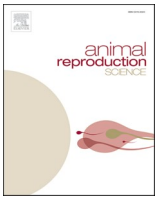
Animal Reproduction Science 259 (2023) 107374

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci



Effect of crocin supplementation in the extender on the quality of chilled canine semen

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ARTICLE INFO

Keywords:
Antioxidant
Spermatozoa
Semen storage
Dog

ABSTRACT

The aim of the study was to evaluate the effects of crocin on canine sperm quality parameters during prolonged storage at 4 °C. Ejaculates from 10 dogs were diluted in a TRIS- egg yolk extender supplemented with 0 (control group), 0.5, 1, and 2 mM crocin and stored at 4 °C. Sperm membrane functional integrity, motility, and kinetics were assessed after 3 h, 24 h, 4 days and 7 days of storage. Based on the results, the more efficient concentration of crocin (0.5 mM) was chosen to evaluate sperm intracellular ROS levels, lipid peroxidation, and DNA fragmentation vs. the control. Semen with the addition of 0.5 mM crocin with respect to the control exhibited: i) increased ($P < 0.05$) sperm membrane functionality at 4 and 7 days of storage; ii) higher ($P < 0.05$) average path (VAP), straight-line velocities (VSL), and beat cross frequency (BCF) at 4 d of storage at 4 °C; iii) decreased ($P < 0.05$) intracellular ROS levels after 3 and 24 h storage. No differences in lipid peroxidation and DNA fragmentation were recorded between the control and C0.5 groups at any time point. Lipid peroxidation did not increase over time, while DNA fragmentation increased ($P < 0.05$) in both groups after 4 days of storage. The results demonstrated that the enrichment of extender with crocin improves to a certain extent canine semen quality, particularly after 4 days of storage at 4 °C.

1. Introduction

In the last decades, there has been an increasing interest in artificial insemination (AI) in dogs, to overcome mating inability and improve breeding programs. Regarding the latter, the shipment of chilled or frozen semen allows the movement of semen from genetically superior dogs across countries, avoiding the risks of animal transportation and improving genetic variability. Both processing and shipping procedures are easier and cheaper with chilled compared to frozen semen whose transport requires expensive equipment (Verstegen et al., 2005). Moreover, vaginal insemination with chilled semen results in higher pregnancy rates and larger litter sizes compared to frozen semen, which requires intrauterine deposition (Linde-Forsberg, 2001, Pinto et al., 1999, Rota et al., 1995).

A major limiting factor of chilled semen is the limited lifespan of spermatozoa after prolonged storage, as quality deteriorates at

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https://doi.org/10.1016/j.anireprosci.2023.107374
Received 8 August 2023; Received in revised form 20 September 2023; Accepted 4 November 2023
Available online 8 November 2023
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increasing times, imposing the use of chilled semen within 4.9 days (England and Ponzio, 1996). It follows the importance of developing strategies to extend the lifespan of sperm during storage at 4 °C. It is known that the reduced sperm longevity is due to oxidative stress, resulting from increased production of reactive oxygen species (ROS) and reduced antioxidants (Aitken, 2017; Henkel, 2005; Silvestre et al., 2021). The presence of unsaturated fatty acid in the plasma membrane increases its elasticity but makes spermatozoa highly sensitive to the attack of free radicals during cooling (Tapia et al., 2012). Oxidative stress (OS) affects sperm motility, membrane fluidity, and DNA integrity, due to ROS-induced lipid peroxidation (Verstegen et al., 2005), and hence enrichment of the semen extender with antioxidants has been proposed to prevent OS during prolonged storage at 4 °C.

The spice saffron (*Crocus sativum*) has anti-inflammatory, anti-proliferative, and anti-apoptotic properties (Hashemzaei et al., 2020), mainly due to its known antioxidant function (Assimopoulou et al., 2005). Crocin, one of the carotenoids responsible for the antioxidant capacity of saffron, is known to protect cells from oxidative damage by scavenging ROS (Rahaiee et al., 2015). It has been reported that crocin protects spermatozoa from OS and consequent DNA damage in deer, ram and goat (Domínguez-Rebolledo et al., 2010, Longobardi et al., 2002; Mata-Campuzano et al., 2015). Furthermore, sperm incubation with crocin improved motility, viability, and membrane integrity, as well as blastocyst yields in cattle (Sapanidou et al., 2015).

To the best of our knowledge, the effects of crocin on dog semen have not yet been investigated. We hypothesized that the enrichment of semen extender with crocin could improve dog sperm quality during storage at 4 °C, through its antioxidant action. The aim of this study was to evaluate the effects of crocin on sperm quality parameters, such as motility, kinetics, and membrane functionality, as well as on sperm intracellular ROS levels, lipid peroxidation, and DNA fragmentation during prolonged storage at 4 °C.

2. Materials and methods

2.1. Experimental design

In Experiment 1, ejaculates from 10 dogs were split into four aliquots that were diluted in a TRIS-egg yolk extender supplemented with 0 (control group), 0.5, 1, and 2 mM crocin (C0.5, C1, and C2 groups) and stored at 4 °C. The concentrations were chosen according to previous studies (Longobardi et al., 2020, 2021; Sapanidou et al., 2022). Sperm membrane functionality, motility, and kinetics were assessed after 3 h, 24 h, 4 days, and 7 days (4 d and 7 d) of storage.

Based on the results, in Experiment 2 the more efficient concentration of crocin (0.5 mM) was chosen to evaluate sperm intracellular ROS levels, lipid peroxidation, and DNA fragmentation compared to the control. To do so, 10 ejaculates from the same dogs were diluted in the absence (control) and presence of 0.5 mM crocin and stored for up to 4 d. The analyses were carried out at 3 h, 24 h, and 4 d.

2.2. Animals

Semen samples were collected from 10 dogs of the "FOOF" breeder center located in the province of Caserta (Italy) through their routine practice in the framework of breeding programs. The study included 9 small breed dogs (2 French bulldogs, 1 Jack Russel, 1 Pug, 1 Shih Tzu, 1 Poodle, and 1 Cavalier King Charles Spaniel) and 1 large breed dog (Golden Retriever), with ages ranging from 1.5 to 8 years (median age was 6). Dogs received a standard commercial dog food twice daily and water ad libitum. All dogs received routine deworming treatments and vaccinations and shared the same environment for at least six months before the study.

2.3. Semen collection and processing

In both experiments, semen collection (n = 10 per each experiment) was performed with an artificial vagina. All ejaculates collected were white and milky in consistency. Raw semen was evaluated for volume, color, and concentration using a Burker's counting chamber. The volume of sperm-rich fraction was 3.1 ± 1.2 (mean ± SD) mL and sperm concentration was 211 ± 56 × 10^6 sperm/mL. Only ejaculates with ≥ 70% motility and ≥ 70% morphologically normal spermatozoa were included.

Each ejaculate was split into aliquots that were diluted to reach a final concentration of 100 × 10^6 sperm/mL respectively in egg-yolk TRIS-citrate glucose (EYT-G: Tris 2.4 g, Citric Acid 1.4 g, Glucose 0.8 g, Penicillin G Sodium Salt 0.06 g, Streptomycin 0.1 g, 20 mL of egg yolk and distilled water to 100 mL), i.e., the control group, and in YET-G supplemented with different concentrations of crocin according to the experimental design. All aliquots were placed in a syringe without air, transported to the laboratory at 4 °C within 3 h, stored at 4 °C and analyzed at different time points according to the experimental design.

2.4. Membrane functionality (Hypo-Osmotic Swelling Test)

The hyposmotic swelling test (HOST) was carried out at each time point to assess the functionality of the sperm plasma membrane in control and treated groups. Twenty microliters of semen were incubated at 37 °C for 45 min with 80 μL of pre-warmed HOST solution (0.73 g sodium citrate and 1.35 g fructose in 100 mL of distilled water, 150 mOsm). After incubation, a volume of 10 μL was placed on a glass slide and covered with a coverslip. Evaluations were conducted under phase-contrast microscopy (40 ×; Eclipse E200, Nikon, Tokyo, Japan) by operators unaware of the experimental design. The cells were classified as positive (damaged membrane) or negative (intact membrane) according to the presence or absence of coiled tails, respectively. A total of 200 spermatozoa were counted.

2.5. Motility assessment

Sperm motility parameters (total and progressive motility, sperm subpopulations, and semen kinetic parameters) were assessed by a Sperm Class Analyzer (SCA) system (Microptic SL, Veterinary Edition, Barcelona, Spain) installed on a camera-equipped light microscope system (Eclipse E200, Nikon, Tokyo, Japan). The following parameters were considered for the assessment: total motility (%), progressive motility (%), the percentage of sperm subpopulations (rapid and medium progressive), average path velocity (VAP; μm/s), straight-line velocity (VSL; μm/s), curvilinear velocity (VCL; μm/s), straightness (STR; %), linearity (LIN; %), amplitude of lateral head displacement (ALH; beats/s) and beat cross frequency (beats/s).

SCA system settings for dog semen classified as spermatozoa, all the particles sized between 10 and 80 μm^2, and as progressively motile spermatozoa those with 75% STR. The minimum velocity values considered for slow-medium and rapid spermatozoa subpopulations were 50 and 100 μm/s; spermatozoa with VCL below 10 μm/s were considered static and spermatozoa with VCL $>$ 150 μm/s and ALH $>$ 3.5 μm as hyperactive. Sixty frames per second with a minimum contrast of 35 were acquired.

For the evaluation, an aliquot of control or treated (C0.5, C1, and C2) semen at each time point was diluted 1:3 with TRIS-glucose-citrate in order to reach a concentration of 30×10^6 sperm/mL, as required by SCA system and incubated at 37 °C for 10 min before evaluation. Then, 5 μL were spotted onto a pre-warmed glass microscope slide, covered with a glass coverslip (22 mm × 22 mm). A minimum of five randomly selected microscopic fields with at least 100 sperm cells were evaluated in each sample, for a total of 500.

2.6. Intracellular ROS levels

Sperm intracellular H_2O_2 levels were assessed with the fluorescent probe 2′,7′-Dichlorofluorescein diacetate (DCHF-DA) as previously described (Benedetti et al., 2022). The sperm (control and 0.5 mM Crocin groups) were incubated with 10 μM DCFH-DA for 30 min in the dark at 37 ℃. After the incubation, 200 μL aliquots of these solutions were added to wells of a black-sided, clear-bottomed 96-well plate in replicates of three. The conversion of DCHF to the fluorescent product DCF was measured using a fluorescence spectrophotometer (GloMax®-Multi Detection System, Promega, Madison, WI) with excitation at 485 nm and emission at 535 nm. ROS production was quantified from a DCHF standard curve and results were expressed as the intensity of DCHF fluorescence (Arbitrary Units, A.U.).

2.7. Lipid peroxidation

Sperm lipid peroxidation of control and 0.5 mM crocin-treated samples was determined by assaying the Malondialdehyde (MDA) concentration using the thiobarbituric acid (TBA) test (Esterbauer and Cheeseman, 1990). To precipitate proteins, 100 μL of each sample was treated with 0.5 mL of cold 30% (w/v) trichloroacetic acid and centrifugated. One millimeter of supernatant was reacted with 1.3 mL of 0.5% (w/v) TBA at 85 °C for 40 min. In the TBA test reaction, each molecule of MDA reacts with two molecules of TBA with the production of a pink pigment having maximal absorbance at 532–535 nm. After cooling, the fluorescence was read at wavelengths of 536 nm for excitation and 557 nm for emission using a SPEX Fluoromax spectrophotofluorimeter (GloMax®-Multi Detection System, Promega, Madison, WI, USA). Concentrations of MDA were calculated using a calibration curve ranging between 0.5 and 2 pmol/mL and were expressed as nmol/L of proteins.

2.8. DNA fragmentation

The Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP Nick-End Labeling (TUNEL) assay was performed using the In Situ Cell Death Detection Kit (Roche Diagnostic, Mannheim, Germany) following the protocol previously described (Longobardi et al., 2020). Briefly, after fixation, cooled sperm (control and 0.5 mM Crocin groups) were permeabilized with 0.1% Triton X-100 containing 0.1% (w/v) sodium citrate for 10 min. Samples were then incubated with 50 μL TdT enzyme for 1 h at 37 °C in a dark and humidified atmosphere. After incubation, sperm were stained with bisbenzimide (Hoechst 33342; 1 mg/mL) for 30 min. Sperm cells were examined using a fluorescent microscope (Eclipse E-600; Nikon, Japan). The total sperm population was determined under blue fluorescence (DAPI filter, ex 330–380) revealing the nuclei of living or dead cells. Sperm with intense green fluorescence (FITC filter, ex 465–495) represent TUNEL-positive cells with DNA strand breaks in their nuclei. At least 200 spermatozoa of each sample were analyzed randomly to evaluate the percentage of TUNEL-positive sperm cells.

2.9. Statistical analysis

Data were first recorded using a computerized spreadsheet (Microsoft® Excel® 2021, Redmond, WA, USA) and then imported into Statistical Package for Social Sciences (SPSS IBM® Statistics version 27.0, IBM Corporation, Armonk, NY, USA) for statistical analysis. The normality of the data and the homogeneity of variances were assessed using the Shapiro-Wilk and Levene tests, respectively. Accordingly, parametric and non-parametric tests were used in Experiment 1 and 2, respectively. Data are expressed as mean ± Standard Error when normally distributed, and as median and interquartile ranges (IQR) when not normally distributed. For Experiment 1, the Wilcoxon signed-rank test was used for comparisons among groups (control, C0.5, C1, and C2) at each time point and among storage time points within each group. In experiment 2, data on sperm intracellular ROS levels, lipid peroxidation, and DNA fragmentation were analyzed by a multivariate ANOVA (general linear model) with time points and groups as fixed factors and post hoc comparisons were carried out by Least Square Difference (LSD). Differences were considered statistically significant when $P \leq$

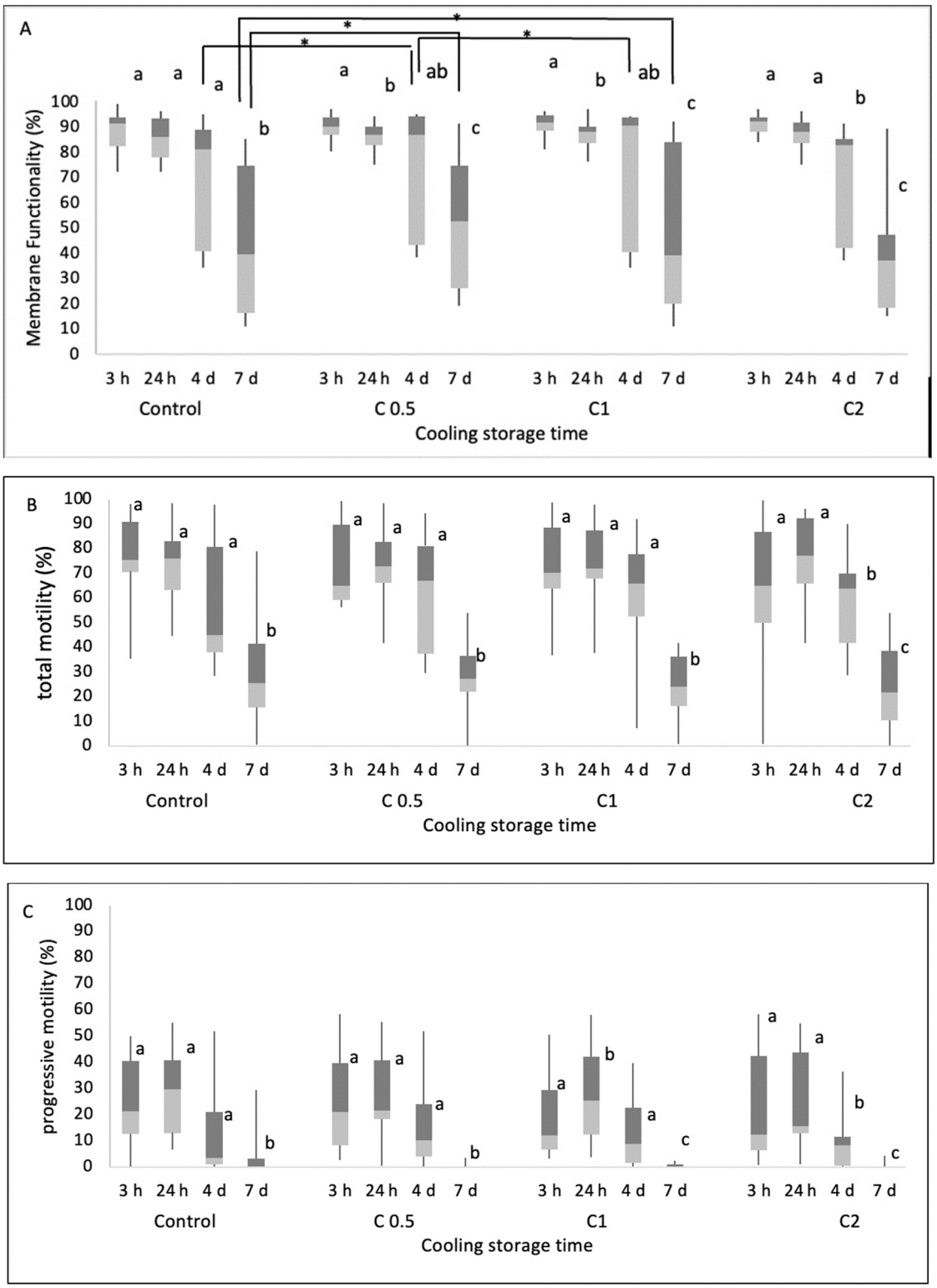

Fig. 1. Membrane functionality (A), total (B) and progressive motility (C) of canine semen (N = 10) diluted with egg-yolk tris-citrate glucose (YET-G; control) or with YET-G supplemented with 0.5, 1, and 2 mM of crocin (C0.5, C1 and C2) after storage at 4 °C for 3 h, 24 h, 4, and 7 days (4 d and 7 d). For each box, the central line represents the median, the edges represent the IQR (25th and 75th percentiles), the whiskers represent the extreme points. Asterisks indicate significant difference at $P \leq 0.05$ among groups within each time point. [a,b,c] Different letters indicate significant differences among time points within each group ($P < 0.05$).

0.05.

3. Results

3.1. Membrane functionality (HOST)

The results of sperm membrane functionality during storage time are shown in Fig. 1. A decrease ($P \leq 0.05$) of membrane functional integrity was observed at 7 days in all groups. Moreover, in the semen treated with 2 mM crocin (C2) a decrease ($P \leq 0.05$) was already recorded at 4 days. About the treatment effect, sperm membrane functionality was higher ($P \leq 0.05$) in C0.5 compared to both control and C1 groups at 4 days of storage. Furthermore, a higher ($P \leq 0.05$) proportion of sperm with functional intact membranes was recorded in the C0.5 and C1 groups compared to the control group after 7 days of storage. At any time point, no differences were detected between C2 and the other groups.

3.2. Sperm kinematics

Results of total and progressive motility are depicted in Fig. 1. No differences were observed in total and progressive motility among groups at any time point (Fig. 1). During storage at 4 °C, total and progressive sperm motility decreased ($P \leq 0.05$) at 7 days in the

Table 1
Trends of hyperactivated spermatozoa (%) and semen kinetic parameters of canine (n = 10) semen of control (CTRL) and Crocin groups (C0.5, C1 and C2) during storage at 4 °C for 7 days. Data are expressed as median and interquartile ranges (IQR).

Variables	*Storage time*			
	3 h	24 h	4d	7d
Hyperactivated(%)				
CTRL	2.9 (0–8)^{a}	2.5 (0–8.2)^{a}	0.3 (0–4.1)^{a}	0 (0–1.1)^{a}
C0.5	1.6 (0.2–11.4)^{a}	1.9 (0.6–4.1)^{a}	0.5 (0–4.3)^{a}	0 (0–0)^{b}
C1	2.5 (0.2–7.8)^{a}	3.5 (0–7.3)^{a}	0.9 (0–3.4)^{ab}	0 (0–0)^{b}
C2	2.2 (0.7–7.8)^{a}	2.6 (1.2–9.8)^{a}	0 (0–2.7)^{a}	0 (0–0.4)^{b}
VCL (μm/s)				
CTRL	73 (56.4–91.4)^{a,xy}	77.7 (55.4–128.6)$^{a,\ xy\ z}$	34.2 (19.3–91.2)$^{ab,\ xy}$	24.1 (17.3–73.2)^{b}
C0.5	84.2 (54.3–104.3)^{a,x}	70.5 (49.5–107.1)^{a,x}	65.5 (27.1–91.6)^{a,x}	19.8 (16.2–30.3)^{b}
C1	64.2 (40.7–95.2)^{a,y}	83 (63.3–122.7)^{b,y}	48.1 (26.9–71.8)^{a,y}	19.8 (17.4–42.2)^{c}
C2	68.7 (37.7–118.6)$^{a,\ xy}$	81.6 (48.3–116.8)^{b,xz}	44.4 (19.4–73.7)$^{a,\ xy}$	17.5 (10.3–21.6)^{c}
VSL (μm/s)				
CTRL	37.1 (26.4–49)^{a}	39.3 (34–73.8)$^{a,\ xy}$	13.7 (2.3–40.5)^{b,x}	6.4 (3.2–24.3)^{b,x}
C0.5	38 (20.2–57.5)^{a}	39.2 (24.8–57.3)$^{a,\ xy}$	26.8 (7.3–52.8)^{a,y}	2.2 (1.3–7.4)^{b,y}
C1	27.4 (21.5–43–1)^{a}	39.9 (29.3–57.6)^{b,x}	20.7 (8.4–29.3)$^{a,\ xy}$	3.3 (1.5–13.6)$^{c,\ xy}$
C2	34 (14.8–46)^{ab}	35.5 (24.7–46.2)^{a,y}	19.7 (3.4–29.7)$^{b,\ xy}$	1.7 (0.7–4.8)^{c,y}
VAP (μm/s)				
CTRL	49.2 (42.2–63.2)^{a}	50.9 (40–92.7)$^{a,\ xy}$	19.8 (5.7–52)^{b,x}	11.2 (6.9–41.2)^{b,x}
C0.5	53.2 (40.4–74.4)^{a}	48 (32–75.7)^{a,x}	36.6 (12.5–61.4)^{a,y}	6.2 (5.1–14.2)^{b,y}
C1	44.3 (28.2–61.2)^{a}	56.7 (43–84.5)^{b,y}	31.7 (13.8–41.8)$^{a,\ xy}$	6.8 (3.4–23.7)^{c,y}
C2	44.8 (24–81.2)^{a}	53 (33.2–75.9)^{a,x}	28.4 (6.7–44.9)$^{b,\ xy}$	6.3 (3.4–10.3)^{c,y}
LIN (%)				
CTRL	47.6 (40–54.7)^{a}	46.7 (41.4–55.4)^{a}	29.8 (14.9–41.5)^{b}	26.4 (17.3–33.2)^{b,x}
C0.5	47 (40–48.7)^{a}	41.8 (35.3–52.2)^{a}	35.8 (27.4–44.8)^{a}	14.2 (8.3 −24)^{b,y}
C1	42.2 (40.1–52.5)^{a}	46.3 (40.5–51.7)^{a}	33 (31.1–40.5)^{b}	17.3 (10–24.9)$^{c,\ xy}$
C2	39.8 (35.1–45.1)^{a}	41.5 (35.7–46.4)^{a}	26.4 (17.3–33.2)^{a}	9.9 (4.5–22.2)^{b,y}
STR (%)				
CTRL	66.4 (60–74)^{a}	70.1 (65.2–73.1)^{a}	53.8 (44.2–70.9)^{b}	48.6 (42.3–62.5)^{b,x}
C0.5	66.9 (61.2–71.2)^{a}	64.1 (55.5–72.1)^{a}	62.2 (49.8 −71.4)^{a}	48.6 (36.4–54.5)$^{b,\ xy}$
C1	65.4 (61–69.4)^{a}	68 (60–71)^{a}	60.2 (53.2–68.5)^{ab}	59.3 (44.3–57.1)^{b,x}
C2	63.4 (55.2–69.4)^{a}	65.9 (57.7–71.3)^{a}	60 (46.4–68.2)^{a}	33.4 (11.8–50.7)^{b,y}
BCF (beats/s)				
CTRL	8.8 (5.5–13.4)^{a,x}	10.8 (6.4–13.7)^{a}	3.8 (0.7–10.6)^{b,x}	2 (1–8.2)^{bb,x}
C0.5	9.1 (4.9–12.9)$^{a,\ xy}$	7.5 (5.6–13.2)^{a}	6.9 (2.2–11.2)^{a,y}	0.9 (0.6–2.5)$^{b,\ xy}$
C1	6.8 (4.2–12)^{a,y}	9.9 (6.8–14)^{b}	5.5 (2.6–10.4)$^{a,\ xy}$	0.9 (0.4–5)$^{c,\ xy}$
C2	7.8 (4–14.2)$^{a,\ xy}$	7.5 (4.7–13.6)^{a}	6 (0.8–11)$^{b,\ xy}$	0.8 (0.4–1.5)^{c,y}
ALH (beats/s)				
CTRL	1.8 (1.2–2.1)^{ab}	1.9 (1.4–2.3)^{a}	1.1 (0.8–2.3)$^{ab,\ xy}$	0.9 (0.7–1.9)^{b}
C0.5	1.9 (1.3–2.5)^{a}	1.7 (1.4–2.3)^{a}	1.8 (1–2.1)$^{a,\ x}$	0.8 (0.7–1.9)^{b}
C1	1.6 (1.3–2.3)^{a}	2 (1.6–2.7)^{a}	1.3 (0.9 −1.9)$^{a,\ y}$	0.8 (0.7–1.3)^{b}
C2	1.7 (1.3–2.6)^{a}	1.9 (1.3–2.7)^{a}	1.3 (0.8–1.7)$^{a,\ y}$	0.7 (0.5–0.8)^{a}

$^{a,\ b,\ c}$ Values with different superscripts within rows are significantly different; $P < 0.05$;
^{x,y} Values with different superscripts within columns are significantly different; $P < 0.05$.
Abbreviations: VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; BCF, beat cross frequency; ALH, amplitude of lateral head displacement.

control, C0.5 and C1 groups, while a reduction ($P \leq 0.05$) was already observed at 4 days in C2 group. Within the C1 group, progressive motility was higher ($P \leq 0.05$) after 24 h compared to the other times.

The results of kinetic parameters are shown in Table 1. The percentage of hyperactivated spermatozoa was similar among groups at all time points (Table 1). This parameter decreased at 7 d of storage in all groups even if the difference was not statistically different in the control group. No differences in VCL were detected between the control and treated groups. After 4 days of storage, the VCL was higher in C0.5 than in C1 ($P \leq 0.05$) and the control group but in the latter case, the difference was not significant ($P > 0.07$). In all groups, VCL dropped ($P < 0.05$) only after 7 days of storage. The semen treated with 0.5 mM crocin (C0.5) exhibited higher ($P < 0.05$) VSL and VAP than the control group ($P \leq 0.05$) at 4 d of storage at 4 °C. Extending storage to 7 d resulted in a decrease of VAP in all groups; all treated groups, however, had lower ($P < 0.05$) values than the control.

No differences in LIN and STR were observed among groups at 3 h, 24 h, and 4 d of storage, whereas differences ($P < 0.05$) were detected at 7 d, with values tendentially higher in the control, as shown in Table 1. In relation to storage time, however, both parameters decreased ($P < 0.05$) in the control group at 4 d compared to earlier times, whereas this decrease ($P < 0.05$) was only detected in the C0.5 group at 7 d. BCF was higher ($P < 0.05$) in C0.5 group compared to the control at 4 d, with intermediate values in the other treated groups. The temporal pattern of BCF was similar to that of LIN and STR, with high values preserved up to 4 d and 7 d, respectively in the C0.5 and the control groups. Finally, ALH was higher ($P < 0.05$) in C0.5 compared to C1 and C2 groups at 4 d.

3.3. ROS levels, lipid peroxidation, and DNA fragmentation

As shown in Table 2, 0.5 mM crocin decreased ($P < 0.05$) the sperm intracellular ROS levels after 3 and 24 h storage compared to the control, while no differences were detected after 4 d. In the control group, ROS levels did not change at increasing storage times, while in the C0.5 group an increase ($P < 0.05$) was registered at 4 d. No differences in lipid peroxidation, indicated by the MDA concentrations, were recorded between the control and C0.5 groups at any time points, and unexpectedly no increase in lipid peroxidation was observed at prolonged storage times.

Finally, the percentages of sperm with fragmented DNA, i.e. TUNEL+ , were similar in the control and C0.5 groups and increased ($P < 0.05$). only after 4 days.

4. Discussion

This study hypothesized that supplementation of the extender with crocin, an active constituent of saffron with antioxidant properties, would improve the quality of canine semen stored at 4 °C. The rationale behind this work originates from the evidence of OS occurring after prolonged refrigeration and of beneficial effects of crocin on sperm quality parameters reported in cattle, goats and buffalo (Longobardi et al., 2020, 2021; Sapanidou et al., 2022). To the best of our knowledge, this is the first study to assess the effects of crocin on chilled canine semen.

In order to evaluate the effects of crocin on sperm quality a dose-response trial was carried out, using concentrations (0, 0.5, 1 and 2 mM) previously tested (Longobardi et al., 2020, 2021; Sapanidou et al., 2022), showing that the most effective concentration for canine chilled semen was the lowest tested (0.5 mM), while the highest (2 mM) exerted in part a deleterious effect. The effects of the treatment were assessed at different times, such as 3 h, 24 h, 4 days, and 7 d. A deterioration of semen quality was recorded after 7 d of storage, the time at which sperm membrane functionality, total and progressive motility, as well as kinetic parameters, were significantly reduced in all groups. Meanwhile, the addition of the highest concentration of crocin (2 mM) to the extender caused a decrease of membrane functionality, total motility, and progressive motility earlier, i.e., at 4 d storage, indicating a potential toxic effect. The worsening of semen quality at prolonged storage time at 4 °C agrees with a previous study suggesting that chilled canine semen should be used for AI within 4.9 days (England and Ponzio, 1996). It has been previously shown that over extended storage at 4 °C spermatozoa switch from aerobic to anaerobic metabolism, due to oxygen consumption, and that the activation of glycolysis results in

Table 2
Sperm lipid peroxidation, measured as malondialdehyde (MDA) concentration, intracellular reactive oxygen species (ROS) levels and DNA fragmentation (TUNEL+ sperm), of canine semen (n = 10) during storage at 4 °C in the absence (control) and presence of 0.5 mM crocin for 4 days. Data are expressed as mean ± Standard Error (SE).

	Storage time		
	3 h	24 h	4 d
ROS (A.U.)			
Control	903.5 ± 116.5^{x}	967.0 ± 176.8^{x}	933.2 ± 100.0
C 0.5	$613.6 \pm 42.8^{y,\ a}$	578.0 ± 29.2^{ya}	1130.0 ± 133.8^{b}
MDA (mM)			
Control	5.2 ± 0.5	5.4 ± 0.4	5.1 ± 0.3
C 0.5	5.5 ± 0.4	5.2 ± 0.4	4.9 ± 0.4
TUNEL + (%)			
Control	2.9 ± 0.6^{a}	6.9 ± 1.4^{a}	19.1 ± 1.7^{b}
C 0.5	3.7 ± 0.5^{a}	6.0 ± 2.0^{a}	18.3 ± 2.5^{b}

$^{s,\ b}$ Values with different superscripts within rows are significantly different; $P < 0.05$
^{x,y} Values with different superscripts within columns are significantly different; $P < 0.05$

lactate production and hence reduced pH of the medium, leading in turn to decreased metabolism, ATP production and motility (Mann, 1964).

The most interesting findings of Experiment 1 regard the improvement of semen quality obtained with 0.5 mM crocin. Indeed, the addition of 0.5 mM crocin in the extender significantly increased sperm membrane functionality at both 4 and 7 d compared to the control group. Moreover, despite similar values of total motility and progressive motility, after 4 d of storage, most of the sperm kinetic parameters improved in the C0.5 group, compared to the control. Furthermore, in the C0.5 group, most of the kinetic parameters were preserved up to 4 d, whereas a decrease was already observed at this time in the control group. Motility is one of the most important indicators of the potential fertilizing ability of spermatozoa (Vijayaraghavan, 2003) and sperm kinetics is associated with fertility in various species (Broekhuijse et al., 2012; Marshburn et al., 1992).

It is known that sperm quality deteriorates at increasing storage times due to the occurrence of OS, resulting from an unbalance between ROS production and antioxidant systems (Silvestre et al., 2021). A correlation between OS and semen quality parameters has been demonstrated in dogs (Del Prete et al., 2018). Indeed, an improved semen quality of chilled canine semen has been previously obtained by supplementing extender with several antioxidants such as maca, lycopene, cysteamine, and vitamins (Del Prete et al., 2022; Michael et al., 2009; Sheikholeslami et al., 2020).

In this study, the enrichment of the extender with 0.5 mM crocin improved sperm membrane functionality and sperm kinetics after 4 d of storage at 4 °C. To evaluate whether the beneficial effect of crocin was related to its antioxidant properties, this concentration was used in Experiment 2 to assess intracellular ROS levels, lipid peroxidation, and DNA fragmentation up to 4 d of storage. The reduced intracellular ROS levels detected in the C0.5 group after 3 and 24 h storage of cooled canine semen are in line with the known ROS scavenger function of crocin (Singla and Bhat, 2011; Sapanidou et al., 2015). This effect was unexpectedly lost after 4 d of storage, when the improvement of semen quality traits was more evident. Furthermore, he reduced intracellular ROS levels detected at 3 and 24 h in the C0.5 group were not associated with either increased lipid peroxidation or decreased DNA fragmentation. Regardless of the treatment, prolonged cooling of canine semen to 4 d was associated with increased DNA fragmentation, while lipid peroxidation was not affected by storage time. It is worth noting that the MDA levels in spermatozoa were relatively low in all groups and hence, due to the limitation of the assay and the high variability, small differences among groups could not be detected.

There are several reports on the beneficial effects of crocin on semen quality in different domestic species, in most of which, though crocin was added to the semen extender before freezing rather than chilling, and hence, results cannot be compared. In the goat supplementation of crocin in the extender decreased OS, improving sperm motility and DNA integrity of frozen-thawed sperm (Longobardi et al., 2020). In cattle, crocin improved sperm viability, motility, and kinetic parameters after thawing but a reduction of lipid peroxidation was only observed after 2 h post-thawing incubation (Sapanidou et al., 2022). In another study the incubation of frozen-thawed buffalo semen with crocin improved sperm membrane integrity and decreased DNA fragmentation and ROS levels (Longobardi et al., 2021). Undoubtedly, OS is more severe after freezing-thawing than cooling processes (Chatterjee and Gagnon, 2001). Moreover, there are fundamental differences between those two processes: during cryopreservation, OS increases the rigidity in the hydrophobic portion of the sperm membrane and consequently the susceptibility to lipid peroxidation (Chatterjee and Gagnon, 2001). Beneficial effects of the antioxidant supplementation in semen extenders were observed during long storage periods, when the concentration of ROS dramatically increases (Silvestre et al., 2021). Based on our results, the improvement of membrane functionality and sperm kinetics observed in the C0.5 group after 4 d of storage is not associated with lower ROS levels, which decreased though during short-term storage; it is speculated that the antioxidant effect is lost over time due to reduced crocin bioavailability. Nevertheless, the improved protection from OS at early storage times seems to make spermatozoa more resistant during prolonged storage. Indeed, spermatozoa in the C0.5 group are exposed to high ROS levels for a shorter time. It would be interesting to evaluate whether re-exposing sperm to crocin during cooling would extend the beneficial effect on sperm quality beyond 4 d. The beneficial effects may also be related to other functions of crocin, such as the capability to increase intracellular detoxifying enzymes and to modulate membrane fluidity, leading to changes in its permeability to oxygen and other molecules (Assimopoulou et al., 2005).

5. Conclusions

In conclusion, we demonstrated that the enrichment of extender with crocin improves to a certain extent canine semen quality, particularly after 4 d of storage at 4 °C. At this time point crocin increased the percentage of sperm with intact membrane and most of the kinetic parameters. The treatment was not effective at further extending the lifespan of spermatozoa under chilling conditions, as shown by the deterioration of semen quality observed at 7 d. It was also demonstrated that crocin decreased sperm intracellular ROS levels at 3 and 24 h cooling, without affecting lipid peroxidation and DNA fragmentation. Further studies are required to further elucidate the mechanism of action of the compound, and validate these results, by assessing other fertility-associated parameters including fertilizing ability. Finally, from a future perspective, it would be interesting to assess the effect of crocin on the quality of frozen semen.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical statement

The experiment was conducted in accordance with the code of ethics (D.lgs. 26—04/03/2014), and it was approved by the Ethics

Committee of the Department of Veterinary Medicine and Animal Productions at the University of Naples Federico II, Italy (prot. no. PG/2021/0057934 of 07/06/2021).

CRediT authorship contribution statement

Alfonso Calabria, Chiara Del Prete: Conception and design, analysis, interpretation, data collection, writing – original draft. **Valentina Longobardi, Stefano Spada, Maria Teresa Alfano, Daniela De Felice**: Analysis, and interpretation, data collection. **Bianca Gasparrini**: Conceptualization, data analysis and interpretation, writing – review and editing. **Natascia Cocchia**: Conceptualization, Methodology, writing – review and editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

NA.

References

Aitken, R.J., 2017. Reactive oxygen species as mediators of sperm capacitation and pathological damage. Mol. Reprod. Dev. 84 (10), 1039–1052. https://doi.org/10.1002/mrd.22871.

Assimopoulou, A.N., Sinakos, Z., Papageorgiou, V., 2005. Radical scavenging activity of *Crocus sativus L.* extract and its bioactive constituents. Phytother. Res. 19 (11), 997–1000. https://doi.org/10.1002/ptr.1749.

Benedetti, S., Catalani, S., De Stefani, S., Primiterra, M., Fraternale, A., Palma, F., Palini, S., 2022. A microplate-based DCFH-DA assay for the evaluation of oxidative stress in whole semen. Heliyon 8 (9), e10642. https://doi.org/10.1016/j.heliyon.2022.e10642.

Broekhuijse, M.L.W.J., Šoštarić, E., Feitsma, H., Gadella, B.M., 2012. Application of computer-assisted semen analysis to explain variations in pig fertility. J. Anim. Sci. 90 (3), 779–789. https://doi.org/10.2527/jas.2011-4311.

Chatterjee, S., Gagnon, C., 2001. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. Mol. Reprod. Dev.: Inc. Gamete Res. 59 (4), 451–458. https://doi.org/10.1002/mrd.1052.

Del Prete, C., Ciani, F., Tafuri, S., Pasolini, M.P., Della Valle, G., Palumbo, V., Abbondante, L., Calamo, A., Barbato, V., Gualtieri, R., Talevi, R., 2018. Effect of superoxide dismutase, catalase, and glutathione peroxidase supplementation in the extender on chilled semen of fertile and hypofertile dogs. J. Vet. Sci. 19 (5), 667–675. https://doi.org/10.4142/jvs.2018.19.5.667.

Del Prete, C., Calabria, A., Longobardi, V., Palumbo, V., Merlo, B., Iacono, E., Tafuri, S., Carotenuto, D., Ciani, F., Damiano, S., Ciarcia, R., Cocchia, N., 2022. Effect of aqueous extract of maca addition to an extender for chilled canine semen. Animals 12 (13), 1638. https://doi.org/10.3390/ani12131638.

Domínguez-Rebolledo, Á.E., Fernández-Santos, M.R., Bisbal, A., Ros-Santaella, J.L., Ramón, M., Carmona, M., Martínez-Pastor, F., Garde, J.J., 2010. Improving the effect of incubation and oxidative stress on thawed spermatozoa from red deer by using different antioxidant treatments. Reprod. Fertil. Dev. 22 (5), 856–870. https://doi.org/10.1071/RD09197.

England, G.C.W., Ponzio, P., 1996. Comparison of the quality of frozen-thawed and cooled-rewarmed dog semen. Theriogenology 46 (1), 165–171. https://doi.org/10.1016/0093-691X(96)00151-3.

Esterbauer, H., Cheeseman, K.H., 1990. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. In Academic Press. Methods Enzymol. 407–421.

Hashemzaei, M., Mamoulakis, C., Tsarouhas, K., Georgiadis, G., Lazopoulos, G., Tsatsakis, A., Asrami, E.S., Rezaee, R., 2020. Crocin: A fighter against inflammation and pain. Food Chem. Toxicol. 143, 111521 https://doi.org/10.1016/j.fct.2020.111521.

Henkel, R., 2005. The impact of oxidants on sperm function. Andrologia 37 (6), 205–206. https://doi.org/10.1111/j.1439-0272.2005.00699.x.

Linde-Forsberg, C., 2001. Regulations and recommendations for international shipment of chilled and frozen canine semen. A1209.0501. In: Concannon, P.W., England, G.C.W., Verstegen, J. (Eds.), Recent Advances in Small Animal Reproduction. Intenational Veterinary Information Service, Ithaca. A1209.0501. http://www.ivis.org/.

Longobardi, V., Zullo, G., Cotticelli, A., Salzano, A., Albero, G., Navas, L., Rufrano, D., Claps, S., Neglia, G., 2020. Crocin improves the quality of cryopreserved goat semen in different breeds. Animals 10 (6), 1101. https://doi.org/10.3390/ani10061101.

Longobardi, V., della Valle, G., Iannaccone, F., Calabria, A., Di Vuolo, G., Damiano, S., Ciarcia, R., Gasparrini, B., 2021. Effects of the antioxidant crocin on frozen-thawed buffalo (*Bubalus bubalis*) sperm. In: Ital. J. Anim. Sci, 20, pp. 2095–2101. https://doi.org/10.1080/1828051X.2021.1997653.

Mann, T., 1964. Metabolism of semen: fructolysis, respiration, and sperm energetics. The Biochemistry of Semen and the Male Reproductive Tract. Wiley,, New York, USA, pp. 265–307.

Marshburn, P.B., McIntire, D., Carr, B.R., Byrd, W., 1992. Spermatozoal characteristics from fresh and frozen donor semen and their correlation with fertility outcome after intrauterine insemination. Fertil. Steril. 58 (1), 179–186. https://doi.org/10.1016/S0015-0282(16)55157-7.

Mata-Campuzano, M., Álvarez-Rodríguez, M., Álvarez, M., Tamayo-Canul, J., Anel, L., de Paz, P., Martínez-Pastor, F., 2015. Post-thawing quality and incubation resilience of cryopreserved ram spermatozoa are affected by antioxidant supplementation and choice of extender. Theriogenology 83 (4), 520–528. https://doi.org/10.1016/j.theriogenology.2014.10.018.

Michael, A.J., Alexopoulos, C., Pontiki, E.A., Hadjipavlou-Litina, D.J., Saratsis, P., Ververidis, H.N., Boscos, C.M., 2009. Effect of antioxidant supplementation in semen extenders on semen quality and reactive oxygen species of chilled canine spermatozoa. Anim. Reprod. Sci. 112 (1–2), 119–135. https://doi.org/10.1016/j.anireprosci.2008.04.007.

Rahaiee, S., Moini, S., Hashemi, M., Shojaosadati, S.A., 2015. Evaluation of antioxidant activities of bioactive compounds and various extracts obtained from saffron (*Crocus sativus L.*): a review. J. Food Sci. Technol. 52, 1881–1888. https://doi.org/10.1007/s13197-013-1238-x.

Rota, A., Ström, B., Linde-Forsberg, C., 1995. Effects of seminal plasma and three extenders on canine semen stored at 4C. Theriogenology 44 (6), 885–900. https://doi.org/10.1016/0093-691X(95)00278-G.

Sapanidou, V., Taitzoglou, I., Tsakmakidis, I, Kourtzelis, I., Fletouris, D., Theodoridis, A., Zervos, I., Tsantarliotou, M., 2015. Antioxidant effect of crocin on bovine sperm quality and in vitro fertilization. Theriogenology 84 (8), 1273–1282. https://doi.org/10.1016/j.theriogenology.2015.07.005.

Sapanidou, V., Lavrentiadou, S.N., Errico, M., Panagiotidis, I., Fletouris, D., Efraimidis, I., Zervos, I., Taitzoglou, I., Gasparrini, B., Tsantarliotou, M., 2022. The addition of crocin in the freezing medium extender improves post-thaw semen quality. Reprod. Domest. Anim. 57 (3), 69–276. https://doi.org/10.1111/rda.14049.

Sheikholeslami, S.A., Soleimanzadeh, A., Rakhshanpour, A., Shirani, D., 2020. The evaluation of lycopene and cysteamine supplementation effects on sperm and oxidative stress parameters during chilled storage of canine semen. Reprod. Domest. Anim. 55 (9), 1229–1239. https://doi.org/10.1111/rda.13770.
Silvestre, M.A., Yániz, J.L., Peña, F.J., Santolaria, P., Castelló-Ruiz, M., 2021. Role of antioxidants in cooled liquid storage of mammal spermatozoa. Antioxidants 10 (7), 1096. https://doi.org/10.3390/antiox10071096.
Singla, R.K., Bhat, G.V., 2011. Crocin: an overview. Indo-Glob. Res. J. Pharm. Sci. 1 (4), 281–286.
Tapia, J.A., Macias-Garcia, B., Miro-Moran, A., Ortega-Ferrusola, C., Salido, G.M., Pena, F.J., Aparicio, I.M., 2012. The membrane of the mammalian spermatozoa: much more than an inert envelope. Reprod. Domest. Anim. 47, 65–75. https://doi.org/10.1111/j.1439-0531.2012.02046.x.
Verstegen, J.P., Onclin, K., Iguer-Ouada, M., 2005. Long-term motility and fertility conservation of chilled canine semen using egg yolk added Tris–glucose extender: In vitro and in vivo studies. Theriogenology 64 (3), 720–733. https://doi.org/10.1016/j.theriogenology.2005.05.035.
Vijayaraghavan, S., 2003. Sperm motility: patterns and regulation. Introduction to Mammalian Reproduction. Springer, US, Boston, MA, pp. 79–91.