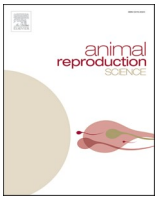
Animal Reproduction Science 274 (2025) 107787

Contents lists available at ScienceDirect

Animal Reproduction Science

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



Incorporating olive (*Olea europaea L*) fruit extracts in a tris-based extender improves buffalo semen cryotolerance by reducing oxidative stress

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ARTICLE INFO

Keywords:
Antioxidants
Frozen-thawed spermatozoa
Hydroxytyrosol
Olive-derived products
Buffalo

ABSTRACT

This work aimed to evaluate whether supplementing the freezing extender with olive fruit extract (OFE) would improve the antioxidant defense of buffalo sperm, resulting in improved post-thaw semen quality. Ejaculates (two per 16 Murrah buffalo bulls) were split into four aliquots that were diluted in an extender supplemented with different doses of OFE (0, D50, D100, and D150, based on μM concentrations of hydroxytyrosol, the most represented polyphenol) and frozen according to standard procedures. At thawing, sperm motility, kinetics, viability, acrosome integrity, and membrane functionality were evaluated. Based on the dose-response results, biological antioxidant potential (BAP) and reactive oxygen metabolites (ROMs) were assessed after thawing in D50 and control groups. The pre-freezing supplementation of the extender with D50 OFE showed higher ($P < 0.05$) total and progressive sperm motility, as well as straight-line velocity compared to the control. Treatment with D50 OFE of buffalo semen also improved ($P < 0.01$) post-thaw sperm viability, membrane functionality, and acrosome integrity compared to the control. The enrichment of the extender with D50 OFE increased ($P < 0.01$) the post-thaw BAP and reduced ($P < 0.05$) the ROMs levels. The highest concentration tested (D150 OFE) negatively affected ($P < 0.05$) total and progressive motility, and the percentage of sperm with functional membranes and intact acrosomes, compared to the control. In conclusion, low doses of OFE added to the extender significantly improved post-thawing buffalo semen quality by protecting the spermatozoa from cryopreservation-induced oxidative stress. Further studies should investigate its effectiveness on *in vivo* and *in vitro* fertility, for potential commercial applications.

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https://doi.org/10.1016/j.anireprosci.2025.107787
Received 20 November 2024; Received in revised form 22 January 2025; Accepted 23 January 2025
Available online 26 January 2025

1. Introduction

The world buffalo population has steadily increased over the years, due to its economic relevance as an animal protein producer, particularly in tropical countries (Zicarelli, 2016). In the current scenario, the profitability of buffalo breeding depends on genetic improvement, which can be achieved through a wider application of advanced reproductive technologies. Semen cryopreservation, by ensuring long-term storage and transport of sperm, is pivotal to planning artificial insemination and in vitro embryo production programs., Cryopreservation, however, results in a reduction in sperm fertility, including decreased viability and motility, as well as capacitation-like changes (Andrabi et al., 2001; Rasul et al., 2001; Elkhawagah et al., 2014).

During cryopreservation, cold and osmotic shock can lead to physical damage to sperm cells due to ice crystal formation and oxidative stress (OS) (Khan et al., 2021). Buffalo sperm are highly sensitive to cryopreservation-induced OS due to the lipid composition of the sperm membrane, i.e., the lower cholesterol: phospholipids ratio and high concentration of long-chain polyunsaturated fatty acids (Andrabi, 2009; Rajoriya et al., 2016; Longobardi et al., 2021). It is known that cryopreservation leads to excessive ROS production, and reduces the semen antioxidant defense, as indicated by the decreased superoxide dismutase (Len et al., 2019; Hungerford et al., 2023). The supplementation of the freezing extender with antioxidants such as cysteine, glutamine (Topraggaleh et al., 2014), sericin (Kumar et al., 2015), taurine, trehalose (Reddy et al., 2010), as well as carnitine and resveratrol (Longobardi et al., 2017a; 2017b) has been reported toimprove buffalo sperm cryotolerance, by reducing OS during cryopreservation.

Recently, the focus of research has moved to using natural products rich in substances with antioxidant activity, such as polyphenols, flavonoids, carotenoids, gallic acid, tannins, and essential oils. The interest in plant-based products has also increased due to their lower cytotoxicity compared to synthetic antioxidants (Gupta and Sharma, 2006; Nagulendran et al., 2007; Sen et al., 2010). It has been demonstrated that the enrichment of semen extender with rosemary extract (*Rosmarinus officinalis*; Hussain et al., 2022), date palm (*Phoenix dactylifera*) pollen grains (El-Sheshtawy et al., 2016), and green tea (*Camelia sinensis*) extract (Ahmed et al., 2020) enhances post-thawing buffalo semen quality. The olive fruit contains several phenolic compounds (Mateos et al., 2001; Fernández-Bolaños et al., 2008) whose antioxidant function can account for the well-known beneficial effects of olives and olive-derived products on human health (de Roos et al., 2011). Interestingly, olive fruit extract (OFE) can be obtained from processing waste, which is sustainable and reduces the environmental impact of production. The hydroxytyrosol (HT), one of the main phenols of OFE, is known for its antioxidant properties: it acts as a natural ROS scavenger, reduces oxidation of low-density lipoproteins, protects from hydrogen peroxide-induced cytotoxicity, and minimizes the lactate dehydrogenase activity (Roche et al., 2009; Cicerale et al., 2010). Beneficial effects of HT on sperm quality have also been reported during cooling of boar semen (Li et al., 2022) and during semen freezing in rams (Krishnappa et al., 2018), bucks (Arando Arbulu et al., 2021), and poor-freezer bulls (Sharafi et al., 2022). Despite the demonstrated benefits of HT and the richness in various antioxidant compounds of OFE, only a few recent studies have explored the use of olive-derived products in freezing-thawing buffalo semen (Khalil et al., 2023; Benitez Mora et al., 2024). Recently, it was demonstrated that incubating frozen-thawed buffalo semen with OFE improves sperm quality and fertilization ability by increasing its biological antioxidant potential (Benitez Mora et al., 2024). To our knowledge, however, the effects of adding OFE before semen freezing have not yet been investigated. We hypothesized that the enrichment of semen extender with OFE would improve sperm cryo-tolerance, due to the synergic antioxidant action of its phenolic compounds. The work aimed to evaluate whether supplementing the freezing extender with OFE would increase the antioxidant defense of buffalo sperm, resulting in improved post-thawing semen quality.

2. Material and methods

The experimental design and animal treatments were approved by the Ethical Animal Care and Use Committee of the Universities involved (PG/2024/0127146–10/10/2024).

Unless otherwise stated, reagents were purchased from Sigma-Aldrich-Merck (Milano, Italy).

2.1. Experimental design

A dose-response trial was designed based on the concentrations of the most represented polyphenol, i.e. HT, determined by high-pressure liquid chromatography, as described below and HT concentrations previously tested (Krishnappa et al., 2018; Arando Arbulu et al., 2021; Li et al., 2022; Sharafi et al., 2022). Semen was collected from 16 Murrah buffalo bulls (two ejaculates/bull), and each ejaculate was split into four aliquots that were diluted in a freezing extender containing 0, 72, 143, and 214 mL/L of OFE, corresponding to 0, 50, 100, and 150 μM HT, respectively, and referred to as D0 (control), D50, D100, and D150. The diluted semen was frozen as described below. At collection and after thawing, sperm quality (viability, acrosome integrity, membrane functionality, motility, and kinetics) was evaluated to compare the effects of various OFE concentrations. Based on these results, the concentration of 50 μM HT (D50) was chosen to assess the Reactive Oxygen Metabolites (ROMs) levels and the biological antioxidant potential (BAP) after thawing, compared to the control group, with no supplement.

2.2. Extraction of phenolic compounds from olives

The preparation of OFE employed a newly described method [Patent No102023000021246 “Processo di estrazione di idrossitirosolo da foglie di ulivo e suo utilizzo in ambito funzionale nutraceutico” filed at the UIBM on 12/10/2023] (Benitez Mora et al., 2024). In brief, 50 g of olives (*Olea europaea* cultivar Carolea) gathered in the Calabria region (Vibo Valencia, Italy) were desiccated at 55 °C

for six h, cooled, and ground with a blender. The powder was suspended in 0.4 % (v/v) HCl solution under agitation, autoclaved for 30 min, cooled, and filtered by gravity with a Whatman No. 4 paper filter. After washing with deionized water, the filtrates underwent a further vacuum filtration on a Millipore system to obtain a clear solution that was subjected to spray drying (BUCHI Mini Spry Dryer B-191, Labortechnik AG, Flawil, Switzerland).

The qualitative and quantitative profiles of polyphenol esters were analyzed using High-Pressure Liquid Chromatography, as previously described (Benitez Mora et al., 2024). The OFE used in this study contained high concentrations of phenolic compounds, with 10.8 % and 1.5 % of the crude extract given by HT and tyrosol, respectively.

2.3. Semen collection and manipulation

The experiment was carried out on a farm located in Paraguay (Cordillera Department, Arroyos y Esteros); two ejaculates from 16 Murrah buffalo bulls aged 5 ± 1.4 (mean ± standard deviation) years were collected by electroejaculation (e320 - Eporvac®, Buenos Aires, Argentina) under respectful welfare conditions, with equipment 0.5 volt upward discharging to a maximum of 16 volts, according to manufacturer's instructions (García-Paloma et al., 2017). Briefly, a partially hydraulic and iron operating stall (cattle treatment cage) was used for the safety and welfare of the operators and animals. The rectum was emptied of fecal matter, and the accessory glands were massaged before electroejaculation. The ejaculator's probe was inserted in the rectum and placed above the prostate gland. The ejaculation was induced using the automatic mode on curve two of the device, in which the electrical pulses increase rapidly during the first few cycles, then decrease and rise again linearly up to the maximum. On the collection day, the volume of each ejaculate was recorded, and after determining sperm concentration using a Burker chamber, each ejaculate was split into four aliquots that were diluted at 37°C with Tryladil® extender (Minitube®, Tiefenbach, Germany), containing different concentrations of OFE, to a final concentration of 30×10^6 spermatozoa per mL.

The diluted semen was cooled at 0.6 °C/min and maintained at 4°C for 4 h. Semen samples were then packaged in appropriately identified 0.5-mL straws, frozen with a vapor freezing method in a Styrofoam box, and then plunged in the LN_2 for storage (Buranaamnuay et al., 2016). After at least one week, the samples were thawed in a 37°C water bath for two min. Fresh and frozen-thawed semen were evaluated for sperm viability, acrosome integrity, membrane functionality, motility, and kinetics.

2.4. Sperm motility and kinetic parameters

Sperm motility and kinetics and sperm subpopulations were evaluated by a computer-assisted sperm analysis (AndroVision®, Minitube, Tiefenbach, Germany) installed on a camera-equipped light microscope system (CX43, Olympus, Tokyo, Japan) provided with a warmer stage. A pre-programmed Casa system setting for buffalo was applied and all the particles sized between 5 and 70 μm_2 were classified as spermatozoa. Progressive motility and kinetics were defined as sperm with VCL ≥ 48 μm/s and VSL≥ 24 μm/s. Sixty frames per second with a minimum contrast of 35 were acquired. More specifically, the following parameters were assessed: total motility (%), progressive motility (%), curvilinear velocity (VCL; μm/s), average path velocity (VAP; μm/s), straight-line velocity (VSL; μm/s), straightness (STR; %), linearity (LIN; %), the amplitude of lateral head displacement (ALH, beats/s,), beat cross-frequency (BCF, beats/s) and wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL, %). For the evaluation, after collection and after thawing, an aliquot of semen five μL of semen were put on a pre-warmed glass microscope slide, covered with a glass coverslip. A total of 500 spermatozoa in five randomly selected fields (100/field) were evaluated.

2.5. Sperm viability and acrosome integrity

Sperm viability and acrosome integrity were evaluated by Trypan Blue/Giemsa staining, as previously reported (Longobardi et al., 2017a). In brief, five μL of semen were mixed with five μL of 0.27 % Trypan blue on a slide; then, slides were fixed in 2 % paraformaldehyde solution for two min, washed, and stained with 7.5 % Giemsa overnight. Spermatozoa were microscopically evaluated (magnification: X 40; Nikon Eclipse E200, Tokyo, Japan) and classified as acrosome intact live (AIL), acrosome intact dead (AID), acrosome-lost live (ALL), and acrosome-lost dead (ALD). Only sperm showing both head and tail viable (pink colored) were recorded as viable, while those with either the head or the tail unviable (black-dark violet colored) were recorded as dead. A total of 200 spermatozoa were analyzed per slide.

2.6. Assessment of sperm membrane functionality

Sperm membrane functionality was assessed by the hypoosmotic swelling (HOS) test, as previously described (Domínguez-Rebolledo et al., 2010). Five μL of semen were added to 45 μL of a hypo-osmotic solution (0.73 g sodium citrate and 1.35 g fructose in 100 mL of distilled water, 150 mOsm) and incubated at 37 ℃ for 45 min. After incubation, a drop of diluted semen was put on a clean slide and covered with a cover slip. A total of 200 spermatozoa were counted in different fields (magnification: X 100) under a phase contrast microscope (Nikon Eclipse E200, Tokyo, Japan), and the percentage of HOS-positive spermatozoa (having coiled tails), i.e., those with intact membrane, was determined.

2.7. Evaluation of ROMs and BAP

After thawing, samples of D0 and D50 ODE were centrifuged at 700 x g for 10 min to obtain a sperm-free semen sample for

measuring BAP and ROMs. The BAP was measured spectrophotometrically by the FREE Carpe Diem® reader (Diacron International srl., Grosseto, Italy) using the Bap test kit (Diacron International srl, Grosseto, Italy) according to the manufacturer's protocol. In detail, 50 μL of chromogenic substrate (R2 kit reagent) were gently mixed in a cuvette with one mL of reactive solution (R1 kit reagent), and absorbance at 505 nm was read. Then 10 μL of semen samples were quickly added to the cuvette that was immediately incubated in the thermostatic block of the analyzer for five min at 37°C, and absorbance at 505 nm was recorded. The BAP results were expressed as mmol/L of reduced ferric ions. Three technical replicates per ejaculate were carried out.

The ROMs were evaluated using a free radical elective evaluator that included a spectrophotometric device reader (FREE Carpe Diem®, Diacron International srl., Grosseto, Italy), and specific kits (d-ROMs test, Diacron International srl, Grosseto, Italy) following the manufacturer's instructions. Briefly, 20 μL of semen samples diluted 1:10 with PBS were first placed in a cuvette containing one mL buffered solution (R2 kit reagent, pH 4.8), to which 20 μL of the chromogenic substrate (R1 kit reagent) were then added. After blending, the cuvette was immediately incubated in the thermostatic block of the analyzer for five min at 37°C, and absorbance was recorded at 505 nm. The ROMs were expressed in arbitrary units (U/CARR), with one unit corresponding to 0.8 mg/L of hydrogen peroxide. Three technical replicates per ejaculate were carried out.

2.8. Statistical analysis

Statistical analysis was carried out using SPSS (29.0.1) for Windows 10 (SPSS Inc., Chicago, IL).

The normal distribution and the homogeneity of variance of data were verified using the Shapiro-Wilk and Levene's tests, respectively. Data were log-transformed when necessary. Post-thawing fertility parameters of semen (viability, acrosome integrity, membrane functionality, motility, and kinetics) were compared among groups (D0, D50, D100, and D150 OFE) by analyses of variance (ANOVA) for repeated measures (generalized linear mixed model) with the group as a fixed effect and ejaculate as a repeated measure. Tukey's HSD procedure was used to make multiple post hoc comparisons among different groups. Differences in ROMs and BAP values between the control and the D50 OFE group were analyzed by Student's *t*-test. In both analyses, the significance level was set at $P < 0.05$.

3. Results

At the time of semen collection, the percentage of sperm with a functional membrane (HOS+), total viable sperm, and AIL sperm were 61.8 ± 3.1, 61.0 ± 2.5, and 59.0 ± 2.6, respectively (mean ± SE). Progressive motility was 60.3 ± 1.9. Regardless of the treatment, freezing-thawing decreased ($P < 0.01$) the percentages of HOS+ sperm, total viable sperm, AIL, and progressive motility.

3.1. Effects of OFE addition to the freezing extender on post-thawing sperm quality

As shown in Fig. 1, the addition of D50 and D100 OFE to the extender increased the post-thawing total and progressive motility compared to the control ($P < 0.01$) and to D150 OFE ($P < 0.01$). Higher ($P < 0.01$) post-thawing total and progressive motility were observed in D50 than in D100 OFE.

Concerning the kinetic parameters, higher values of VCL were recorded in the D50 group but a significant difference ($P < 0.05$) was only found with D100 (Table 1). In the D50 OFE group, VAP was higher ($P < 0.05$) than in the other treated groups. Semen treated with D50 OFE exhibited higher ($P < 0.05$) VSL than the other groups. At the higher concentration (D150), LIN was higher ($P < 0.05$) than in the control and BCF was higher ($P < 0.01$) than in the D100 group.

Semen treated with D50 OFE showed a higher ($P < 0.01$) proportion of total viable sperm, sperm with a functional membrane (HOS+), and intact acrosome compared to the control (Fig. 2). The addition of D100 OFE also gave higher ($P < 0.05$) sperm viability and HOS+ sperm than the control. Supplementation of the extender with D150 OFE decreased ($P < 0.01$) the percentage of sperm with

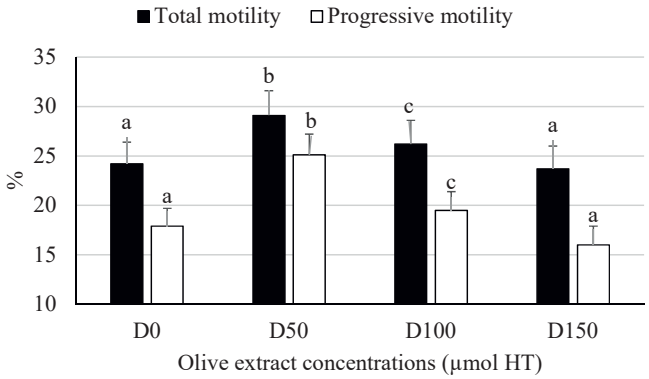

Fig. 1. Effect of olive fruit extract (OFE) supplementation in the freezing extender on post-thawing total and progressive sperm motility of buffalo semen (n = 32 ejaculates/group). Data are expressed as mean ± SE.

Table 1
Post-thawing kinetic parameters of buffalo semen frozen (32 samples/group) with different concentrations of olive fruit extracts (OFE). Data are expressed as mean ± SE.

Kinetic parameters	OFE concentrations (μmol HT)			
	0 (Control)	50	100	150
VCL (μm/s)	49.0 ± 2.3^{ab}	56.8 ± 5.6^{a}	47.9 ± 1.9^{b}	47.3 ± 3.1^{ab}
VAP (μm/s)	25.1 ± 2.3^{ab}	30.1 ± 4.0^{a}	24.5 ± 1.9^{b}	24.0 ± 2.5^{b}
VSL (μm/s)	30.8 ± 2.3^{a}	35.0 ± 3.2^{b}	30.6 ± 1.9^{a}	29.9 ± 2.6^{a}
STR (%)	44.0 ± 4.3^{ab}	42.4 ± 3.7^{ab}	39.5 ± 2.8^{a}	47.4 ± 4.8^{b}
LIN (%)	58.9 ± 2.4^{a}	57.2 ± 2.4^{ab}	61.2 ± 1.7^{ab}	61.3 ± 1.7^{b}
ALH (beats/s)	1.5 ± 0.2	1.9 ± 0.3	1.3 ± 0.1	1.6 ± 0.2
BCF (beats/s)	10.6 ± 1.5^{ab}	9.3 ± 1.1^{ab}	8.3 ± 0.8^{a}	12.1 ± 1.8^{b}
WOB (%)	56.3 ± 2.5	55.2 ± 2.5	52.8 ± 2.0	56.7 ± 3.2

$^{a, b}$ Within rows values with different superscripts are significantly different; $P < 0.05$

a functional membrane (HOS+), and intact acrosome compared to all other groups.

3.2. Effects of OFE addition to the freezing extender on OS markers

The enrichment of the extender with D50 OFE increased ($P < 0.01$) biological antioxidant potential (Fig. 3A) and reduced ($P < 0.05$) the ROMs levels (Fig. 3 B) in the post-thawing semen.

4. Discussion

The results of this study confirmed the hypothesis that adding OFE to the freezing extender would improve post-thawing buffalo semen quality by reducing cryopreservation-induced OS. For this reason, the strategy to enrich the extender with a natural plant-derived antioxidant additive such as OFE was effective in counteracting excessive ROS formation during cryopreservation, which is known to be a major cause of sperm injury (Yánez-Ortiz et al., 2022).

The low proportion of buffalo sperm surviving the freezing-thawing procedure and hence the lower fertilizing ability of frozen semen (Singh et al., 2018; Hassan et al., 2022) impose the adoption of corrective strategies to improve semen cryopreservation efficiency in this species. It is known that during cryopreservation an excess of ROS is generated, leading to OS (Bucak et al., 2007; 2008; 2009; 2012; Longobardi et al., 2021). Although physiological levels of ROS are required for many reproductive functions, such as capacitation, acrosome reaction, and zona pellucida binding (Bucak et al., 2007; 2008; 2009; 2012; Longobardi et al., 2021), higher concentrations exert detrimental effects (Bansal and Bilaspuri, 2011). The lipid composition of the sperm membrane (Rajoriya et al., 2016) makes buffalo sperm particularly susceptible to lipid peroxidation in the presence of high levels of ROS (Andrabi et al., 2009). The sperm's endogenous antioxidant systems cannot effectively counteract stress-induced overproduction of ROS, and hence the inclusion of antioxidants in the semen extender is a potential corrective strategy to improve cryopreservation efficiency.

The increasing awareness of the reduced toxicity of natural antioxidants and the importance of recycling product wastes has recently switched the focus of researchers to plant-derived antioxidants. Olive cultivation is an ancient agricultural activity in Mediterranean countries, and the recovery of by-products from the oil industry is significant for a sustainable, regenerative economy. Olive oil and its derivates contain a high concentration of bioactive compounds, such as polyphenols (oleuropein, HT, and tyrosol) and flavonoids (luteolin and apigenin), with antioxidant properties (Fernández-Bolaños et al., 2008; Banihani, 2017), to which beneficial effects on human health have been attributed (de Roos et al., 2011).

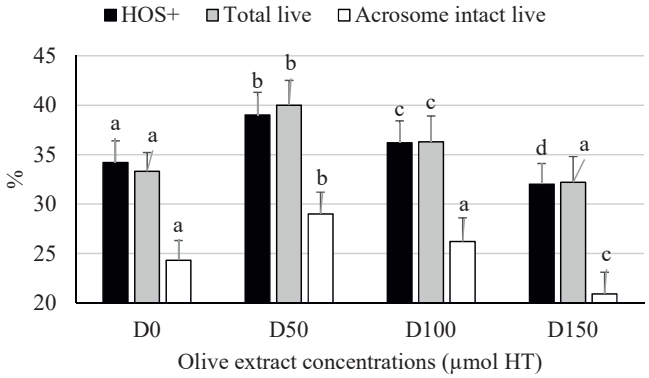

Fig. 2. Effect of olive fruit extract (OFE) supplementation in the freezing extender on the percentages of sperm with functional membrane (HOS+), total viable sperm, and acrosome intact live (AIL) sperm recorded after thawing (n = 32 ejaculates/group). Data are expressed as mean ± SE. $^{a, b}$ Bars of the same color with different letters are significantly different; $P < 0.05$.

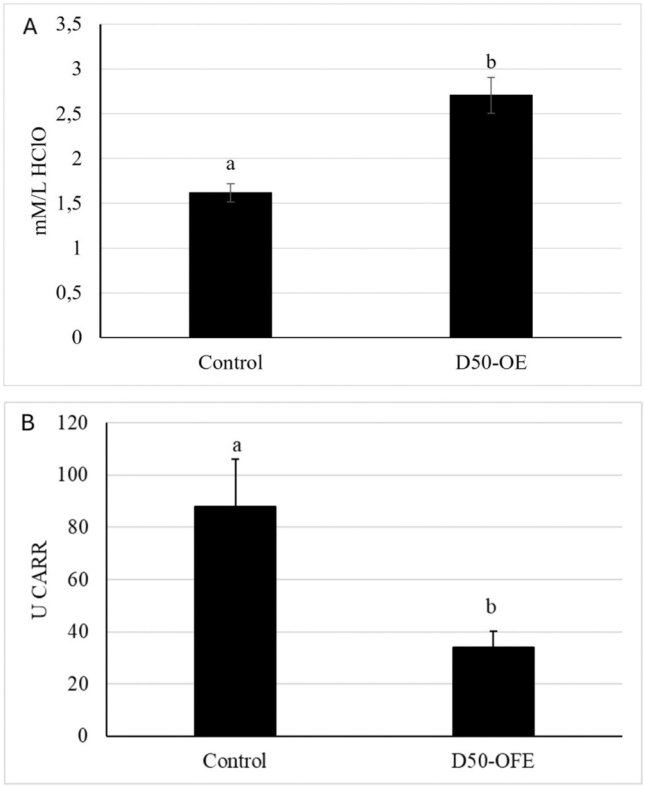

Fig. 3. Post-thawing biological antioxidant potential (A) reactive oxygen metabolites (ROMs) levels (B) of semen frozen in the presence or absence of D50 olive fruit extract (D50-OFE). Data are expressed as mean ± SE. [a,b] Bars with different letters are significantly different; $P < 0.0$.

In this study, we first evaluated the effect of different concentrations of OFE on qualitative parameters of post-thawing sperm. The concentrations of OFE were chosen based on the main constituent, i.e. HT, known to affect sperm function in other species (Krishnappa et al., 2018; Arando Arbulu et al., 2021; Li et al., 2022; Sharafi et al., 2022) and on the results of a previous study in which we used OFE as a post-thawing treatment (Benitez Mora et al., 2024). The results demonstrated a dose-dependent effect, with the lower dose (D50) exerting beneficial effects on semen quality, whereas higher concentrations (D150) were harmful to sperm. This partially disagrees with the previous study in which the beneficial effect was obtained with higher doses (100 μm HT); in that case, though, OFE was added after freezing, i.e. on semen that was already subjected to stress caused by cryopreservation procedures. Similar to the other study, higher dosages of OFE showed aa negative effect.

An interesting finding of the work was that the supplementation of the extender with D50 OFE before freezing improved both total and progressive sperm motility compared to the control, which were instead reduced with D150. This is a relevant finding as motility is one of the most reliable predictive markers of sperm fertilizing ability (Vijayaraghavan, 2003). Although OFE has not been used before as an additive to semen extender before freezing, a previous study showed a beneficial effect of the supplementation of this extract after freezing (Benitez Mora et al., 2024). In particular, OFE preserved sperm motility over post-thawing incubation times and enhanced some kinetic parameters such as VAP and WOB (Benitez Mora et al., 2024). The results of this study support the hypothesis that OFE can not only rehabilitate sperm after cryoshock but also prevent sperm damage during the freezing-thawing process. Furthermore, the preventive effects appear to require lower doses than the rehabilitative effects.

Previous studies have tested the effect of HT, which is the main bioactive constituent of OFE, known for its antioxidant properties (Cicerale et al., 2010). Treatment with HT improved post-thawing sperm motility in bucks (Arando Arbulu et al., 2021) and in bulls with poor cryotolerance (Sharafi et al., 2022). In the ram, the supplementation of the extender with HT also increased post-thawing total motility (Krishnappa et al., 2018), although, in another study, motility was not affected (Arando et al., 2019). Improved sperm motility was also recorded during the long-term preservation of pig semen at 17 °C (Li et al., 2022). Incubation of human semen with HT did not affect its motility after centrifugation (Kedechi et al., 2017). Concerning kinetic sperm parameters, semen treated with D50 OFE exhibited higher post-thawing VSL, while the other kinetic parameters remained unaffected.

Interestingly, treatment with D50 OFE of buffalo semen significantly improved post-thaw sperm viability, membrane integrity, and acrosome integrity compared to the control, while D150 decreased the proportion of sperm with intact acrosome and membrane integrity, confirming a dose-dependent effect. When OFE was supplemented after thawing, an improvement in the percentage of spermatozoa with intact acrosome was observed, but the treatment did not affect the percentage of spermatozoa with a functional membrane (Benitez Mora et al., 2024). The addition of antioxidants to the freezing extender may stabilize proteins and enzymes,

helping to prevent functional membrane alterations during the freezing-thawing process. An improvement in post-thawing membrane and acrosome integrity has also been reported in the buck semen after the addition of low doses of HT in ram frozen-thawed semen (Arando Arbulu et al., 2021). The treatment with phenolic antioxidants or oleic acid (olive oil-derived) has also improved acrosome integrity in rooster (Al-Daraji, 2012) and ram sperm (Hashem et al., 2017). The addition of HT in the incubation medium improved human sperm viability following centrifugation (Kedechi et al., 2017). Supplementation of the extender with HT reduced post-thawing membrane damage in bad freezer bulls, without affecting acrosome integrity (Sharafi et al., 2022). In another study, the treatment with HT failed to improve viability, acrosome integrity, and membrane integrity in ram frozen-thawed semen (Arando et al., 2019). Furthermore, when a mixture of HT and 3,4-dihydroxyphenylglycol (DHPG), another olive-derived phenol, was used, sperm viability and acrosome integrity were reduced compared to the control (Arando et al., 2019). This is in agreement with the deterioration of semen quality parameters found with the higher concentration used in the present study, i.e. D150. Although antioxidant supplementation is required to provide sperm protection against cryopreservation-induced OS, it is difficult to identify the appropriate dose, which also depends on the starting endogenous antioxidant levels (Gvozdjáková et al., 2015). An excess of antioxidants, indeed, may affect the cellular redox balance (Bouayed and Bohn, 2010) and enhance membrane fluidity, increasing sperm susceptibility (Shoae and Zamiri, 2008).

The notable enhancement in BAP and the reduction in ROMs levels observed in buffalo semen treated with D50 OFE, in comparison to the control group, suggest that the positive effects of OFE on the post-thawing semen quality are due to its antioxidant properties. The reduced ROMs levels are likely due to the presence in the OFE of HT, a known powerful ROS scavenger, and other bioactive compounds (Tripoli et al., 2005; Hamden et al., 2009). The increased antioxidant capacity may also be due to the effect of HT on the preservation of antioxidant enzymes, as previously hypothesized (Li et al., 2022). An effective antioxidant activity, indicated by reduced ROS levels, decreased lipid peroxidation, and increased antioxidant capacity, has also been recently demonstrated for an olive oil nano-emulsion added to the freezing extender of buffalo semen, which was associated with reduced apoptosis and improved in vivo fertility (Khalil et al., 2023).

The reduced ROMs levels detected in semen treated with D50 OFE account for the improved sperm motility, as a negative association between these parameters has been previously reported (Kadirvel et al., 2009). The decline in sperm motility recorded in the presence of high levels of ROS has been attributed to lipid peroxidation, which affects membrane integrity and permeability, leading to impaired regulation of the intracellular ions involved in sperm movement (Aitken et al., 1993). Furthermore, the decreased motility induced by OS may be related to ATP depletion, alterations in mitochondrial function, and reduction of phosphorylation of axonemal proteins (de Lamirande and Gagnon, 1992, Armstrong et al., 1999). The antioxidant action of OFE (D50) also accounts for the improved sperm membrane functionality and acrosome integrity, which are other important predictive markers of frozen-thawed sperm fertilizing ability (Brito et al., 2003), as the plasma membrane and the acrosome, highly sensitive to OS, are primary targets of cryopreservation-induced damages (Hammerstedt et al., 1990).

This is the first work reporting the beneficial effects of OFE supplementation to the extender on post-thawing semen quality. The administration of extracts of olive leaf has been previously reported to counteract chlorpyrifos and lead-induced reproductive toxicity, by attenuating OS, in laboratory animal models, such as rats (Ahmed et al., 2021; Hassan et al., 2022). A protective role of the olive leaf extract, due to the antioxidant action, was also found against type II diabetes mellitus and cisplatinum-induced testicular damage in rats (Almeer and Abdel Moneim, 2018; Soliman et al., 2019).

Overall treatment with low doses of OFE (D50) improved post-thawing semen qualitative parameters, by reducing OS, as indicated by increased total antioxidant capacity and reduced ROS levels. Using the entire OFE, which contains various bioactive phenolic components, may be more effective than HT or other specific compounds used individually (Zielinska et al., 2007; Bouayed and Bohn, 2010). Another advantage of using OFE rather than individual phenols to improve post-thaw semen quality is the cost-effectiveness and, more importantly, the possibility of recycling olive wastes in respect of a circular economy.

5. Conclusions

In conclusion, it was demonstrated that low doses of OFE (corresponding to 50 μM HT) added to the freezing extender significantly improve post-thawing sperm motility and some kinetic parameters, viability, as well as membrane functionality and acrosome integrity. It was also demonstrated that the treatment with OFE before freezing increases the semen's total antioxidant activity and reduces ROS levels. These results indicate that the inclusion of OFE in the extender is a valid strategy to improve the cryotolerance of buffalo sperm. In perspective studies, it will be worth investigating the effect of OFE supplementation of buffalo semen on *in vivo* and *in vitro* fertility, for potential commercial applications.

Funding

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

CRediT authorship contribution statement

Gasparrini Bianca: Writing – review & editing, Methodology, Conceptualization. **Aiudi Giulio Guido:** Investigation. **Cocchia Natascia:** Methodology. **Benitez Mora Maria Paz:** Investigation, Conceptualization. **Kosior Michal Andrzej:** Writing – original draft. **Fedele Francesca Luisa:** Investigation. **Staropoli Alessia:** Investigation. **Longobardi Valentina:** Methodology, Investigation, Data curation. **Del Prete Chiara:** Writing – original draft, Supervision.

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.

Data availability

All data generated or analyzed during this study will be available on request.

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Glossary

AID: acrosome intact dead spermatozoa
AIL: acrosome intact live spermatozoa
ALD: acrosome-lost dead
ALH: the amplitude of lateral head displacement
ALL: acrosome-lost live spermatozoa
BAP: biological antioxidant potential
BCF: beat cross-frequency

D0: control
D100: 100 μM hydroxytyrosol
D150: 150 μM hydroxytyrosol
D50: 50 μM hydroxytyrosol
HOS: hypoosmotic swelling
HT: hydroxytyrosol
LIN: linearity
OFE: Olive fruit extract
OS: oxidative stress
ROMs: reactive oxygen metabolites
ROS: reactive oxygen species
STR: straightness
VAP: average path velocity
VCL: curvilinear velocity
VSL: straight-line velocity
WOB: wobble of the curvilinear trajectory