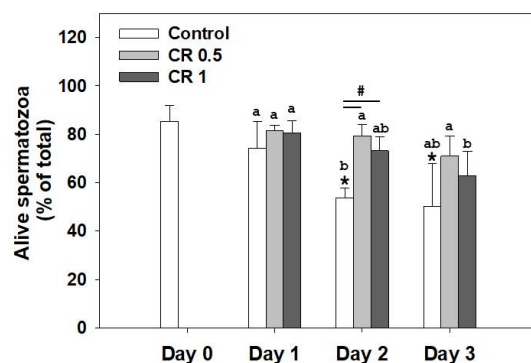


Figure 2. Alive ram spermatozoa (% of total) stored for 1, 2, or 3 days at 5 °C in the presence of 0.5 mM (CR 0.5) or 1 mM (CR 1) crocin. Values constitute means $\pm$ S.D. ($n = 5$). Statistically significant differences compared to control (Day 0) are denoted by asterisk (*), whereas differences between experimental groups of the same day are denoted by hashtag (#). Lowercase letters denote statistically significant changes of each parameter between different days for each group ($p \leq 0.05$).

3.3. Intracellular Glutathione Levels

Both concentrations of crocin (CR 0.5 and CR 1) prevented the depletion of GSH compared to their corresponding control, while crocin tended to upregulate the production of GSH (Figure 3). On Day 1, control (EMM: 0.0155, 95% CI: -0.0044 – 0.0362), CR 0.5 (EMM: 0.0622, 95% CI: 0.054 – 0.0715), and CR 1 (EMM: 0.086, 95% CI: 0.045 – 0.1255) groups exhibited the following: control vs. CR 0.5 diff: -0.046 , $p = 0.0026$; control vs. CR 1 diff: -0.071 , $p = 0.0069$.

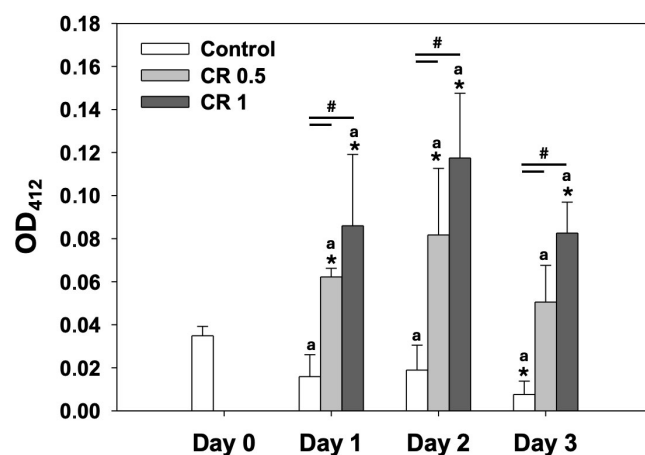


Figure 3. Reduction of DTNB radical levels to 2-nitro-5-thiobenzoate by GSH in ram spermatozoa stored for 1, 2, or 3 days at 5 °C, in the presence of 0.5 mM (CR 0.5) or 1 mM (CR 1) crocin. Values constitute means of O.D. at 412 nm $\pm$ S.D. ($n = 5$). Statistically significant differences compared to control (Day 0) are denoted by asterisk (*), whereas differences between experimental groups of the same day are denoted by hashtag (#). Lowercase letters denote statistically significant changes of each parameter between different days for each group ($p \leq 0.05$).

On Day 2, control (EMM: 0.0189, 95% CI: -0.0131 – 0.0247), CR 0.5 (EMM: 0.0817, 95% CI: 0.0662 – 0.0971), and CR 1 (EMM: 0.1175, 95% CI: 0.0125 – 0.13255) groups exhibited the following: control vs. CR 0.5: diff: -0.063 , $p = 0.0001$; control vs. CR 1: diff: -0.098 , $p = 0.00033$.

On Day 3, control (EMM: 0.0076, 95% CI: 0.0045 – 0.0106), CR 0.5 (EMM: 0.0505, 95% CI: 0.0419 – 0.0591), and CR 1 (EMM: 0.0826, 95% CI: 0.0754 – 0.0897) groups exhibited the following: control vs. CR 0.5 diff: -0.043 , $p = 0.0008$; control vs. CR 1: diff: -0.075 , $p = 0.0005$.

The LMM revealed a significant main effect of exposure time ($p < 0.001$), concentration $\times$ exposure time interaction ($p < 0.001$), whereas no main effect was observed for concentration ($p > 0.05$).

3.4. Heat Shock Response (HSR)

Hsp70 levels in ram spermatozoa were increased within 24 h at 5 °C, but returned to basal values within the next 24 h (Day 2), and they significantly ($p \leq 0.05$) decreased during the 3rd day of storage. The administration of crocin at both concentrations [0.5 mM (CR 0.5) or 1 mM (CR 1)] significantly increased Hsp70 levels compared to all control samples of Day 0, 1, 2, and 3. CR 1 resulted in significantly higher Hsp70 levels on Day 1 (EMM: 1.2723, 95% CI: 1.2345–1.3101) and Day 2 (EMM: 1.5405, 95% CI: 1.5027–1.5784) compared to CR 0.5 on Day 1 (EMM: 1.1399, 95% CI: 1.1021–1.1777) and Day 2 (EMM: 1.2726, 95% CI: 1.2347–1.3104), respectively (Day 1 CR 0.5 vs. CR 1 diff: -0.1323 , $p = 0.0001$; Day 2 CR 0.5 vs. CR 1 diff: -1.14 , $p = 0.0001$), while on Day 3, no statistically significant differences between concentrations were observed. The higher Hsp70 levels were detected on Day 2 at the concentration of 1 mM (Figure 4). The LMM revealed a significant main effect of concentration ($p > 0.01$), exposure time ($p < 0.001$), and concentration $\times$ exposure time interaction ($p < 0.001$).

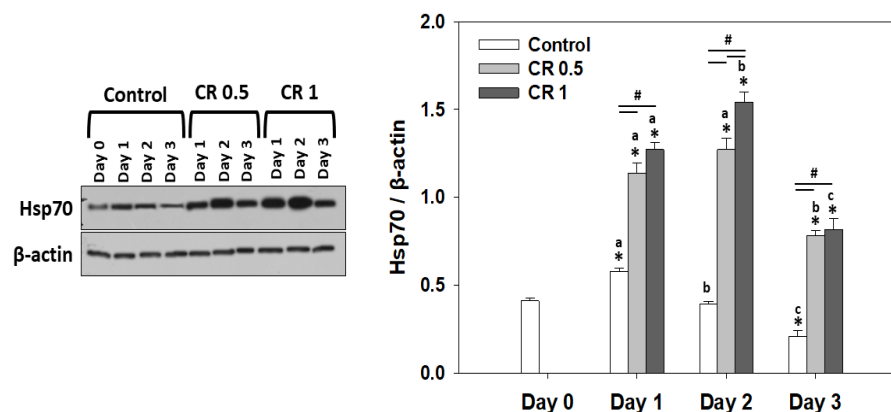


Figure 4. Hsp70 levels in ram spermatozoa stored for 1, 2, or 3 days at 5 °C in the presence of 0.5 mM (CR 0.5) or 1 mM (CR 1) crocin. Values constitute means $\pm$ S.D. ($n = 5$). Representative blots are shown. Statistically significant differences compared to control (Day 0) are denoted by asterisk (*), whereas differences between experimental groups of the same day are denoted by hashtag (#). Lowercase letters denote statistically significant changes of each parameter between different days for each group ($p \leq 0.05$).

3.5. Apoptosis

Figure 5 illustrates the effect of crocin on the apoptotic response of chilled ram spermatozoa during a 3-day storage at 5 °C. The levels of the pro-apoptotic protein Bax were increased after 2 days of storage and remained increased during the 3rd day (Figure 5B). Both concentrations of crocin (CR 0.5 EMM: 1.1874, 95% CI: 1.1397–1.2351; CR 1 EMM: 1.2113, 95% CI: 1.1636–1.2589) significantly (control vs. CR 0.5 diff: -0.6615 , $p = 0.0001$; control vs. CR 1 diff: -0.6854 , $p = 0.0001$) induced Bax levels on Day 1 compared to the respective control (EMM: 0.5258, 95% CI: 0.4782–0.57351), but they decreased to baseline levels on Day 2 (CR 0.5 EMM: 0.5027, 95% CI: 0.4551–0.5503; CR 1 EMM: 0.5622, 95% CI: 0.5145–0.6098) and Day 3 (CR 0.5 EMM: 0.4467, 95% CI: 0.3991–0.4944; CR 1 EMM: 0.4587, 95% CI: 0.4111–0.5064). The LMM revealed a significant main effect of concentration ($p > 0.01$), exposure time ($p < 0.001$), and concentration $\times$ exposure time interaction ($p < 0.001$).

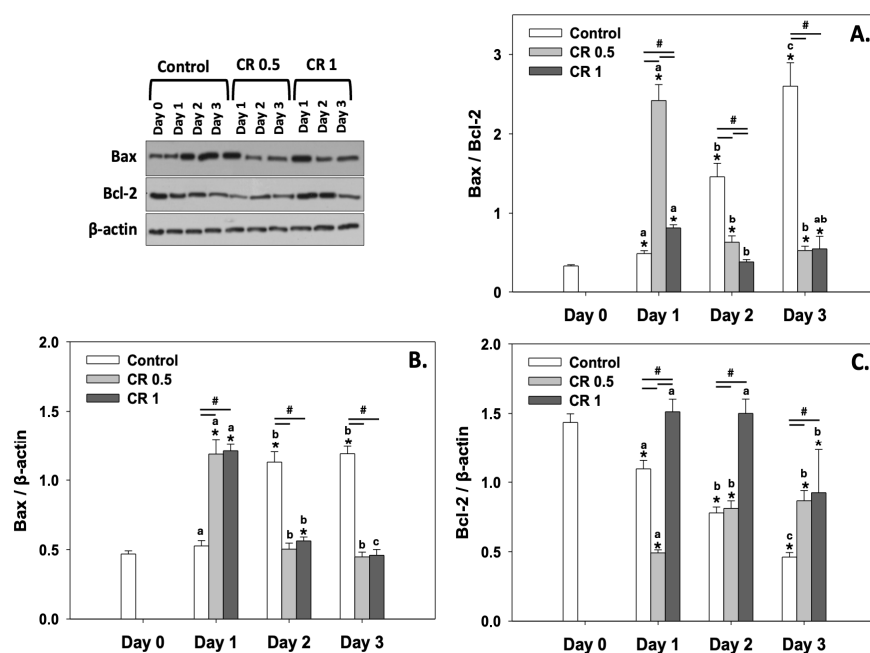


Figure 5. Bax/Bcl-2 ratio (A), Bax (B), and Bcl-2 (C) levels in ram spermatozoa stored for 1, 2, or 3 days at 5 °C, in the presence of 0.5 mM (CR 0.5) or 1 mM (CR 1) crocin. Values constitute means $\pm$ S.D. of $n = 5$. Representative blots are shown. Statistically significant differences compared to control (Day 0) are denoted by asterisk (*), whereas differences between experimental groups of the same day are denoted by hashtag (#). Lowercase letters denote statistically significant changes of each parameter between different days for each group ($p \leq 0.05$).

The Bcl-2 levels were progressively decreased in the control group towards Day 3. The low concentration of crocin CR 0.5 (EMM: 0.4909, 95% CI: 0.3895–0.5923) group significantly (diff: 0.6049, $p = 0.0001$) decreased Bcl-2 levels on Day 1 compared to the respective control (EMM: 1.0957, 95% CI: 0.9943–1.1971) (Figure 5C). However, the higher concentration of crocin (CR 1) retained Bcl-2 at levels similar to those observed on Day 0 for 2 days. Only on Day 3 were the levels of Bcl-2 significantly ($p \leq 0.05$) decreased in the CR 1 group compared to the control of Day 0. Both crocin concentrations retained higher levels of Bcl-2 compared to the respective control (Figure 5C). The LMM revealed a significant main effect of concentration ($p > 0.01$), exposure time ($p < 0.001$), and concentration $\times$ exposure time interaction ($p < 0.001$).

The fate of the cell (survival or apoptosis) is determined by the Bax/Bcl-2 ratio. In the present study, the Bax/Bcl-2 ratio gradually increased in the control group during the 3-day storage at 5 °C, indicating the induction of apoptosis. Interestingly, on Day 1, both crocin concentrations (CR 0.5 EMM: 2.4198, 95% CI: 2.3013–2.5383; CR 1 EMM: 0.8022, 95% CI: 0.6837–0.9207) significantly (control vs. CR 0.5 diff: -1.9391 , $p = 0.0001$; control vs. CR 1 diff: -0.3216 , $p = 0.0001$) increased the Bax/Bcl-2 ratio compared to the respective control (EMM: 0.4807, 95% CI: 0.3621–0.5991). However, on Day 2, this ratio decreased (CR 0.5 EMM: 0.6229, 95% CI: 0.5044–0.7415; CR 1 EMM: 0.3758, 95% CI: 0.2573–0.4943) at levels significantly (control vs. CR 0.5 diff: 1.0823 , $p = 0.0001$; control vs. CR 1 diff: 0.2471 , $p = 0.0036$) lower compared to those of the respective control (EMM: 1.4582, 95% CI: 1.3397–1.5767) and Day 3 (control EMM: 2.6007, 95% CI: 2.4822–2.7192; CR 0.5 EMM: 0.5191, 95% CI: 0.4005–0.6375; CR 1 EMM: 0.5399, 95% CI: 0.4214–0.6584; control vs. CR 0.5 diff: 2.0817 , $p = 0.0001$; control vs. CR 1 diff: 2.0608 , $p = 0.0081$). The LMM revealed a significant main effect of exposure time ($p < 0.001$), concentration $\times$ exposure time interaction ($p < 0.001$), whereas no main effect was observed for concentration ($p > 0.05$).

4. Discussion

AI in sheep presents significant limitations and technical difficulties. In addition to the anatomical peculiarities of the ewe's reproductive system, spermatozoa are particularly sensitive to low temperatures in this animal species [2,3,6]. Chilled ram semen is generally used within 24 h from collection to obtain acceptable pregnancy rates [10]. The fertilizing capacity of ram spermatozoa is retained during storage at 15 °C for up to 8–16 h, but prolonged preservation periods necessitate storage at 5 °C [10,34]. The latter requires the use of chemically defined, animal protein-free extenders, such as the soybean lecithin-based extenders, which have been employed for sperm cryopreservation in ruminants, such as bovine [35] and ovine species [36,37]. It has been suggested that these extenders either create a protective film around the cells [38], or the soy lecithin phospholipids may permeate into sperm membranes or replace membrane phospholipids [39]. Preserving sperm at 5 °C, on one hand, diminishes the risks for structural and functional damage of ram spermatozoa, and on the other hand, it offers many practical advantages [10,11]. Ram semen storage at 5 °C for more than one day allows breeders to maximize the potential for successful AI by extending the window of time that semen remains viable, offering flexibility in scheduling and maintaining high fertility rates. It also enables breeders to take advantage of animals with superior genetics without rushing insemination, and decreases the risk of missed opportunities due to timing or transportation issues [10].

Despite the cryoprotective properties of soybean lecithin-based extenders, prolonged storage of spermatozoa at 5 °C triggers ROS production, especially from dead spermatozoa [40]. As expected, and in line with previous studies [34,37,41,42], the present results depict a progressive decay of sperm motility and CASA kinematic parameters during sperm storage at 5 °C for 3 days. The discrepancies observed among these studies could be attributed to factors such as ram breed, animal nutrition, experimental setup, and extender type. In the present study, on Day 1, sperm viability, TM, and PM varied within the normal range suggested by the extender producer, but after 24 h, the viability declined significantly, with GSH depletion and Bax upregulation at Days 2 and 3. While these results indicate that the extender maintains the parameters mentioned above for 24 h, for longer periods of storage, the addition of a compound for the maintenance of spermatozoa fertilizing capacity seems to be necessary. Few studies have focused on the addition of antioxidants (e.g., trehalose, Trolox, GSH) to soybean lecithin extenders of chilled ram semen [42,43]. Following this rationale, chilled ram spermatozoa could benefit from the presence of crocin in the extender, as crocin has established antioxidant properties, and its impact on sperm physiology has been documented [22–24]. Although in a previous study no effect of crocin was identified on chilled canine semen [29], this could be attributed to species differences or to the different type of extender used (Tris-egg yolk). In the present study, crocin supplementation preserved RM and TM during short-term storage (up to Day 2). This can be attributed to the antioxidant properties of crocin, as indicated by the preserved glutathione levels. Crocin (0.5 mM) retained rapidly motile spermatozoa at levels significantly higher than those observed in the control group or the higher concentration of crocin (CR 1 group) on Day 2. On Day 3, both crocin concentrations had similar effects and preserved RM compared to the respective control on Day 3. Similar observations were identified for TM, which indicates that crocin may be most effective in the short-term (up to Day 2), and its efficacy may weaken after prolonged storage. However, the crucial factor of progressive motility was not affected by crocin. Furthermore, crocin maintained VCL, VAP, and ALH at Day 3. These CASA kinematic parameters have been correlated with fertility *in vivo* [44]; specifically, semen of high quality is characterized by significantly higher CASA parameters compared to semen of low quality [45]. VCL and VAP are positively correlated with the ability of spermatozoa to migrate through the cervical mucus in the ewe [46]. Moreover, the

maintenance of ALH due to crocin supplementation indicates preserved flagellar beating patterns and membrane functionality, which are critical for fertilization competence [47].

In terms of viability, the present results highlighted the fact that crocin supplementation at both concentrations significantly improved sperm viability up to Day 2, compared to control. However, viability levels declined over time, reaching their lowest on Day 3 in all experimental groups regardless of the presence of crocin. It should be underlined that although not statistically significant, viability rates were higher in the crocin-treated groups on Day 3 compared to the untreated control. The above are attributed to crocin's role as an antioxidant agent, which is also indicated by the elevated GSH concentrations [26]. GSH plays a crucial role in protecting spermatozoa, especially from oxidative stress [48]. Both crocin concentrations effectively preserved intracellular GSH levels, a fact which is in line with previously published data [24], emphasizing crocin's ability to modulate the antioxidant defense systems of ruminant spermatozoa.

These results are further supported by the effect of crocin on the Bax/Bcl-2 ratio, a key determinant of sperm survival [49]. Notably, this effect became evident after Day 2 of storage, when the CR 1 group consistently exhibited the lowest Bax/Bcl-2 ratio compared to the control. This suggests that this concentration may protect spermatozoa from apoptosis during the later stages of liquid storage, and verifies that crocin exerts an anti-apoptotic effect similar to its effect on cryopreserved bovine spermatozoa [24].

A very interesting observation is that after 24 h of storage, the CR 0.5 group exhibited higher Bax/Bcl-2 ratio, although the motility parameters and viability were not affected. A possible explanation for this is that cooling can generate a mild and transient stimulus (possibly through ROS accumulation), which can act as a "warning" signal that activates several early adaptive and protective stress responses, such as a significant increase in intracellular Hsp70 levels. Hsp70 is a key modulator of HSR, providing protection against cold stress. Crocin reinforces this protection by further inducing Hsp70 levels in chilled spermatozoa. Therefore, while in the control group, Hsp70 was reduced and the Bax/Bcl-2 ratio gradually increased by Days 2 and 3, in the crocin-treated groups, Hsp70 levels increased, and the Bax/Bcl-2 ratio decreased significantly. Bax expression was suppressed, and GSH and Hsp70 remained consistently higher, indicating that the initial stimulus induced a beneficial adaptive adjustment of cellular homeostasis. The early increase in Bax expression does not necessarily reflect an irreversible progression towards apoptosis, since the phenomenon is much more complex. The early, transient elevation of Bax likely may reflect an acute mitochondrial stress response induced by cold storage, potentially associated with early redox imbalance and membrane perturbation. This upregulation could be consistent with a reversible and adaptive signaling event associated with the initial cellular response to a stressor [49]. We can hypothesize that as crocin progressively attenuated oxidative stress and stabilized mitochondrial function, Bax expression declined on Day 2, probably indicating suppression of the pro-apoptotic signaling. This temporal modulation may suggest that crocin acts upstream of mitochondrial apoptotic pathways, and may limit progressive cellular deterioration during cooling. This pattern of an early, sub-threshold stress signal followed by sustained enhancement of cytoprotective pathways is characteristic of a hormetic response, which could apply to the present results [50]. However, the underlying molecular mechanism remains to be further investigated. To the best of our knowledge, this is the first study to examine the effect of crocin on Hsp70 expression in mature ram spermatozoa. Hsp70 is closely related to the fertilizing capacity in water buffalo [16] and bovine spermatozoa [24,51]. This finding also underscores that direct supplementation of Hsp70 into the freezing medium is a compelling alternative strategy and is worth investigating. In conclusion, crocin not only augmented cellular

antioxidant defenses, but also enhanced the cellular stress response, particularly during short-term preservation (up to Day 2).

This study has certain limitations that should be acknowledged. The pooling strategy applied during sample processing may have masked individual male-level variation. In addition, the absence of direct fertility endpoints, such as artificial insemination (AI) or in vitro fertilization (IVF) outcomes, limits the assessment of functional reproductive performance. Finally, the evaluation of apoptosis and oxidative stress was based on specific molecular markers, which may not fully capture the complexity of the underlying cellular processes.

5. Conclusions

The present findings provide preliminary evidence that crocin may exert protective effects on ram semen during chilled storage by enhancing antioxidant defenses, preserving HSR, and modulating apoptotic pathways. These effects appear to be mediated, at least in part, through increased GSH levels, which may contribute to limiting ROS accumulation. Both tested concentrations (0.5 mM and 1 mM) were associated with significant maintenance of sperm motility, viability, and antioxidant status; however, concentration-dependent differences were observed. The 0.5 mM concentration appeared particularly effective in maintaining motility—especially rapid motility on Day 2—indicating that lower doses may be sufficient to preserve short-term functional parameters. In contrast, the 1 mM concentration showed higher expression levels of Hsp70 and Bcl-2 on Days 1 and 2, suggesting that the higher concentration may more effectively stimulate cellular stress response and anti-apoptotic mechanisms, particularly on Day 3, exhibiting a longer cytoprotection. Overall, from the obtained results, while the 1 mM concentration seems more promising, further studies are required to optimize crocin dosage and storage conditions (e.g., temperature), as well as to evaluate acrosomal integrity and in vivo fertility outcomes, including conception rates in rams.

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Abbreviations

The following abbreviations are used in this manuscript:

AI	Artificial Insemination
ALH	Average Lateral Head displacement
CASA	Computer-Assisted Sperm Analyzer
CAT	Catalase
DTNB	[5,5'-dithiobis (2-nitrobenzoic acid)
GSH	Reduced Glutathione
Hsp	Heat Shock Protein
HSR	Heat Shock Response
IVF	In Vitro Fertilization
LAI	Laparoscopic Intrauterine Insemination
PBS	Phosphate-Buffered Saline
PM	Progressive Motility
PUFA	Polyunsaturated Fatty Acid
ROS	Reactive Oxygen Species
RM	Rapid Motility
TM	Total Motility
VAP	Average Path Velocity
VCL	Curvilinear velocity
VSL	Straight Linear Velocity

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