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Original Research Article

Incubating frozen-thawed buffalo sperm with olive fruit extracts counteracts thawing-induced oxidative stress and improves semen quality

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ABSTRACT

Freezing-thawing procedures and semen manipulation for *in vitro* fertilization induce oxidative stress, which in turn leads to impaired sperm quality. The aim of this study was to evaluate whether incubation of frozen-thawed buffalo semen with olive fruit extracts (OFE), known to contain a high concentration of phenolic antioxidants, would improve semen quality by reducing oxidative stress.

Frozen sperm (4 ejaculates/4 bulls/3 replicates) were thawed and diluted to 30×10^6/mL in IVF medium with 0, 72, 143, and 214 μL/mL of OFE, corresponding to 0 (D0-control), 50 (D50), 100 (D100), and 150 (D150) μM hydroxytyrosol. Sperm viability, acrosome integrity, membrane functionality, motility, and sperm kinetics were evaluated immediately after thawing (T0) and after 1 (T1) and 2 h (T2) of incubation at 38.7 °C. Based on the results, sperm biological antioxidant potential (BAP) and ROS levels (ROMs) were assessed in D0 and D100 groups at T1 and T2. To assess the effect of OFE on fertilizing ability, heterologous penetration rates were also evaluated, using bovine abattoir-derived oocytes.

The treatment with OFE at all concentrations tested increased ($P < 0.05$) the percentage of acrosome intact spermatozoa compared to the D0-control at T1, but the effect was more evident ($P < 0.01$) with D100 (54.5 ± 3.0, 60.5 ± 1.5, 65.2 ± 3.3, and 62.5 ± 1.7, with D0, D50, D100, and D150 OFE, respectively). Total motility, progressive motility, rapid velocity, and progressive velocity decreased ($P < 0.05$) at T2 only in the D0-control group. The percentage of rapidly progressive sperm and the progressive motility tended to increase ($P < 0.10$) at T1 and T2, respectively, in D100 compared to D0 (24.7 ± 4.1 vs 16.4 ± 1.6 and 22.8 ± 2.7 vs 17.0 ± 1.2, respectively). The treatment with D100 OFE of frozen-thawed sperm increased ($P < 0.05$) some kinetic parameters (VAP and WOB). Spermatozoa incubated with D100 OFE exhibited higher ($P < 0.01$) total and normospermic oocyte penetration rates compared to D0 (86.5 ± 1.4 vs 78.5 ± 0.7, and 70.6 ± 1.5 vs 63.8 ± 1.1, respectively). Additionally, D100 OFE increased sperm BAP concentrations at both T1 and T2, while ROS levels were unaffected. These results suggest that incubating frozen-thawed buffalo semen with OFE is an effective strategy for preserving semen quality and *in vitro* fertilization ability by enhancing sperm antioxidant capacity.

1. Introduction

The growing economic importance of buffalo breeding as a livestock species capable of producing animal proteins in disadvantaged production systems is indicated by the steady worldwide increase in the number of heads [1]. Advanced reproductive technologies play a pivotal role in speeding up genetic progress and counteracting the effects of climate change. Semen cryopreservation allows long-term sperm storage, easier exchanges of genetic resources among countries, and appropriate planning of artificial insemination and embryo production technologies.

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Abbreviations

OFE	olive fruit extracts
IVF	*in vitro* fertilization
BAP	biological antioxidant potential
ROS	reactive oxygen species
ROMs	reactive oxygen metabolites levels
VAP	average path velocity
WOB	wobble of the curvilinear trajectory
HT	hydroxytyrosol
OS	oxidative stress
HPLC	high-pressure liquid chromatography
T	tyrosol
SCA	sperm class analyzer system
VSL:	straight-line velocity
VCL:	curvilinear velocity
STR	straightness
LIN	linearity
ALH	amplitude of lateral head displacement
BCF	beat cross-frequency
AIL:	acrosome intact live
AID	acrosome intact dead
ALL:	acrosome-lost live
ALD	acrosome-lost dead
HOS	hypoosmotic swelling
FCS	fetal calf serum
D0-control	0 μM hydroxytyrosol
D50	50 μM hydroxytyrosol
D100	100 μM hydroxytyrosol
D150	150 μM hydroxytyrosol
T0	after thawing
T1	after 1 h of incubation at 38.7 °C
T2	after 2 h of incubation at 38.7 °C

During freezing-thawing procedures and semen manipulation for *in vitro* fertilization (IVF), the thermal and osmotic insults cause an overproduction of reactive oxygen species (ROS) and impairment of the semen antioxidant defense mechanism [2–5]. This condition results in oxidative damage to cellular proteins, lipids, and DNA, leading to impaired sperm function, including reduced viability and motility, as well as premature capacitation, ultimately resulting in decreased fertility [6–10]. The low cholesterol: phospholipids ratio [11] and the rich amount of long-chain polyunsaturated fatty acids [12] in the plasma membrane, make buffalo sperm extremely vulnerable to cryopreservation-induced oxidative stress [13]. It was demonstrated that cryopreservation affects the transcriptome of buffalo sperm, with altered expression of transcripts that regulate the metabolic activities, response to ROS, and fertility-related functions [14]. Moreover, although sperm are naturally equipped with antioxidant systems, the defense machinery is weak to counteract the excess of ROS produced under stressful conditions [15]. Furthermore, the antioxidants present in the seminal plasma or in the semen extender are lost during sperm manipulations required for freezing and IVF [3]. Hence, a potential strategy to counteract oxidative stress (OS) damage in sperm is to supplement frozen-thawed sperm with exogenous antioxidants. This approach aims to enhance sperm quality and improve its *in vitro* fertilizing capability [16–18].

Supplementation of the freezing semen extender with antioxidants has become a common strategy to preserving sperm quality and improving reproductive outcomes in animal reproduction [19]. Various antioxidants have shown promising results in buffalo semen by replenishing depleted endogenous antioxidants, counteracting OS, and minimizing the generation of free radicals [20–26]. In livestock, the addition of antioxidants to semen extenders not only enhances sperm quality but also supports *in vitro* embryo development [17,27,28]. Due to the reduced toxicity and residue, as well as the potential to recycle product wastes within a circular economy, the interest in the use of plant-derived natural antioxidants has recently increased. Several natural antioxidants, like silymarin, spirulina maxima, green tea, and rosemary extracts, have been added to the freezing extender to improve bovine and buffalo sperm cryotolerance [24,29–32]. Only a limited number of studies have examined the impact of natural antioxidants on frozen-thawed buffalo sperm, with promising results [25,33].

Olive fruits and olive oil are well known for their high content of phenolic compounds with antioxidant properties, particularly hydroxytyrosol (HT), tyrosol, and oleuropein [34]. The HT is a powerful ROS scavenger, known to reduce lipid peroxidation and protect cells from ROS toxicity [35,36]. The inclusion of HT in the freezing semen extender improved post-thaw semen quality in small ruminants [37,38], and poor freezer bulls [39]. Beneficial effects of HT have also been recorded on the quality of boar semen stored at 17 °C [40]. Furthermore, *in vitro* treatment with HT has been reported to improve human semen viability, by reducing OS following centrifugation [41].

The olive fruit extract (OFE) is a cocktail of natural antioxidants that can act synergistically to improve cell function. This product can be easily obtained from discarded olives, which are typically wasted during commercialization. Only a few studies have been carried out in laboratory animals, showing the protective effects of olive extracts against induced reproductive toxicity [42–45]. However, the potential of OFE to improve semen quality has not been yet evaluated, neither before freezing nor after thawing. This work aimed to evaluate whether incubation of frozen-thawed buffalo semen with different concentrations of OFE would improve the quality of semen thawed and processed for IVF, by reducing OS.

2. Material and methods

2.1. Experimental design

Sixteen healthy Italian Mediterranean buffalo (*Bubalus bubalis*) bulls (4–6 years of age) maintained at an authorized National Semen Collection Center (Centro Tori Chiacchierini, Civitella D'Arna, Italy) under uniform management conditions, routinely used for semen collection twice per week, were enrolled in the study. Three ejaculates per bull were collected by artificial vagina (IMV, L'Aigle, Cedex, France). Only ejaculates with sperm motility >70 % were diluted at 37 °C in the BioXcell extender (IMV, L'Aigle, Cedex, France) to a final concentration of 30×10^6 spermatozoa per mL, and frozen according to the standard procedures used in the Semen Collection Center, as previously reported by Longobardi et al. [46]. The frozen semen used for the trial was stored in a liquid nitrogen tank for at least 2 months.

On each day of the experiment, frozen sperm from four bulls were thawed in a water bath at 37 °C, pooled, separated by Percoll discontinuous gradient, as described below, diluted to 30×10^6/mL in IVF medium with different concentrations of OFE, and incubated for 2 h at 38.7 °C in a controlled gas atmosphere of 5.5 % CO_2 in humidified air. The range of OFE concentrations tested was chosen based on the most represented polyphenol, i.e. HT, assessed by high-pressure liquid chromatography (HPLC), as described below, and its use in previous studies [37,39,40,47]. More precisely, the IVF medium was supplemented with 0, 72, 143, and 214 μL/mL of OFE, corresponding to 0, 50, 100, and 150 μM HT, termed as D0-control, D50, D100, and D150.

Each pool (4 pools × 4 bulls) was repeated three times. Immediately after thawing (T0) and after 1 (T1) and 2 h (T2) of incubation, sperm

viability, acrosome integrity, membrane functionality, motility, and sperm kinetics were evaluated.

Based on the results, the concentration of 100 μM HT (D100) of OFE was chosen to assess oxidative/antioxidant status by the evaluation of semen biological antioxidant potential (BAP) and ROS levels (ROMs) at T1 and T2, compared to the D0-control group, with no supplement.

To evaluate the effect of OFE on fertilizing ability, heterologous penetration rates, using abattoir-derived bovine oocytes, were assessed: i) by including or not OFE directly into the IVF medium (n = 120/group, over four replicates) or ii) by inseminating *in vitro* matured bovine oocytes with buffalo semen incubated for 1 h with or without D 100 OFE (n = 135 and 140, respectively in the D0-control and D100 OFE, over 4 replicates). In this latter case, the IVF medium did not contain OFE.

2.2. Preparation of OFE

To maximize the recovery of the phenolic compounds, a new extraction method was developed [Patent No102023000021246 "Processo di estrazione di idrossitirosolo da foglie di ulivo e suo utilizzo in ambito funzionale nutraceutico" filed at the UIBM on October 12, 2023]. Briefly, 50 g of olives (*Olea europaea* cultivar Carolea) collected in Vibo Valencia (Calabria region, Italy) were placed in a desiccator at 55 °C for 6 h, cooled, and grounded using a blender. Two hundred mL of a 0.4 % (v/v) HCl solution was added to the resulting powder under agitation. The suspension was autoclaved at 121 °C for 30 min at a pressure of 1.1 bar, cooled, and filtered by gravity with a Whatman No. 4 paper filter. The solid residue was washed with 100 mL of deionized water. The combined filtrates were subjected to a second vacuum filtration on a Millipore system to remove the remaining finely dispersed suspended particles. The clear solution was subjected to spray drying (BUCHI Mini Spry Dryer B-191).

2.3. Determination of concentration of polyphenol esters

Qualitative and quantitative profiles of polyphenol esters have been obtained using HPLC Agilent Technologies 1200 Series, equipped with a diode array detector (DAD), and a column Agilent Eclipse C18 (5 μm x 4,6 × 150 mm) with pre-column Phenomenex. The injection volume was 5 μL. The eluent phases were: A) consisting of H_2O and 0.2 % of formic acid, and B) composed of a mixture acetonitrile:methanol (60:40 v/v). All polyphenols were eluted using a 0.8 mL/min flow rate with a gradient programmed as follows: 0–6 min −20 % of phase B, 16 min −40 % of phase B, 21 min −50 % of phase B, 32 min −90 % of phase B, 35 min −90 % of phase B, 38 min −20 % of phase B, and it stays in this condition for the next 5 min for equilibration of the column. The HPLC profile reported in Fig. 1 shows high concentrations of phenolic compounds specifically HT (10.8 % of the crude extract) and Tyrosol (T, 1.5 % of the crude extract). The HT and T concentrations, 1296.2 ppm and 138.8 ppm respectively, have been obtained with a calibration curve using authentic standards.

2.4. Sperm motility and kinetic parameters

Sperm motility parameters (total and progressive motility, sperm subpopulations, and semen kinetic parameters) were assessed by a sperm class analyzer system (SCA, Sperm Class Analyzer; Microptic SL, Veterinary Edition, Barcelona, Spain) installed on a camera-equipped light microscope system (Eclipse E200, Nikon, Tokyo, Japan) fitted with a warmer stage. The following parameters were considered: total motility (%), progressive motility (%), the percentage of sperm subpopulations (rapid and medium progressive spermatozoa and rapid, medium and slow spermatozoa), average path velocity (VAP; μm/s), straight-line velocity (VSL; μm/s), curvilinear velocity (VCL; μm/s), straightness (STR; %), linearity (LIN; %), wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL, %), amplitude of lateral head displacement (ALH, beats/s) beat cross-frequency (BCF, beats/s) and hyperactive (%). SCA system settings for buffalo semen classified as spermatozoa all the particles sized between 5 and 70 μm^2 and as progressively motile spermatozoa with 50 % STR. The VCL values considered for slow-medium and rapid spermatozoa subpopulations were 25 and 50 μm/s; spermatozoa with VCL below 10 μm/s were considered static. Sixty frames per second with a minimum contrast of 35 were acquired. For the evaluation, 3 μL of semen after thawing and at T1 and T2 were loaded onto a pre-warmed glass microscope Leja slide (Leja, 4-chamber counting slides; 20 μm nominal chamber depth, Leja Products BV, Nieuw-Vennep, The Netherlands). At least 500 sperm cells in five randomly selected fields were evaluated.

2.5. Sperm morphology

For sperm morphology, pre-stained SpermBlue slides (Microptic, Barcelona, Spain) were used. Briefly, 10 μL of semen were dispensed on top of the slide, covered with a 22 × 22 coverslip and visualized by a camera-equipped light microscope system (Eclipse E200, Nikon, Tokyo, Japan) with 60× objective, using the morphology module of the SCA system. This system automatically detects alterations in head, midpiece and tail, as well as cytoplasmic droplets. At least 100 sperm were analyzed per each sample and the percentage of anomalies was calculated out of the total sperm analyzed.

2.6. Sperm viability and acrosome integrity

Sperm viability and acrosome integrity were assessed by Trypan Blue/Giemsa technique as reported by Longobardi et al. [22,46] with slight modifications. Briefly, on a clean slide, 5 μL of semen and 5 μL 0.27 % Trypan blue were spread, fixed in paraformaldehyde solution 2

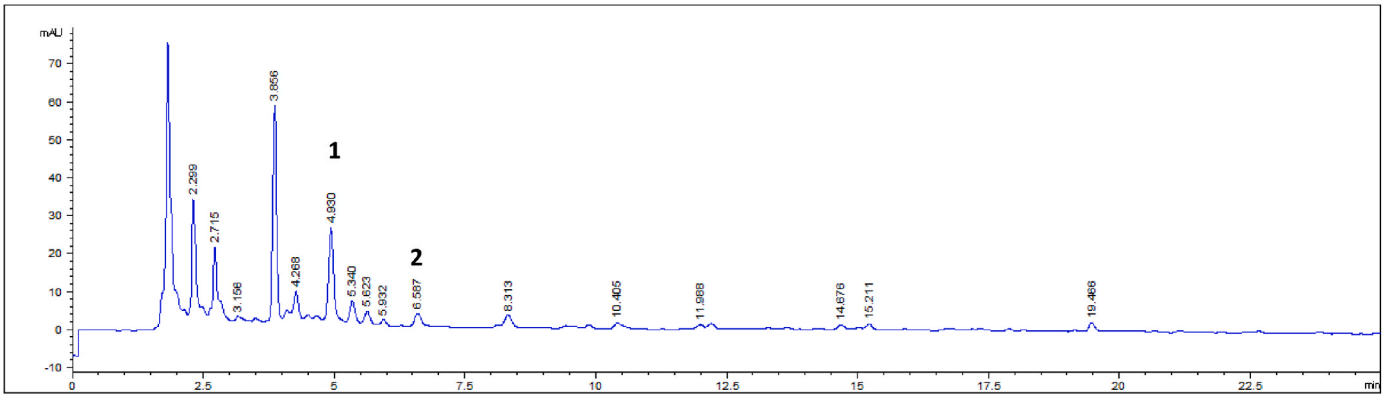

Fig. 1. High-Pressure Liquid Chromatography profile of olives extract (diode array detector at 280 nm). Peak at 4.930 min = hydroxytyrosol (**1**). Peak at 6.587 min = tyrosol (**2**).

% in phosphate buffered saline (PBS) for 2 min, and stained with 7.5 % Giemsa overnight. Sperm cells were microscopically evaluated (magnification: ×40; Nikon E200) and differentiated as acrosome intact live (AIL), acrosome intact dead (AID), acrosome-lost live (ALL), and acrosome-lost dead (ALD). A total of 200 spermatozoa were analyzed per slide.

2.7. Sperm membrane functionality

Sperm membrane functionality was assessed by the hypoosmotic swelling (HOS) test, as previously described [48]. Five μL of semen were mixed with 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 °C for 45 min. A drop of diluted semen was placed on a clean slide and covered with a cover slip. A total of 200 spermatozoa were counted in different fields (magnification: ×100) under a phase contrast microscope (Nikon E200) and the percentage of spermatozoa positive to HOS test (having coiled tails), i.e. those with intact membrane, was determined.

2.8. Sperm penetration

Sperm penetration was assessed by the presence of sperm chromatin in the oocyte cytoplasm after heterologous IVF, as previously described [49]. Bovine ovaries were collected from a local abattoir and transported to the laboratory within 4 h of slaughter at approximately 30–35 °C in physiological saline supplemented with 150 mg/L kanamycin (Gibco, Paisley, UK). Cumulus-oocyte complexes (COCs) were recovered by aspiration of 2–8 mm follicles using an 18-gauge needle under vacuum (40–50 mmHg), and only those surrounded by at least three layers of cumulus cells were selected and matured in 50 μL drops (10/drop) of TCM199 buffered with 25 mM sodium bicarbonate and supplemented with 10 % fetal calf serum (FCS), 0.2 mM sodium pyruvate, 0.5 μg/mL FSH, 5 μg/mL LH, 1 μg/mL 17β-estradiol, 50 μg/mL kanamycin, 100 μM β mercaptoethanol, and 50 μg/mL gentamycin for 22 h at 38.7 °C in a controlled gas atmosphere of 5 % CO_2 in air.

Frozen-thawed buffalo sperm (previously tested for IVF) were selected by centrifugation (1500 rpm × 25 min) on a Percoll discontinuous gradient (Nidacon, Mölndal, Sweden). The sperm pellet obtained after centrifugation was re-suspended to a final concentration of 2×10^6/mL in the IVF medium, consisting of TALP buffered with 25 mM sodium bicarbonate and supplemented with 0.2 mM penicillamine, 0.1 mM hypotaurine, and 0.01 mM heparin. Oocytes were fertilized either in the IVF medium in the absence of OFE (D0-control) and with D100 OFE, or with semen pre-incubated for 1 h with or without D100 OFE. Gametes were co-incubated for 20 h at 38.7 °C, in 5 % CO_2 in air.

At the end of gamete co-incubation, the presumptive zygotes were vortexed for 2 min to remove cumulus cells, and after zona removal by protease (2 mg/mL) digestion, fixed with 60 % methanol in PBS for 30 min, stained with 2.5 μg/mL Hoechst 33342 for 15 min, and mounted on glass slides for subsequent evaluation of nuclear status using a Nikon Diaphot 300 inverted microscope equipped with fluorescence filters. The incidence of normospermic and polyspermic fertilization was assessed. Oocytes with two synchronous pronuclei (2 PN) were considered as normally fertilized, whereas those with >2 PN or sperm heads as polyspermic.

2.9. Oxidative/antioxidant status

ROMs and BAP levels were assessed using specific measurement kits (d-ROMs test and BAP test kit, Diacron International srl, Grosseto-Italy) following the manufacturer's protocol. Measurements were conducted using a spectrophotometric device reader (FREE Carpe Diem®, Diacron International srl, Grosseto-Italy). For ROMs, 20 μL of seminal plasma sample (diluted 1:10 with PBS) and 1 mL buffered solution (R2 kit reagent, pH 4.8) were gently mixed in a cuvette, and 20 μL of the chromogenic substrate (R1 kit reagent) was then added to the cuvette. After mixing well, the cuvette was immediately incubated in the thermostatic block of the analyzer for 5 min at 37 °C, and absorbance at 505 nm was recorded. The results were expressed in arbitrary units (U/CARR), one unit corresponds to 0.8 mg/L of hydrogen peroxide.

For BAP, 50 μL of chromogenic substrate (R2 kit reagent) and 1 mL of reactive solution (R1 kit reagent) were gently mixed in a cuvette, and absorbance at 505 nm was recorded. A 10 μL seminal plasma sample was then immediately added to the cuvette. After mixing, the cuvette was quickly incubated in the thermostatic block of the analyzer for 5 min at 37 °C, and absorbance at 505 nm was recorded. The results were expressed as mmol/L of reduced ferric ions.

2.10. Statistical analysis

Statistical analyses were carried out using SPSS (29.0.1) for Windows 10 (SPSS Inc., Chicago, IL). The normal distribution and the homogeneity of variance of data was verified using the Shapiro-Wilk and Levene's tests, respectively. Data were log-transformed when necessary. The post-incubation sperm quality parameters of frozen-thawed semen were analyzed 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 for post hoc multiple comparisons between different incubation times (T1 and T2) and between groups at the same incubation time. The differences in ROMs and BAP values, as well in penetration rates, between the D0-control and D100 OFE group were analyzed by Student's *t*-test, setting the significance level at $P < 0.05$.

3. Results

3.1. Effects of OFE treatment on frozen-thawed buffalo sperm quality at different incubation times

The results of OFE treatment of frozen-thawed sperm on viability, membrane integrity, and morphology are reported in Table 1. No differences were observed in the percentages of total live sperm, sperm with functional membrane (HOS+), and sperm anomalies among groups and between incubation times. The treatment with OFE at all concentrations tested increased ($P < 0.05$) the percentage of AIL sperm compared to the D0-control at T1, but the effect was more evident with D100. No differences among groups were detected at T2, as, within OFE-treated groups, the percentage of AIL sperm decreased after 2 h incubation.

As shown in Table 2, both total and progressive motility decreased ($P < 0.01$) between T1 and T2 only in the D0-control group; the same

Table 1
Effects of different concentrations (D50, D100, and D150) of olive fruit extracts (OFE) treatment on the percentages of frozen-thawed buffalo total live sperm, acrosome-intact live (AIL) sperm, sperm with a functional membrane (HOS+), and total anomalies immediately after thawing (T0) and at different incubation times (T1 and T2). Data are expressed as mean ± SE.

Time	Treatment	Total Live	AIL	HOS+	Anomalies
T0	–	73.2 ± 3.7	67.0 ± 2.8	53.5 ± 3.7	18.6 ± 3.6
T1	D0-control	70.3 ± 4.6	54.5 ± 3.0^{x}	43.9 ± 3.4	12.3 ± 2.2
	D50	76.0 ± 3.2	60.5 ± 1.5^{ay}	42.4 ± 3.1	12.8 ± 2.3
	D100	73.6 ± 2.9	65.2 ± 3.3^{ay}	41.4 ± 3.2	13.1 ± 2.7
	D150	74.8 ± 3.9	62.5 ± 1.7^{ay}	43.3 ± 2.5	13.6 ± 2.7
T2	D0-control	68.7 ± 4.1	54.7 ± 3.4	39.4 ± 2.8	10.2 ± 1.7^{x}
	D50	67.9 ± 4.5	50.5 ± 3.8^{b}	42.7 ± 3.4	12.5 ± 2.4
	D100	67.5 ± 4.4	51.6 ± 2.9^{b}	37.4 ± 2.5	14.7 ± 2.4
	D150	68.1 ± 3.8	51.1 ± 3.2^{b}	39.9 ± 2.5	15.7 ± 2.2^{y}

^{a,b} Values with different superscripts are significantly different between incubation times; $P < 0.05$.
^{x,y} Values with different superscripts are significantly different among groups within incubation time; $P < 0.05$.

Table 2
Effects of different concentrations (D50, D100, and D150) of olive fruit extracts (OFE) treatment on the percentages of frozen-thawed buffalo sperm total motility, progressive motility, rapid, and rapid progressive spermatozoa immediately after thawing (T0) and at different incubation times (T1 and T2). Data are expressed as mean ± SE.

Time	Treatment	Total Motility	Progressive motility	Rapid	Rapid progressive
T0	–	51.3 ± 4.2	30.6 ± 2.9	25.4 ± 2.8	21.4 ± 2.6
T1	D0-control	43.6 ± 3.4 [a]	24.6 ± 1.4 [a]	19.4 ± 1.8 [a]	16.4 ± 1.6 [a#]
	D50	43.2 ± 4.5	27.6 ± 3.0	21.6 ± 3.1	19.5 ± 3.0 *
	D100	49.1 ± 5.4 [#]	32.4 ± 4.1 [x]	27.8 ± 4.4 [#]	24.7 ± 4.1 [x#]
	D150	35.7 ± 3.4 [#]	22.6 ± 1.5 [y]	17.7 ± 1.9 [#]	15.2 ± 1.0 [y]
T2	D0-control	30.2 ± 4.2[b]	17.0 ± 1.2 [bx#]	13.3 ± 1.8 [b]	11.2 ± 1.1 [b]
	D50	36.8 ± 5.0	23.7 ± 3.4	18.3 ± 3.8	16.1 ± 3.5 *
	D100	35.8 ± 3.9	22.8 ± 2.7 [#]	18.2 ± 2.5	16.1 ± 2.3
	D150	34.5 ± 4.0	22.6 ± 1.8 [y]	16.7 ± 1.5	15.2 ± 1.5

[a, b] Values with different superscripts are significantly different between incubation times; $P < 0.05$.
[x, y] Values with different superscripts are significantly different among groups within incubation times; $P < 0.05$.
*,[#] Indicate a tendency to significant differences between incubation times and among groups, respectively; $P < 0.10$.

pattern was observed for the percentage of rapid and rapid progressive spermatozoa ($P < 0.05$). At T1, progressive motility and the percentage of rapid progressive sperm were higher in semen treated with D100 compared to D150 OFE. The percentage of rapidly progressive sperm tended to increase ($P < 0.10$) in D100 OFE compared to the D0-control. At T2, progressive motility was higher ($P < 0.05$) in the D150 OFE group than in the D0-control and tended ($P < 0.10$) to be higher in D100 OFE.

The effect of OFE treatment on sperm kinetics parameters are reported in Table 3. The treatment with D100 OFE of frozen-thawed sperm increased ($P < 0.05$) VAP and WOB and tended to increase ($P < 0.10$) STR compared to the D0-control at T1.

3.2. Effects of OFE treatment on in vitro sperm penetration

The inclusion of D100 OFE into the IVF medium did not influence the total penetration rate (86.7 ± 4.7 and 82.7 ± 4.2, respectively in the D0-control and D 100 OFE), as well as the normospermic (78.9 ± 1.1 and 82.8 ± 1.3, respectively in the D0-control and D 100 OFE) and polyspermic penetration rates (21.0 ± 1.0 and 17.3 ± 1.3, respectively in the D0-control and D 100 OFE). Instead, the 1 h incubation of frozen-thawed sperm with D100 OFE increased ($P < 0.01$) the total and the normospermic penetration rate, without affecting polyspermy, as shown in Fig. 2.

3.3. Effects OFE treatment of frozen-thawed buffalo semen on OS markers at different incubation times

The treatment with D100 OFE increased ($P < 0.05$) BAP of frozen-thawed sperm both at T1 and T2 (Fig. 3A). However, no significant differences were recorded in the ROS levels between groups at both incubation times (T1 and T2), although values in the OFE-treated group were numerically lower (Fig. 3B).

4. Discussion

This work hypothesized that incubating frozen-thawed buffalo sperm with OFE would improve sperm quality by protecting sperm from OS-induced damage during thawing and handling processes. The rationale of the work derives from the high sensitivity of buffalo sperm to oxidative damage during freezing-thawing [11,22,50] and the known antioxidant function of OFE [34]. To the best of our knowledge, this is the first report that investigated the effect of OFE on buffalo frozen-thawed sperm. The results of this study demonstrated that incubating frozen-thawed buffalo sperm with D100 OFE (corresponding to 100 μM HT) improves sperm quality, as indicated by increased acrosome integrity, motility, some kinetic parameters, and *in vitro* fertilizing ability, by enhancing the antioxidant capacity. Hence, the approach of incubating sperm with a natural plant-derived antioxidant additive like OFE was effective in mitigating OS during thawing and sperm handling before IVF, which is a primary contributor to sperm damage.

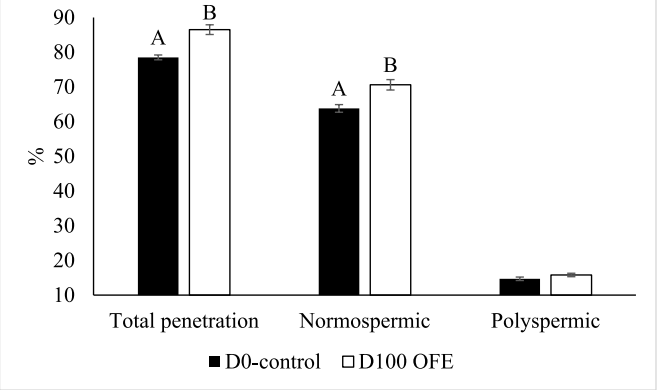

Fig. 2. Total, normospermic, and polyspermic penetration rates of frozen-thawed semen untreated (D0-control) or treated with D100 olive fruit extract (OFE). Data are expressed as means ± SE.
[A, B] Bars with different letters are significantly different; $P < 0.01$.

Table 3
Effects of various concentrations (D50, D100, and D150) of olive fruit extracts (OFE) on frozen-thawed buffalo sperm kinetic parameters at different time points: immediately after thawing (T0) and after incubation times (T1 and T2). Data are expressed as mean ± SE.

Time	Treatment	VCL (μm/s)	VAP (μm/s)	VSL (μm/s)	STR (%)	LIN (%)	WOB (%)	ALH (beats/s)	BCF (beats/s)	Hyp (%)
T0	–	50.7 ± 1.8	31.2 ± 2.3	39.9 ± 2.1	56.9 ± 2.3	70.5 ± 1.6	75.6 ± 1.8	1.6 ± 0.1	6.2 ± 0.4	0.2 ± 0.1
T1	D0-control	46.2 ± 2.8	30.0 ± 2.2[x]	34.3 ± 3.2	60.0 ± 3.3 [a]	65.3 ± 2.8	75.0 ± 2.2[x]	1.5 ± 0.1	6.0 ± 0.5	0.5 ± 0.3
	D50	47.8 ± 2.2	33.9 ± 2.1	37.3 ± 2.8	65.5 ± 2.8	69.8 ± 3.2	78.1 ± 1.6	1.5 ± 0.1	6.0 ± 0.3	0.3 ± 0.3
	D100	50.0 ± 2.1	37.1 ± 2.2[y]	40.1 ± 2.8	67.5 ± 2.1[a]	71.4 ± 2.6	80.6 ± 1.1[y]	1.4 ± 0.1	6.6 ± 0.3	0.1 ± 0.1
	D150	48.3 ± 1.9	33.6 ± 1.7	37.4 ± 2.4	63.8 ± 3.1	68.3 ± 2.4	77.4 ± 2.0	1.5 ± 0.1	6.4 ± 0.4	0.1 ± 0.1
T2	D0-control	45.7 ± 2.0	31.1 ± 2.0	34.8 ± 1.9	62.7 ± 2.8	68.0 ± 2.3	76.7 ± 1.7	1.4 ± 0.1	5.8 ± 0.3	0.4 ± 0.3
	D50	47.3 ± 2.2	33.4 ± 2.3	35.6 ± 2.4	64.5 ± 3.1	67.2 ± 1.9	76.6 ± 1.8	1.4 ± 0.1	6.0 ± 0.3	0.4 ± 0.3
	D100	48.2 ± 2.2	34.0 ± 2.0	38.0 ± 2.3	65.6 ± 3.1	70.3 ± 2.1	79.6 ± 1.5	1.4 ± 0.1	6.2 ± 0.3	0.1 ± 0.1
	D150	47.5 ± 2.1	34.5 ± 2.9	37.3 ± 2.3	65.5 ± 3.4	70.3 ± 2.2	78.7 ± 1.5	1.4 ± 0.1	5.9 ± 0.4	0.3 ± 0.3

[x,y] Values with different superscripts are significantly different among groups, within incubation times; $P < 0.05$.
[a] Indicate a tendency to significant differences among groups, within incubation times; $P > 0.10$.

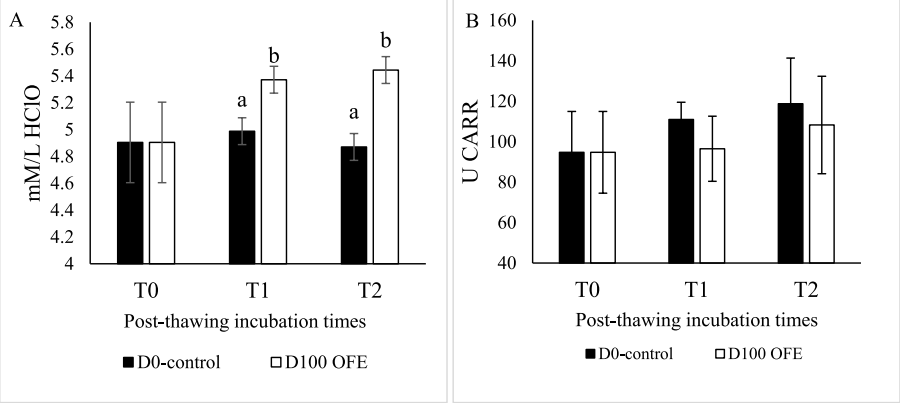

Fig. 3. Biological antioxidant potential (**A**) and ROMs (**B**) of frozen-thawed semen untreated (D0-control) or treated with D100 olive fruit extract (OFE) immediately after thawing (T0) and after 1 (T1) and 2 h (T2) incubation. Data are expressed as means ± SE.
[a, b] Bars with different superscripts differ significantly; $P < 0.05$.

The choice to test the effects of OFE was due to the lower costs of the extract compared to individual phenols, and the potential applications of using olive wastes as pro-fertility products, contributing to a sustainable circular economy. Furthermore, it was hypothesized that the utilization of OFE rather than individual compounds might be more effective due to the synergistic interaction among bioactive phenolic compounds [41,51,52]. To the best of our knowledge, olive extracts have not been previously employed as semen additives in any species, but a protective effect against induced reproductive toxicity, modulated by antioxidant action, has been reported in laboratory animals after oral administration [42–45,53].

In this work, treatment with D100 OFE improved post-thawing semen qualitative parameters and fertilizing ability by preventing OS, as shown by the increased total antioxidant capacity. This may be attributed to the antioxidant properties of OFE phenolic compounds [54–56], and particularly to HT that was the most abundant in the extract here used. However, despite the increased BAP, no significant differences were recorded in the ROS levels between groups at any incubation times, although values in the OFE-treated group were numerically lower. Contrasting effects on ROS levels have been reported for olive derivatives in various types of cells and under different conditions [40,41,57]. Several factors contribute to the efficacy of phenolic antioxidants, including their chemical structure, concentration, and timing of application [57]. It has been previously described that OS in frozen-thawed buffalo semen is not primarily due to an increase in ROS levels but rather to a reduction in antioxidant enzyme levels during the freezing-thawing process [58,59]. The decline in the seminal antioxidant profile during cryopreservation exacerbates the depletion of the natural antioxidant capacity of the semen [60]. Therefore, it seems more likely that the improved antioxidant defense in the OFE-treated group is related to the preservation or the restoration of antioxidant enzymes, previously attributed to HT [40,41].

The increased BAP observed in semen treated with D100 OFE may account for the improved proportion of AIL sperm after 1 h incubation and the preserved motility observed in the present study, as a positive association between these parameters has been previously reported [61,62]. An improved antioxidant capacity may counteract the deleterious effects of OS [62]. The sperm plasma membrane and acrosome are the primary sites of injury in spermatozoa during the freezing-thawing process, affecting sperm viability, acrosome integrity, and motility [63–65]. The proportion of AIL sperm is a reliable predictive marker of sperm fertilizing capability [65], as a positive correlation was previously found with the blastocyst yields in buffalo [49]. However, this beneficial effect was transient, as a significant decrease was recorded after 2 h of incubation, reaching values similar to the control.

A positive effect of OFE was also recorded on sperm motility, one of the most reliable indicators of sperm fertilizing potential [66]. Particularly, sperm treatment with OFE was effective at preserving total and progressive motility, as well as the percentages of rapid and progressive sperm over incubation time, suggesting a delay in the temporal decline of motility, that was, in contrast, observed at T2 in the D0-control group, with no supplement. Furthermore, the significant increase of VAP and WOB and a tendency to higher STR recorded in the D100 OFE group suggest a beneficial effect on sperm kinetics. The positive impact of OFE on sperm quality may be attributed to its antioxidant properties. The ability of HT, tyrosyl, and their derivatives to effectively inhibit lipid peroxidation and maintain membrane stability [67] could explain the preservation of acrosome and plasma membranes by OFE. Moreover, HT is known to enhance cell mitochondrial function and ATP production [68,69] suggesting a potential role in providing the energy necessary to maintain post-thawing sperm motility.

Sperm viability was not affected by the treatment, but remained particularly high after incubation, indicating the good quality of the semen. It is known that the efficacy of antioxidant treatments may depend on the semen quality [70], with better protection observed in low-quality semen, due to their lower antioxidant status [39,71]. Interestingly, it has been reported that the variation in cryotolerance among buffalo bulls is related to lysine succinylation and mitochondrial lipid metabolism [14]. Therefore, we cannot rule out that the effects of OFE would be more evident on low-quality semen, suggesting further investigations.

It is worth noting that OFE has not been previously used as an additive to semen extender before cryopreservation and as a post-thawing treatment before IVF, making it challenging to directly compare our results with previous findings. In the majority of these studies, HT has been added in the extender before freezing [37–39,47]. A positive effect of the addition of HT on sperm motility has been reported after cryopreservation in bucks, rams [37,47], and bad freezer bulls [39], or after prolonged preservation at 17 °C in swine semen [40]. However, human semen incubation with HT improved sperm viability and DNA integrity without affecting motility [41,57]. Neither OFE nor HT have been used to improve the quality of frozen-thawed semen. However, treatment of sperm with antioxidants after thawing has been successfully employed in various species [16,17,25,72–75] and can be a suitable strategy to prevent OS due to this procedure, as washing results in the removal of additives, including antioxidants, from the extender [76].

In the present study, D100 OFE was chosen to assess sperm IVF ability by heterologous IVF on bovine oocytes, due to the shortage of homologous oocytes. This was tested either including OFE during IVF or by short-term incubation of sperm prior IVF. When IVF was carried out in the presence of D100 OFE total and normospermic penetration rates were not affected, while a 1 h incubation of sperm with D100 OFE prior

IVF significantly increased total penetration and, more importantly, the percentage of normospermic penetration. The observed increase in penetration rate following heterologous IVF is noteworthy, as previous studies have shown a strong correlation between this parameter and cleavage and blastocyst yields achievable through homologous IVF [10]. These results suggest that a short treatment of sperm is more effective in increasing IVF capability. Therefore, a 1 h treatment with OFE protects frozen sperm from the thawing/washing-induced OS caused by the removal of antioxidants from the extender, as suggested by increased BAP, whereas a long-term treatment, i.e. the addition of OFE during the entire gametes co-incubation time, was ineffective. It can be speculated that providing OFE throughout the entire IVF process may result in an excess of antioxidants. Excessive levels of antioxidants could disrupt the cellular redox balance, potentially increasing sperm susceptibility by enhancing membrane fluidity [52,77]. Instead, moderate levels of ROS are required for successful capacitation, acrosome reaction, and oocyte fusion [78].

In conclusion, it was demonstrated that treatment with D100 OFE (100 μM HT) of frozen-thawed buffalo sperm improved semen quality, as indicated by an increased percentage of AIL sperm, enhanced kinetic parameters such as VAP and WOB, preserved motility over incubation time, and increased total and normospermic penetration rates after IVF. It was also demonstrated that the improved semen quality was associated with increased semen antioxidant capacity. Therefore, the treatment with OFE may be an effective strategy to improve IVF ability of frozen-thawed buffalo sperm. As this study was performed on semen collected from bulls with good fertility, it will be worth evaluating the effect of OFE also on buffalo low-quality semen. Furthermore, it will be worth investigating in future studies the effect of OFE treatment of buffalo semen on *in vitro* fertility, by assessing blastocyst rates after homologous IVF, for potential commercial applications.

Declarations of competing interest

None.

Data availability

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

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CRediT authorship contribution statement

Maria Paz Benitez Mora: Writing – original draft, Investigation, Conceptualization. **Chiara Del Prete:** Writing – original draft. **Valentina Longobardi:** Methodology, Data curation. **Natascia Cocchia:** Supervision. **Riccardo Esposito:** Investigation. **Federica Piscopo:** Investigation. **Andrea Sicari:** Investigation. **Francesco Vinale:** Methodology. **Alice Carbonari:** Methodology. **Bianca Gasparrini:** Writing – review & editing, Methodology, Conceptualization.

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