Article

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Olea europaea Leaf Extract: Antioxidant Properties and Supplement in Human Sperm Cryopreservation

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Background: Semen handling and cryopreservation increase reactive oxygen species (ROS) production, exposing spermatozoa to oxidative stress (OS) that can be minimised by antioxidant supplementation. We studied if *Olea europaea* leaf extract (OE) could have antioxidant and protective activity during sperm manipulation.
Methods: The extract was characterized by high-performance liquid chromatography with diode array detection (HPLC-DAD) and antioxidant activity was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC) assays. Then, spermatozoa of 35 normozoospermic donors were treated with OE. First, swim-up selected spermatozoa were incubated with OE (1:100–1:400) and sperm motility and DNA integrity (acridine orange test) were assessed. Then, swim-up selected sperm were treated with 100 µM H_2O_2 to induce OS with and without OE; motility, DNA integrity and F_2-Isoprostanes (F_2-IsoPs quantified by gas chromatography/negative ion chemical ionization tandem mass spectrometry analysis), an OS marker, were assessed. Finally, sperm were frozen with and without OE. Beside the previous endpoints, acrosome shape was evaluated by Tetramethylrhodamine (TRITC)-conjugated *Pisum sativum* agglutinin (PSA).
Results: OE was enriched in polyphenols (0.34%) and triterpenes (0.72%) and the synergistic action of phytocomplex components was responsible for antioxidant activity. Since OE was not toxic for spermatozoa, 1:100 dilution was used for the other experiments. OE protected motility, DNA integrity and reduced F_2-IsoPs ($p < 0.001$) in *in vitro* experiment with OS induction. OE treated frozen-thawed spermatozoa showed increased motility, DNA integrity, reduced F_2-IsoP concentration ($p < 0.001$) and normal acrosomes *versus* non-supplemented samples.
Conclusions: OE is characterized by high concentration of polyphenols and exhibits protective properties against oxidative damage induced by H_2O_2 in human ejaculated sperm. After cryopreservation, the samples supplemented with the extract showed increased sperm quality. OE could represent a supplement of culture media during semen handling where OS is exacerbated.

Keywords: antioxidant activity; cryopreservation; human sperm parameters; F_2-IsoProstanes; *Olea europaea* leaf extract

Introduction

Olea europaea L., olive tree, is one of the most known species in the plant kingdom as regards its nutritional properties [1], especially (extra-virgin) olive oil, which is also known for its health effects. The leaves of *O. europaea* are an interesting low-cost by-product from which bioactive compounds useful in nutraceutical, industrial, biomedical, and cosmetic fields can be obtained. This is in line with the sustainable circular economy model where wastes and by-products are not discarded but considered as reusable and recyclable resources [2,3]. Recently, *O. europaea* leaves have attracted attention for their antioxidant, antibacterial [4], and anti-inflammatory effects [5,6], as well as for their activity against many diseases such as cardiovascular problems [7,8]. In addition, leaf extract plays a role in lowering blood pressure, plasma lipids, and inflammatory markers [9], as well as stimulating thyroid activity [10]. These beneficial effects are due to the substances contained in the leaves, especially the phenolic compounds that exert antioxidant activity such as oleuropein, tyrosol, hydroxytyrosol [11–14] and many others. Recently, the protective effects of *O. europaea* leaf extract (OE) were also tested in the male reproductive system. For this purpose, animal models, mainly mice and rats, were treated with toxic compounds such as rotenone [15], busulfan [16], cyclophosphamide [17], lead acetate [18], chlorpyrifos (organophos-

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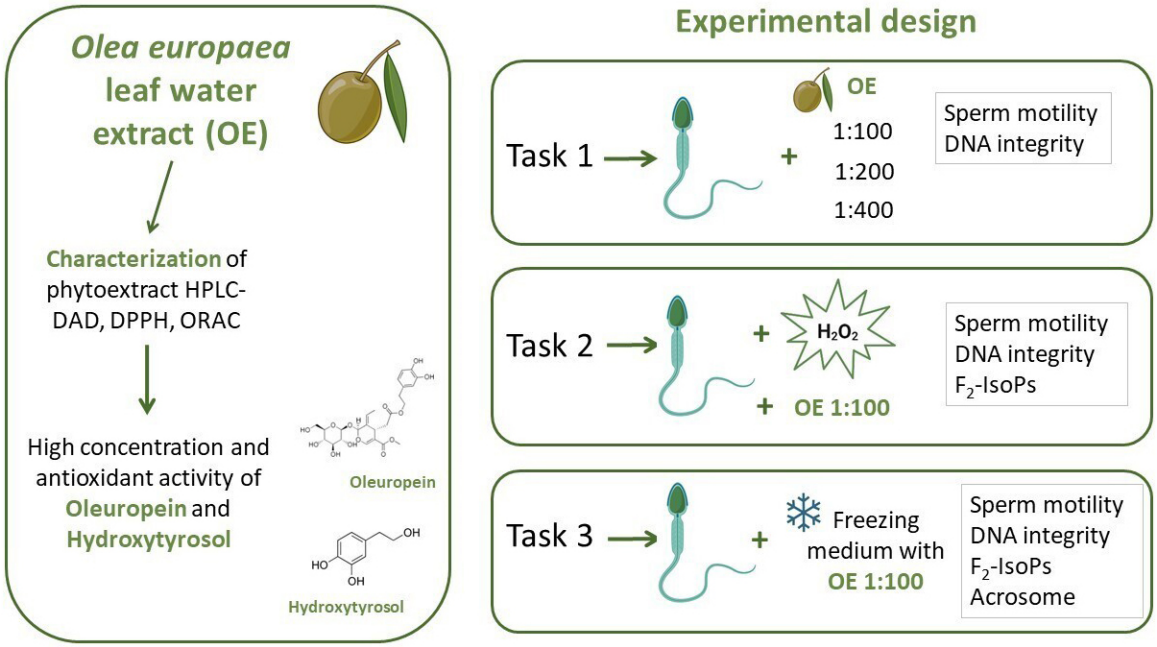


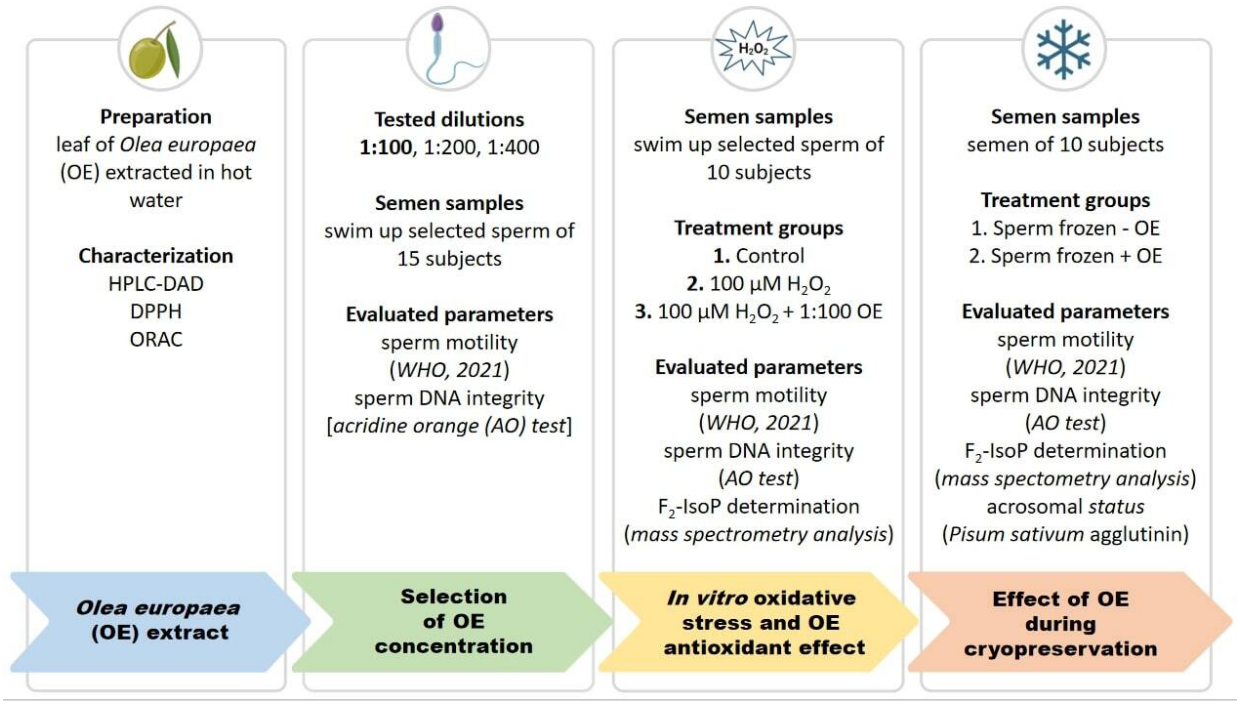
Fig. 1. Experimental design that includes *O. europaea* leaf extract (OE) production, characterization and use in experiments (Tasks 1–3) with human spermatozoa. The phytoextract was characterized with high-performance liquid chromatography with diode array detection (HPLC-DAD), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC) assays. In Task 1 swim-up selected human spermatozoa were treated with OE diluted 1:100, 1:200, 1:400 to find an OE concentration to be used for the other Tasks. In this case, sperm motility and DNA integrity represented the endpoints evaluated. In Task 2, swim-up selected human spermatozoa were treated with 100 µM H_2O_2 to induce oxidative stress with OE diluted 1:100 or without OE. The OE protective effect was determined by evaluating sperm motility, DNA integrity and F_2-IsoP concentration, a marker of oxidative stress. In Task 3, human semen was frozen with OE diluted 1:100 or without OE. The OE protective and antioxidant effect was assessed analyzing the following endpoints: sperm motility, DNA integrity, F_2-IsoP concentration, and the acrosome. F_2-IsoP, F_2-Isoprostane.

phorus insecticide [19] and with OE. Overall, the results of the different studies concur that olive tree leaf extracts improve sperm parameters and antioxidant status of the testes and play a protective role against oxidative stress (OS) and apoptosis in testicular tissue.

It is known that reactive oxygen species (ROS) play a dual role in sperm function. When produced at low levels, they regulate motility, capacitation, acrosome reaction, and sperm-oocyte interaction. However, an overdose of ROS and/or an imbalance between the ROS concentration and the antioxidant scavenging system affect normal sperm function by damaging DNA, proteins, and sperm membranes, causing lipid peroxidation (LPO) due to the high content of polyunsaturated fatty acids [20,21]. LPO is measured by end-products of lipid peroxidation such as aldehydes [22], but also isoprostanoids. For example, F_2-Isoprostanes (F_2-IsoPs), formed *in vivo* via a non-enzymatic mechanism involving free radical-triggered peroxidation of arachidonic acid, are one of the most reliable biomarkers for the assessment of LPO in sperm and semen [23–25].

The high level of ROS may be due to exogenous factors such as centrifugation, exposure to light, pH and temperature fluctuations as well as cryopreservation [26–28]. Although cryopreservation has a prominent role in the treatment of infertility, particularly in the preservation of sperm before oncological therapies and in patients with spermatogenetic alterations [29], this procedure promotes ROS formation, causes sperm damage [21], and impairs fertilization and early embryonic development [30].

The reason why we conducted this study is that a strategy to minimize OS during sperm manipulation includes supplementation of media with antioxidants. Many compounds with antioxidant activity have been tested *in vitro* on human spermatozoa [31]. The finding of the best antioxidants and their optimal concentrations being used during the *in vitro* supplementation of media for semen handling is an open area of research. In this regards, the characterization and the use of OE in sperm handling could be relevant. Therefore, the aim of the study was first to characterize the aqueous extract of *O. europaea* leaves collected as pruning wastes in Southern Italy, by quantifying the total polyphenols and triterpenes, analysing the polyphenol fraction by high-performance liquid chromatography with diode array detection (HPLC-DAD), and determining the antioxidant activity by cell-free assays. Then, the effect of the extract on OS induced *in vitro* in human spermatozoa was tested by evaluating sperm motility, DNA integrity, and antioxi-



Scheme 1. The scheme shows a roadmap supporting the steps of the study. First, leaves of *Olea europaea* were extracted in hot water and the hydro-extract was characterized by HPLC-DAD, DPPH and ORAC. In the second step, different dilutions of the extract were tested on swim-up selected spermatozoa to evaluate the effects on sperm motility (World Health Organization (WHO) guidelines for semen analysis, 2021) and DNA integrity (acridine orange test, AO). In the third step, swim-up selected spermatozoa were treated with 100 µM H_2O_2 to induce oxidative stress and with or without the extract, then sperm motility, DNA integrity and F_2-IsoP level (mass spectrometry analysis) were assessed. Finally, semen samples were frozen with or without the extract; the analysis of sperm motility, DNA integrity, F_2-IsoP level and the status of acrosome (*Pisum sativum* agglutinin test) represented the endpoints.

dant capacity by measuring F_2-IsoPs as biomarkers of OS. Finally, the extract was directly used in sperm cryopreservation experiments to test its potential protective effect in a procedure that increases ROS production.

Materials and Methods

Fig. 1 shows the experimental design.

First, the extract was produced, characterized and the antioxidant activity was studied with cell-free methods. Then, the extract was used in experiments with human sperm, which included three Tasks:

(1) testing the extract concentrations and possible toxic effect; choice of the concentration for the other Tasks.

(2) OS was induced *in vitro* with and without the extract to test the possible protective effect on sperm damage.

(3) extract was used as supplement in sperm cryopreservation protocol.

A roadmap of the study supporting the experimental design is shown in Scheme 1.

Collection and Preparation of Olive Leaf Extracts

In this work, we considered pruning waste of *Olea europaea* L. cultivar Nocellara collected in March 2022 in Palazzo Adriano (Palermo, Sicily, Italy). The herbal material was completely dried for about 30 days at room temperature in a cool, dry and dark place. Then the leaves were separated from the branches and were semi-finely ground. The samples were identified by Prof. Elisabetta Miraldi and Prof. Claudia Angiolini (Department of Life Sciences, University of Siena). Part of the samples were prepared for the Herbarium Universitatis Senensis (SIENA). The other leaf samples were extracted in hot water. In detail, 1:10 drug/extract ratio (DER) was used: 3 g of cut herbal material was added to 30 mL of double-distilled water in a sealed 50 mL test tube. The sample was sonicated for 30 min, followed by a decoction for 20 min. Finally, the volume was adjusted to 30 mL. Two independent extraction procedures were performed. Phytochemical analyses were carried out on the *O. europaea* leaf aqueous extract and on the standardized *Olea europaea* leaf dry extract used as a reference standard (United States Pharmacopoeia (USP), Merck reference: 1478265 lot. F05120, USP extract).

Phytochemical Analyses

Quantification of Total Polyphenols and Total Triterpenes

The colorimetric Folin-Ciocâlteu (FC) assay was used to quantify total polyphenol content as previously described

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in [32]. Gallic acid was used as an external standard (Lot: #098K0245, Sigma-Aldrich, St., Louis, MO, USA). Total triterpenes were quantified by the vanillin perchloric acid method as described in [33]. Briefly: 190 µL of water and 300 µL of 5% (w/v) vanillin solution in glacial acetic acid were added to 10 µL of OE. After mixing, 1 mL of perchloric acid was added, and the samples were placed at 60 °C for 45 min and then cooled and brought to a volume of 5 mL with glacial acetic acid. The absorbance was measured at 540 nm with a plate reader spectrophotometer (N/S 96501047, Safas Monaco MP96, Montecarlo, France). Water was used as blank, and β-sitosterol was used as a reference standard (Lot: #0001126984, Sigma-Aldrich, St., Louis, MO, USA). Total polyphenols, expressed as gallic acid, and total triterpenes, expressed as β-sitosterol, were quantified by interpolation of absorbance data into standard calibration curves. Analyses were repeated in quadruplicate. Results were expressed as % of dry herbal material (dhm, mean ± standard deviation, SD).

HPLC-DAD Analysis of the Polyphenolic Fraction

OE and United States Pharmacopoeia (USP) extracts were filtered with a 0.45 µm filter. HPLC analyses were performed using a Shimadzu Prominence-i LC-2030C 3D Plus Liquid Chromatograph instrument (N/S L21455510075; Shimadzu Europa GmbH, Duisburg, F. R. Germany).

The following method was used:

A: water + 0.1% formic acid; B: acetonitrile + 0.1% formic acid.

The following gradient was set for the analyses:

B: 10% v/v to 25% v/v in 15 min; 35% v/v at 18 min; then at 50% v/v until the end (at 25 min).

The flow was adjusted to 0.8 mL/min and the injected volume of sample was 10 µL. Absorbance was recorded at 280 nm and 366 nm and the different constituents identified and quantified, based on the retention time (RT) and the UV-vis spectrum: oleuropein (OLE), hydroxytyrosol (HT), tyrosol (TYR), caffeic acid (CA) and luteolin (LUT). The calibration curves were carried out with concentrations from 0.16 µg to 10 µg in all injected standards. Analyses were replicated in quadruplicate. Results were expressed as % in dhm (mean ± SD).

Determination of Antioxidant Capacity of Olive Leaf Extract by Cell-Free Assays

The 2,2-diphenyl-1-picrylhydrazyl (DPPH, Lot: 0000155812, Sigma-Aldrich, St., Louis, MO, USA) assay was performed on OE and on its main constituents OLE, HT, and oleanolic acid (OA), considered as the main triterpene of OE. TYR and LUT were added as references for their derivatives; the assay was performed according to the optimized method published in [34]. Ascorbic acid was used as positive control.

Oxygen radical absorbance capacity (ORAC) assay was tested for OE, OLE and HT according to the data sheet of the kit used (Ref. KF1004, Lot: 10042111; Bioquochem ORAC Antioxidant Capacity Assay Kit, Asturias, Spain).

DPPH-HPLC-DAD Analysis

To accurately monitor the role of the different OE constituents in term of their antioxidant activity, the DPPH assay was coupled with the HPLC-DAD analysis as reported in [35].

Semen Samples

Semen samples were provided by 35 healthy men (21–34 years old) with normal semen parameters. The study was carried out according to the Declaration of Helsinki, and the protocol was approved by the Ethics Committee: ID CEAVSE 18370; Ethics Committee Siena University Hospital. All participants were informed of the study, and they provided an informed written consent before the inclusion on this research. They agreed that their semen could be used for scientific purposes. Samples were collected by masturbation after 3 to 5 days of sexual abstinence and were incubated at 37 °C for 30 min to liquefy. Semen analysis was then performed according to World Health Organization (WHO) guidelines [36] and semen volume, pH, sperm concentration, and motility were measured.

Pre-stained Testsimplets® slides (Ref. 191574, Waldeck GmbH & Co. KG, Münster, Germany) were used to assess sperm morphology.

Selection of Motile Sperm Population

The effect of OE on human spermatozoa (Task 1) and the *in vitro* OS induction experiment (Task 2) were performed on a selected human sperm population that was as homogeneous as possible. Summarizing, 1 mL of Sperm Washing Medium IrvineScientific® (Ref. 9983, Lot: -10-0000015321, Santa Ana, CA, USA) was layered on top of 1 mL of each semen sample in sterile conical centrifuge tubes. The tubes were tilted at a 45° angle and incubated at 37 °C for 45 min. Then, 1 mL of the uppermost medium, rich in motile spermatozoa, was recovered and used for the different experiments *in vitro*.

Treatment of Human Spermatozoa with OE

Human spermatozoa were treated with OE following this experimental design (see Fig. 1 and Scheme 1):

-Task 1: evaluation of the possible OE toxic effects on selected spermatozoa of 15 donors. The endpoints considered were sperm motility and DNA integrity.

-Task 2: induction of LPO and OS in selected human spermatozoa (10 semen samples) and supplementation with OE to test the protective and antioxidant effects of the extract. The endpoints considered were sperm motility, DNA integrity and free F_2-IsoPs.

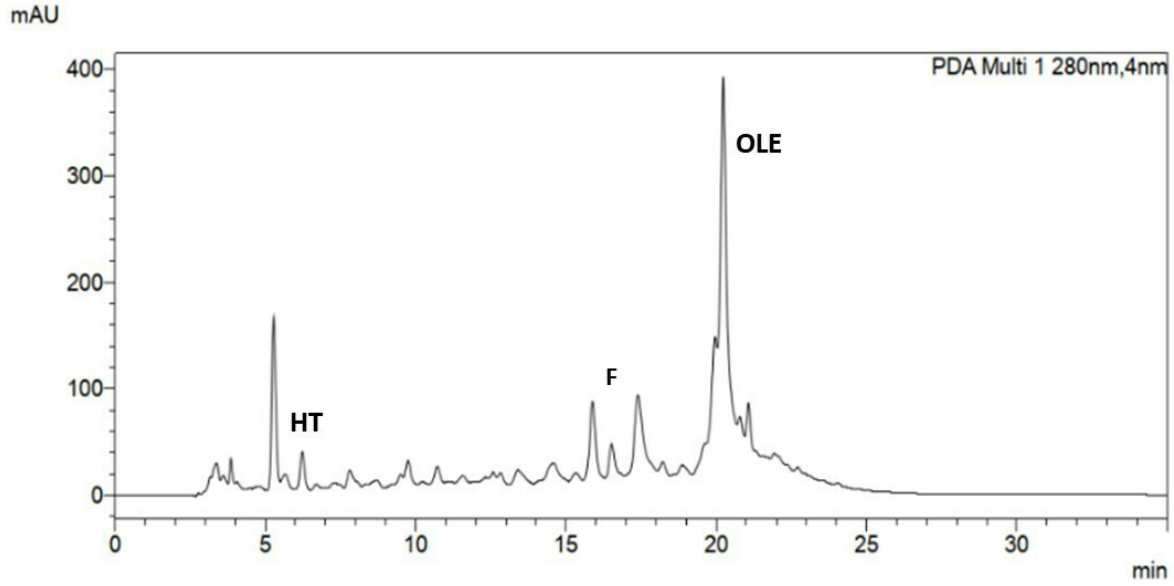

Fig. 2. Chromatogram of OE registered at 280 nm. HT, hydroxytyrosol; F, flavones; OLE, oleuropein.

-Task 3: 10 semen samples were frozen with and without OE supplementation. The endpoints considered were sperm motility, DNA integrity, total F_2-IsoPs, acrosome status.

OE Effects on Human Sperm Motility and DNA Integrity

These experiments were performed to exclude any toxic effect of OE on human sperm and to select the best concentration to use in the other experiments. The 15 selected sperm samples were treated with OE diluted 1:100, 1:200, and 1:400 and incubated at 37 °C for 1 h. Sperm treated under the same conditions but without OE represented the control. Sperm motility was assessed using a Burker counting chamber following WHO guidelines for semen analysis. Sperm DNA integrity was evaluated in the same samples using acridine orange (AO) test as reported in Noto *et al*. [37]. Spermatozoa smeared onto glass slides and stained with AO were analysed and scored with a Leitz Aristoplan fluorescence Microscope (S/N 507404, Leica Microsystems CMS GmbH, Wetzlar, Germany) equipped with a 490 nm excitation light and 530 nm barrier filter. For each sample, at least 300 sperm were analysed at ×1000 magnification. Sperm with double stranded DNA (dsDNA) showed green fluorescence, whereas sperm with denatured DNA showed yellow/orange/red fluorescence. Results were expressed as percentage of sperm with dsDNA (green fluorescence).

OE Effects on Induced Lipid Peroxidation in Human Sperm Samples

For this series of experiments, 10 sperm samples were used. The selected motile sperm population was divided into 3 aliquots and treated as follows:

untreated sperm as control (CTR);

sperm treated with 100 µM H_2O_2 to induce OS (H_2O_2);

sperm treated with both 100 µM H_2O_2 and OE diluted 1:100 to assay the protective effect of the extract (H_2O_2 + OE).

After incubation at 37 °C for 1 h, the samples were centrifuged at 400 g for 15 min. The supernatants, examined under the microscope to verify the absence of spermatozoa, were stored at –80 °C until F_2-IsoP levels were evaluated. Spermatozoa were used to assess motility % and DNA integrity % as previously reported.

F_2-Isoprostane Determination

Free F_2-IsoP level was measured in the media of sperm selection after treatment with 100 µM H_2O_2 and with both 100 µM H_2O_2 and OE 1:100 stored at –80 °C. Untreated samples were used as controls (Task 2).

Total F_2-IsoP content was measured in samples of cryopreservation experiments: an aliquot composed by semen and cryopreservation medium, the other composed by semen, cryopreservation medium and OE 1:100 (Task 3).

F_2-IsoPs are initially formed esterified on phospholipids and then released in free (unesterified) form by phospholipases. Thus, total F_2-IsoPs represent both molecules (esterified and free forms). To perform total F_2-IsoP measure, in each sample basic hydrolysis was carried out in presence of 1N KOH at 45 °C for 45 min. At the end of the incubation, sample acidification was obtained using 1N HCl and 500 pg tetradeuterated prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$-d_4) (Item No. 316010, Cayman, Ann Arbor, MI, USA) was added. For quantification of free F_2-IsoPs, 2 mL acidified water and 500 pg $PGF_{2\alpha}$-d_4 were added in each sample. Afterward, the same purification procedures were car-

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Table 1. Chemical composition of OE compared with USP extract.

	Polyphenols %	Triterpenes %	OLE %	HT %	Flavones %
OE	0.34 ± 0.03 (3.41 ± 0.34 dhm)	0.72 ± 0.06 (7.16 ± 0.57 dhm)	0.28 ± 0.06 (2.77 ± 0.63 dhm)	0.01 ± 0.01 (0.08 ± 0.01 dhm)	0.01 ± 0.01 (0.06 ± 0.01 dhm)
USP extract	19.92 ± 1.88	24.49 ± 3.06	20.73 ± 2.32	0.14 ± 0.01	0.39 ± 0.07

Total polyphenols are expressed as gallic acid; triterpenes as β-sitosterol; flavones as luteolin. OLE, oleuropein; HT, hydroxytyrosol; USP, United States Pharmacopoeia; dhm, dry herbal material. Values are expressed as mean ± standard deviation.

ried out in all samples. Firstly, each sample was applied to an octadecylsilane (C_{18}) cartridge (WAT043395, Sep-Pak® Vac C18, 500 mg, Waters, Milford, MA, USA) and subsequently each C_{18} extract was applied to an aminopropyl (NH_2) cartridge (WAT054560, Sep-Pak® Vac NH2, 500 mg, Waters, Milford, MA, USA). As a further step, the F_2-IsoP carboxylic group was derivatized as the pentafluorobenzyl ester in the presence of pentafluorobenzyl bromide (40 µL, 10% in acetonitrile, WAT054560, Sigma-Aldrich, St., Louis, MO, USA) whereas the hydroxyl groups were converted to trimethylsilyl ethers in the presence of N,O-bis(trimethylsilyl) trifluoroacetamide (50 µL, 10% in acetonitrile, N° CAT.15222, Merck, Sigma-Aldrich, St., Louis, MO, USA). Finally, F_2-IsoP determinations were carried out by gas chromatography/negative ion chemical ionization tandem mass spectrometry (GC/NICI-MS/MS) analysis (TRACE GC and PolarisQ Ion Trap,Thermo Finnigan, San Jose, CA, USA). F_2-IsoPs were quantified by identification/measure of the *m/z* 299 ion produced by ionization of 8-iso-$PGF_{2\alpha}$, the most represented F_2-IsoP isomer [38]. The reference 8-iso-$PGF_{2\alpha}$ compound (Item No. 16350, Cayman Chemical, Ann Arbor, MI, USA) was used for the calibration curve. The experiment was carried out in 10 samples. The results were expressed as pg/mL and ng/mL for free and total F_2-IsoPs, respectively.

Cryopreservation Protocol with OE Supplementation

Ten semen samples were used for this experiment. Each sample was divided into 2 aliquots and the cryopreservation medium (Test Yolk Buffer with gentamicin sulphate; Ref. 90128, Lot: -10-90128211107; FujiFilm, Irvine Scientific, Santa Ana, CA, USA) was added dropwise at a ratio of 1:1 (v/v) and gently mixed. For the treated sample, the cryopreservation medium was supplemented with OE diluted 1:100. Specimens were transferred into cryovials with a final volume of 0.3 mL. Cryovials were stored at 4 °C for 30 min, and then immersed in liquid nitrogen at –196 °C. Two weeks later, the cryovials were thawed at 37 °C for 10 min and sperm motility was assessed, as reported before. Then, the aliquots were centrifuged at 400 g, and the supernatant (medium/semen without spermatozoa) was used for F_2-IsoP determination; the spermatozoa were tested for DNA integrity using AO, as previously described.

Evaluation of Acrosome with *Pisum sativum* Agglutinin in Cryopreserved Spermatozoa

Tetramethylrhodamine (TRITC) conjugated *Pisum sativum* agglutinin (PSA, 21761046, Genelinx International Inc, dba bio WORLD, Dublin, Ireland) is a lectin that recognizes the carbohydrates of glycoproteins and is used for its ability to distinguish acrosome-intact from acrosome-damaged human spermatozoa. Experiments were performed with frozen-thawed spermatozoa both with and without OE 1:100. Samples were washed with PBS, centrifuged at 400 g for 10 min, suspended in PBS and smeared into glass slides. The slides were fixed in methanol for 20 min and in acetone for 5 min at –20 °C. Then the slides were rinsed in PBS for 10 min, incubated for 30 min (in the dark at room temperature) in TRITC-PSA solution diluted 1:1000 in PBS and rinsed again in PBS for 15 min.

The sperm nuclei were stained with a solution of 4′,6-diamidin-2-fenilindole (DAPI, Lot: #097M4033V, Sigma-Aldrich, St., Louis, MO, USA) diluted 1:20,000 in PBS for 10 min (in the dark at room temperature) and then rinsed in PBS. Finally, the slides were mounted with 1,4-diazabicyclo[2.2.2]octane (Lot: #BCBL1642V, Sigma-Aldrich, St., Louis, MO, USA) and observed with a Leica DMI 6000 fluorescence microscope (Leica Microsystems, Wetzlar, Germany). A Leica AF6500 Integrated System for Imaging and Analysis (Leica Microsystems, Wetzlar, Germany) was used for image acquisition. At least 300 spermatozoa were evaluated and classified into the following categories: acrosome-intact spermatozoa showed a homogeneously stained red cap, acrosome-altered spermatozoa include spermatozoa with a shrunken, short, and misshapen acrosome, acrosome-absent spermatozoa include spermatozoa without an acrosome.

Statistical Analysis

The SPSS for Windows software package (SPSS Inc, version 17.0, Chicago, IL, USA) was used to perform statistical analysis. The Kolmogorov-Smirnov test was used to verify the normality in the distribution of the variables. Since the values of the variables were non-normally distributed, specific non-parametric tests were applied. The Kruskal-Wallis test was used to compare the difference among different groups in experiments of OE toxicity (Task 1), *in vitro* assays (Task 2) and frozen samples (Task 3, regarding percentage of sperm motility and dsDNA). When a significant difference was found, the Tukey Post Hoc

test was applied for pairwise post-hoc testing. The Mann-Whitney U test was applied to compare the differences between two groups regarding the F_2-IsoP levels and the percentage of normal, altered, absent acrosomes in frozen samples treated with OE and untreated. Data were reported as median (interquartile range [IQR]). $p < 0.05$ was considered significant.

Results

Phytochemical Analyses

Phytochemical analysis was carried out to determine the main components of the extract. As expected, the OE was enriched in polyphenols and in triterpenes (0.34% and 0.72%, respectively). A prevalence of triterpenes over total polyphenols was also observed in USP extract. It should be noted that the FC reagent also reacts with OLE, although not as pure phenolic compound. Our analyses revealed that OLE reacted with the FC reagent providing 70–75% of the absorbance compared to gallic acid. OLE was the most enriched single compound in both OE and the USP extract. The predominant role of OLE was clearly evident in the chromatogram at 280 nm (RT = 20.2 min). HT was also present in both samples analysed (RT = 6.15 min) (Fig. 2).

TYR and CA were not identified as native compounds, because RTs did not match those of the standards. However, UV spectra recorded for peaks at similar RTs suggested that the presence of close derivatives of TYR and hydroxycinnamic acid derivatives is plausible. The presence of flavones, in glycosylated form, was also highlighted. Table 1 shows the summary of chemical characteristic of OE and, as a comparison, of USP extract.

Comparing USP extract and OE, it can be seen that the ratio between active constituents in the two preparations ranged from 1.75 (HT) to 7.48 (OLE), which is exactly the same as specified by the US Pharmacopoeia (drug:extract ratio 5–60:1). This suggests that OE is a good source of OLE, total polyphenols, and triterpenes, and consequently that by-products and pruining wastes of *O. europaea* could be considered in order to obtain valuable herbal preparations at low cost.

DPPH Assay

DPPH assay was used to determine the antioxidant activity of the whole extract and some components. LUT showed good antiradical activity, with half-maximal inhibitory concentration (IC_{50}) <100 µg/mL. On the other hand, OA showed only weak DPPH radical inhibition capacity (IC_{50} >100 µg/mL). OLE and HT showed the best antiradical capacity, higher than the other OE components and better than the reference compound, the ascorbic acid. Olive leaf aqueous extract showed an antiradical capacity, with IC_{50} at 618.1 µg/mL, corresponding to 61.8 µg/mL referred to the dry herbal material contained in the extract. The effective concentrations of OE and its individual components, as well as the quantitative OE composition, support the presence of synergy between the different components.

Table 2. Antiradical activity of OE and its most active constituents.

	DPPH assay IC_{50} µg/mL
OE	618.12 ± 82.40 (61.81 ± 8.24 dhm)
OLE	6.15 ± 0.67
HT	2.25 ± 0.28
Ascorbic acid	11.05 ± 2.11

IC_{50} of OE, oleuropein (OLE) and hydroxytyrosol (HT), determined with DPPH assay, compared to ascorbic acid is shown. Values are expressed as mean ± standard deviation. IC_{50}, half-maximal inhibitory concentration.

Table 3. ORAC assay results.

	TEAC mM/g
OE	0.72 ± 0.01
OLE	46.19 ± 21.78
HT	158.79 ± 34.26

This assay was used to evaluate the antioxidant activity of OE, oleuropein (OLE) and hydroxytyrosol (HT). Results are expressed as Trolox equivalents antioxidant capacity (TEAC) mean ± standard deviation.

In Table 2 the IC_{50} of OE, OLE, HT and ascorbic acid (as reference) is reported.

ORAC Assay

ORAC assay was performed to further test the antioxidant activity of OE. ORAC assay confirmed the good OE scavenging ability and, at the same time, the importance of the phytocomplex for the antioxidant activity of the extract. Again, OLE and, to a higher extent, HT showed an excellent antioxidant activity as single compounds. In Table 3 the antioxidant capacity of OE, OLE and HT, measured by the ORAC assay, and expressed as µM of Trolox equivalents is reported.

DPPH Assay Coupled to HPLC-DAD Analysis

To understand which components of the phytocomplex are involved in the antiradical activity of OE, we coupled the HPLC analysis with the DPPH assay. By comparing the chromatograms resulting from the analysis before and after the radical reaction (Fig. 3), it is possible to qualitatively and quantitatively identify which peaks are reduced, i.e., which compounds are consumed in the redox reaction. OLE contributed most to the antiradical activity, as indicated by a large decrease in peak area (Fig. 3A). Consistent with the postulated synergistic effect of the phytocomplex in providing the antiradical effect, the assay showed that hydroxycinnamic acid derivatives and flavones were also involved in DPPH radical scavenging activity (Fig. 3B).

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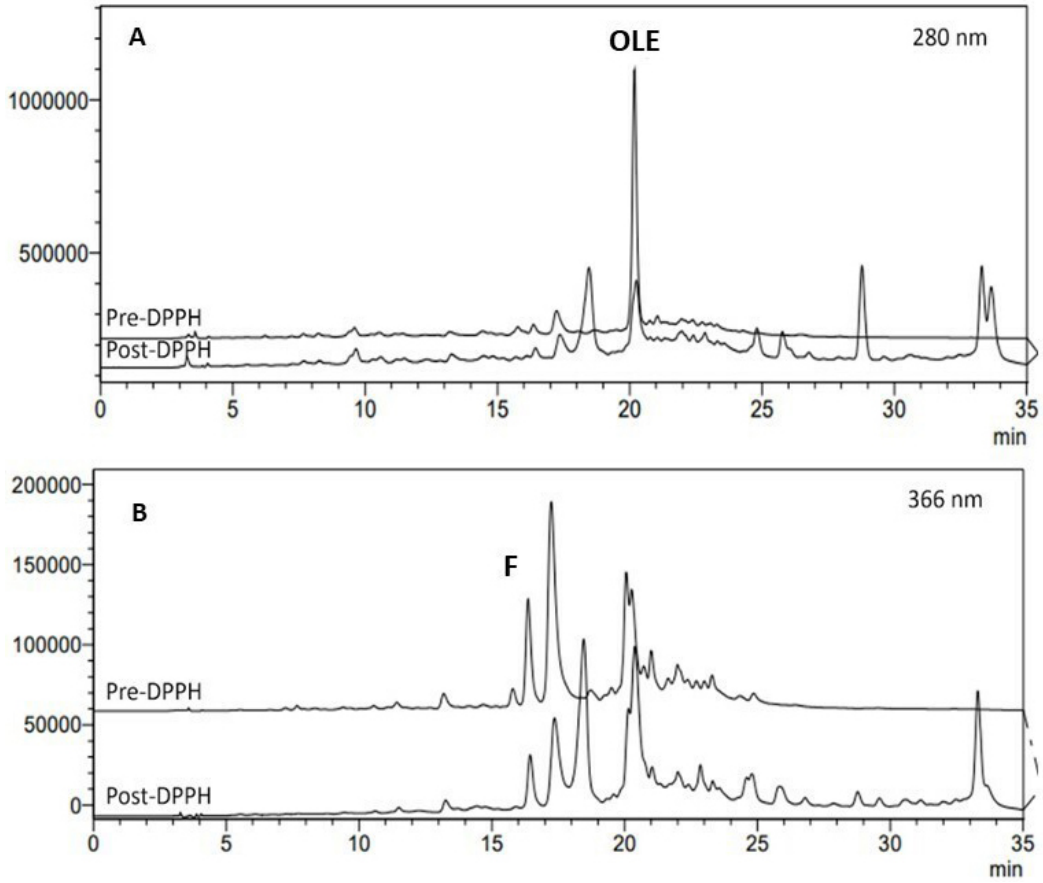

Fig. 3. Comparison of the chromatograms of OE before and after the reaction with the DPPH radical. (A) In the chromatogram recorded at 280 nm the change of the oleuropein (OLE) peak is visible. (B) In the chromatogram recorded at 366 nm the oxidation of the flavones (F) can be observed.

Semen Analysis

Semen analysis was performed according to the WHO guidelines [36]. All samples had normal semen parameters: sperm concentration ranged from 25th to 75th percentile, sperm progressive motility from 25th to 75th percentile, normal morphology from 25th to 75th percentile, and sperm vitality from 50th to 75th percentile.

Task 1. Effect of OE at Different Concentrations on Sperm Motility and DNA Integrity: The Selection of OE Concentration Used in the Other Experiments

Experiments were performed in swim-up selected spermatozoa of 15 different semen samples. The results showed that the OE diluted 1:100, 1:200, and 1:400 had no significant effect on progressive sperm motility and DNA integrity (Table 4).

Due to its tolerability, OE diluted 1:100 was used for the other experiments of the study.

Task 2. Oxidative Stress Induced in Vitro and OE Antioxidant Effect

For this Task, 10 semen samples were used and swim-up selected spermatozoa were treated with 100 µM H_2O_2 to induce LPO and with 100 µM H_2O_2 and OE diluted 1:100 in order to test the potential scavenging activity of the phytocomplex. An untreated sample under the same incubation conditions represented the control. The following endpoints were assessed: progressive sperm motility %, DNA integrity %, and free F_2-IsoPs.

Compared to controls, the sperm motility (Fig. 4) significantly decreased in the samples treated with 100 µM H_2O_2 both supplemented with OE and non-supplemented (both $p < 0.001$). A significant increase in percentage of sperm motility was observed in samples treated with 100 µM H_2O_2 and OE compared to samples treated with only 100 µM H_2O_2 ($p < 0.001$).

Regarding DNA integrity (Fig. 5), the non-supplemented samples treated with 100 µM H_2O_2 showed a lower percentage of spermatozoa with dsDNA than that observed in the control ($p < 0.001$) and in the samples treated with 100 µM H_2O_2 supplemented with OE ($p < 0.001$). No significant differences were found between controls and samples treated with 100 µM H_2O_2 and OE ($p = 0.115$).

Free F_2-IsoPs, marker for LPO and OS, were assessed in the medium. The concentration of F_2-IsoPs (Fig. 6)

Table 4. Percentage of spermatozoa with progressive motility and dsDNA treated with different OE concentrations.

	OE 1:100	OE 1:200	OE 1:400	CTR	Kruskal-Wallis test
Progressive motility%	80.00 [76.00–84.00]	79.00 [71.00–82.00]	79.00 [74.00–82.00]	81.00 [76.00–81.00]	$p = 0.923$
dsDNA%	98.00 [95.00–100.00]	98.00 [91.00–99.00]	95.00 [93.00–98.00]	96.00 [95.00–99.00]	$p = 0.658$

Median [IQR] of the percentages of progressive motility and double stranded (ds)DNA in 15 human sperm samples treated with different OE concentrations and controls (CTR). IQR, interquartile range.

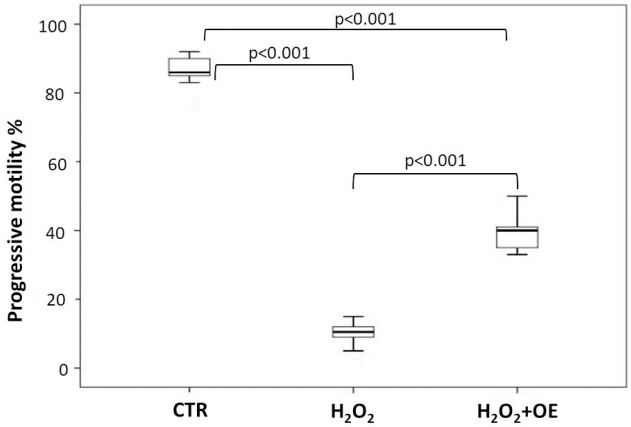

Fig. 4. Percentage of spermatozoa with progressive motility exposed to oxidative stress in presence and in absence of OE. Median [IQR] of progressive motility (%) in selected sperm samples treated with 100 µM H_2O_2 (H_2O_2), 100 µM H_2O_2 + OE 1:100 (H_2O_2 + OE) and control (CTR). The experiment was performed 10 times.

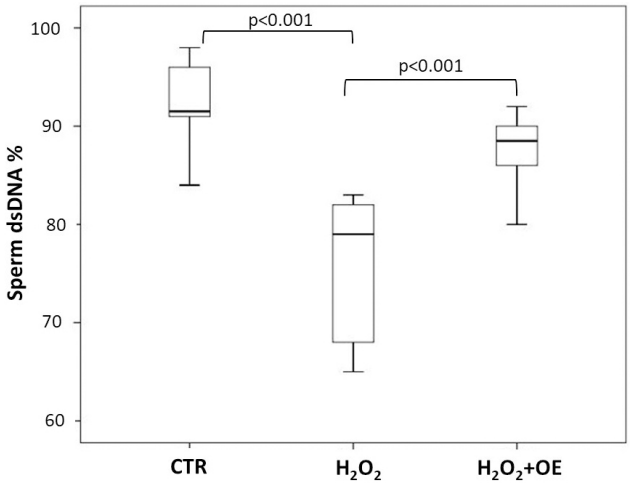

Fig. 5. Percentage of spermatozoa with dsDNA exposed to oxidative stress in presence and in absence of OE. Median [IQR] of percentage of selected sperm with dsDNA treated with 100 µM H_2O_2 (H_2O_2), 100 µM H_2O_2 + OE 1:100 (H_2O_2 + OE) and control (CTR). The experiment was performed 10 times.

was significantly higher in the 100 µM H_2O_2 treated samples (89.20 [81.20–91.50] pg/mL) than in OE supplemented ones (61.00 [54.00–63.60] pg/mL; $p < 0.001$), indicating that the extract exerted an antioxidant and protective effect.

The F_2-IsoP levels measured in controls (50.20 [47.20–55.80] pg/mL) were significantly lower than those detected in 100 µM H_2O_2 treated samples ($p < 0.001$) and slightly reduced compared to those observed in specimens supplemented with OE ($p = 0.054$).

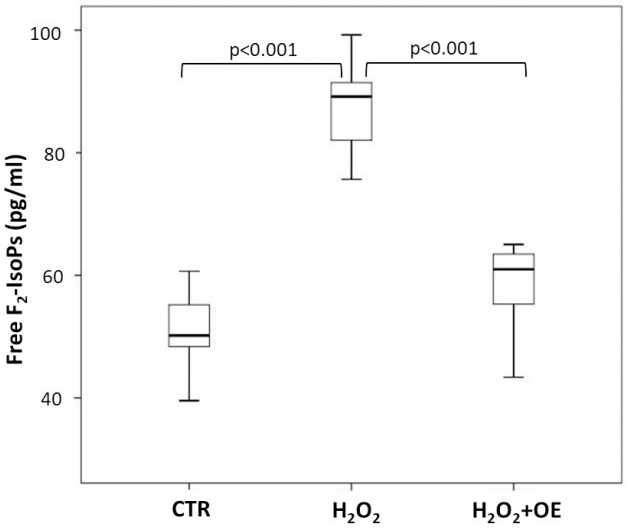

Fig. 6. Free F_2-IsoP levels in samples exposed to oxidative stress in presence and in absence of OE. Median [IQR] of free F_2-IsoP levels (pg/mL) evaluated in cell-free swim-up media after the samples were treated with 100 µM H_2O_2 (H_2O_2), 100 µM H_2O_2 + OE 1:100 (H_2O_2 + OE) and control (CTR). The experiment was performed 10 times.

Task 3. Effect of OE during Cryopreservation Protocol

The raw semen of 10 healthy donors was frozen both with and without OE diluted 1:100. After thawing, sperm progressive motility %, DNA integrity %, total F_2-IsoPs and the status of acrosome were analysed. Sperm motility percentage (Table 5) was significantly increased ($p < 0.001$) in thawed samples supplemented with OE (32.50% [29.00%–35.30%]) compared to non-supplemented samples (19.00% [15.80%–25.00%]). The percentage of sperm motility in raw semen (53.50% [49.30%–59.30%]) was significantly increased respect to both supplemented and non-supplemented frozen samples ($p < 0.001$). The percentage of spermatozoa with dsDNA (Table 5) was higher in thawed samples supplemented with OE (89.50% [89.00%–

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Table 5. Percentage of spermatozoa with progressive motility and dsDNA in semen cryopreservation experiments.

	Raw semen (R)	Frozen non-supplemented semen (NS)	Frozen supplemented semen (S)	Kruskal-Wallis test	Tukey Post Hoc test
Progressive motility %	53.50 [49.30–59.30]	19.00 [15.80–25.00]	32.50 [29.00–35.30]	$p < 0.001$	R vs NS $p < 0.001$ R vs S $p < 0.001$ S vs NS $p < 0.001$
Sperm dsDNA%	90.50 [89.80–93.30]	77.00 [75.00–80.50]	89.50 [89.00–93.30]	$p < 0.001$	R vs NS $p < 0.001$ S vs NS $p < 0.001$

Median [IQR] of percentages of sperm progressive motility and sperm double stranded (ds)DNA in raw semen (R), frozen non-supplemented (NS) and supplemented semen (S). The experiments were performed using 10 human semen samples.

93.30%]) than that detected in non-supplemented samples (77.00% [75.00%–80.50%], $p < 0.001$) and similar ($p = 0.903$) to that observed in raw semen before freezing (90.50% [89.80%–93.30%]). The same variable was significantly increased in raw semen respect to that observed in non-supplemented frozen samples ($p < 0.001$).

Total F_2-IsoP concentration and sperm acrosome were evaluated in frozen-thawed samples; data concerning raw semen are not present. As reported in Fig. 7 the total F_2-IsoP concentration in non-supplemented frozen-thawed samples (33.81 ng/mL [27.13 ng/mL–39.76 ng/mL]) was significantly higher than that evaluated in OE supplemented samples (16.19 ng/mL [13.35 ng/mL–17.82 ng/mL]).

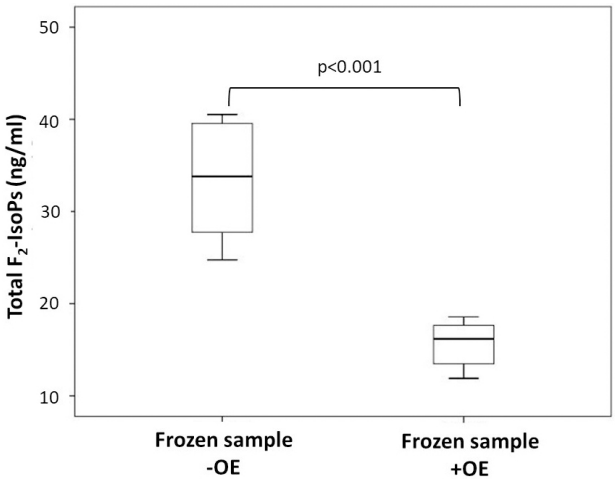

Fig. 7. F_2-IsoP levels in samples frozen with and without OE. Median [IQR] of total F_2-IsoP levels (ng/mL) measured in frozen samples supplemented with OE (frozen sample + OE) and non-supplemented (frozen sample – OE). The experiments were performed 10 times.

The sperm acrosome shape was analysed by PSA test in supplemented and non-supplemented samples after thawing. The percentages of sperm with normal, altered, and absent acrosomes were reported in Table 6.

The percentage of spermatozoa with normal acrosomes in frozen-thawed non-supplemented samples (32.00% [28.80%–34.30%]) was significantly lower ($p < 0.001$) than that detected in frozen-thawed supplemented samples (48.50% [45.50%–50.30%], Table 6, Fig. 8). The percentage of spermatozoa with altered acrosomes (Fig. 8A) in frozen-thawed non-supplemented samples (51.00% [43.80%–59.00%]) was slightly increased (non-significantly, $p = 0.190$) compared to that observed in frozen-thawed supplemented samples (47.00% [42.80%–49.30%], Fig. 8B). It was clear that OE had a protective impact on spermatozoa during semen cryopreservation because the percentage of spermatozoa lacking an acrosome in frozen-thawed non-supplemented samples (17.00% [13.80%–21.50%]) was significantly higher ($p < 0.001$) than that found in supplemented frozen-thawed samples (5.00% [2.80%–8.30%]).

Discussion

Human spermatozoa are a suitable model to test *in vitro* many compounds for the following reasons:

-The easy collection of spermatozoa from fertile men can provide a large amount of cells.

-Spermatozoa are differentiated cells endowed with motility, one of the most important indices of sperm function. The analysis of sperm motility is easy and cheap and provides indications on the effects of the compounds tested *in vitro*.

-Spermatozoa are deeply influenced by the external environment because of their low level of transcription and inability to repair DNA.

-Finally, due to lack of intracellular antioxidant protection, human spermatozoa have a restricted capacity to repair oxidative damage.

For these characteristics, any harmful effects of a tested substance are immediately detectable [31,39].

It is known that semen handling in laboratory and during assisted reproductive technologies (ART) can increase ROS production and cause sperm damage [28,40]. Assuming that laboratory conditions are optimal, a strategy to reduce OS during sperm manipulation could include the supplementation of media with antioxidants.

The ability of antioxidants to scavenge *in vitro* OS in human sperm has been reported in the literature, and many other studies have successfully used antioxidants to

Table 6. Evaluation of acrosome shape in spermatozoa from OE supplemented and non-supplemented frozen samples.

	Normal acrosome %	Altered	Absent
		Acrosome %	Acrosome %
Frozen semen samples + OE	48.50 [45.50–50.30]	47.00 [42.80–49.30]	5.00 [2.80–8.30]
Frozen semen samples – OE	32.00 [28.80–34.30]	51.00 [43.80–59.00]	17.00 [13.80–21.50]
Mann-Whitney U test	$p < 0.001$	*Ns*	$p < 0.001$

Median [IQR] of percentages of spermatozoa with normal acrosome, altered acrosome and without acrosome (absent acrosome) in frozen samples supplemented with OE (+ OE) and non-supplemented (– OE). The experiments were performed using 10 human semen samples. *Ns*, not significant.

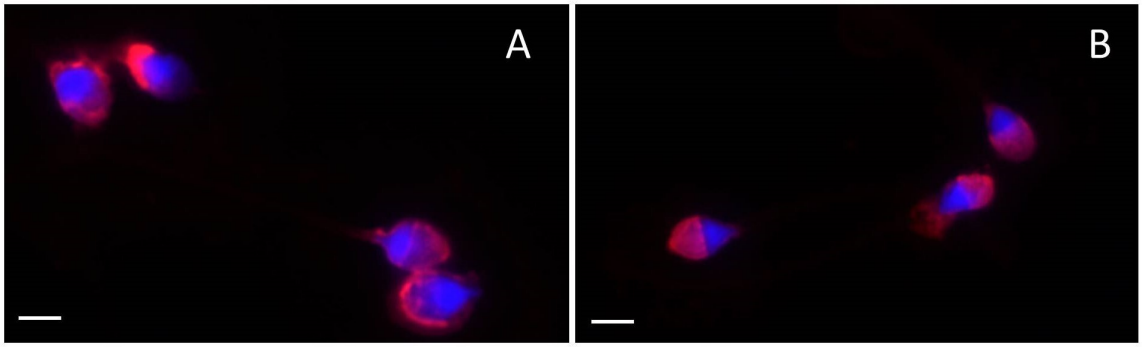

Fig. 8. UV micrographs of frozen-thawed spermatozoa treated with *Pisum sativum* agglutinin (PSA). (A) In non-supplemented samples the spermatozoa showed acrosome alterations. (B) In samples treated with OE 1:100 the spermatozoa had an evident PSA labelling in the well-shaped acrosome. Bars: 6 µM.

supplement the cryopreservation media [41,42]. The pentoxifylline, a methylxanthine derivative, was one of the first, still-used, compounds that showed a positive effect on human sperm respiration and motility [43,44]. In addition, zinc, aspartate and coenzyme Q [45], vitamin E [46], and many other substances [31] showed protective effects against OS in human sperm *in vitro*. Recently, much research has focused on the antioxidant activity of melatonin and inositol/myoinositol in human spermatozoa [47–51]. In the last two decades, natural compounds, either as a single purified molecule or as a phytocomplex, have caught attention. In the first case, polyphenols were used as supplements during cryopreservation. Essentially, these molecules exert a dual action on ROS homeostasis as they can scavenge ROS under normal conditions but also have a pro-oxidant activity [52,53]. Increasing attention is now shifting to natural extracts as whole phytocomplexes because the positive synergic effect between the molecules of the extract rather than isolated constituents can make the supplement more active and tolerated [54,55].

Following this rationale, extracts from many plants were tested *in vitro* on human spermatozoa, revealing a protective effect on sperm parameters and DNA integrity [31,56–59].

The present study that used OE as an antioxidant supplement made sense in this context. It is essential to chemically characterize the extract and assess its composition, antioxidant activity, and toxicity because herbal preparations for research and medical applications contain a variety of bioactive chemicals. We have chosen to use an aqueous extract because it is, at the same time, the cheapest and easiest preparation to produce, which also has a strong medicinal tradition as indicated in the European Medicines Agency (EMA) monograph (European Union herbal monograph on *Olea europaea* L., folium, 2017 [https://www.ema.europa.eu/en/documents/herbal-monograph/final-european-union-herbal-monograph-olea-europaea-l-folium-first-version_en.pdf]). Here we confirmed that the aqueous extract provides high concentrations of OLE, polyphenols and triterpenes, resulting in a well-tolerated preparation for human spermatozoa. Interestingly, the antioxidant activity, recorded in preliminary cell-free tests, has promoted OE as a potent antioxidant; this is due to the particular action of OLE, here in synergy with other polyphenols as HT which has a higher antioxidant capacity than that of reference substances as ascorbic acid [60]. Many compounds are present in the whole extract and most of them play a synergic effect. For this reason, it is difficult to understand the mechanism of action of the extract, unless we focus on the most abundant components. Both OLE and HT have been found to decrease ROS production and elicit free radical scavenging [61]. These compounds exert antioxidant activity against radicals for the redox properties of their phenolic groups and the structural relationship between different parts in their chemical structures [62].

According to the study's findings, sperm motility was not adversely affected by the OE employed at dilutions of

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1:100, 1:200, and 1:400. These results are comparable with those obtained using Propolfenol®, a fixed combination of European propolis and catechins [32] and with *Castanea sativa* Mill. leaf extract [57] used in similar experiments. These findings contrast with some of our group's earlier results using pure polyphenols [52,63], which, at high concentrations, reduced sperm motility and viability and damaged DNA. It appears that a phytocomplex produces a synergistic effect, at least in this type of *in vitro* investigations and it is more tolerated than a single pure molecule. In fact, the use of phytocomplexes in modern phytotherapy is justified by their multiple targets and nonspecific mechanisms.

OE diluted 1:100 revealed a scavenging ability on H_2O_2-induced LPO in *in vitro* tests, restoring similar levels of F_2-IsoPs and the percentage of dsDNA observed in the control samples. The primary phenolic compound of virgin oil, HT, which is also present in the OE, showed antioxidant properties during human sperm centrifugation and *in vitro* incubation [64]. For the first time, Noto *et al*. [37] employed F_2-IsoP level, a well-standardized OS marker [23,25], in these kinds of *in vitro* investigations. In particular, they measured F_2-IsoPs, formed by arachidonic acid oxidation, together with the malondialdehyde, likely the most commonly used LPO by-product for OS assessment, and the results between these two different markers were concordant.

Regarding sperm motility percentage, although this parameter was much higher in samples supplemented with OE than in non-supplemented samples, it was still lower than that observed in controls because the sperm motility is affected by the harmful action of H_2O_2 as demonstrated by other authors [65].

After OE protective and antioxidant properties were demonstrated *in vitro*, we used the extract in a practical application of semen handling such as sperm cryopreservation, which enables the long-term storage of the cells. During the freezing process, different factors such as sudden temperature changes, ice crystal formation, osmotic stress and oxidative insults are responsible for post-thaw poor sperm quality. The main damages occurring during freezing-thawing protocols affect sperm motility, vitality, membrane integrity, DNA fragmentation, and mitochondrial activity [66]. The supplementation of semen cryopreservation media with phytoextracts can improve human sperm quality after thawing [59,67–69].

Although both frozen samples in this study showed a progressive decline in sperm motility compared to the raw semen samples before freezing, the supplementation of cryopreservation medium with OE had a protective impact on sperm motility after thawing. Nijs *et al*. [70] observed that the percentage of motile spermatozoa after cryopreservation decreases from 50.6% to 30.3%, data that agrees with our results.

Other tested natural extracts of *Origanum vulgare* [69] and *Terminalia arjuna bark* [68] showed a positive effect on sperm motility in samples supplemented during cryopreservation. To date, the supplementation during freezing appeared to be the most used method; however, some research reported a sperm motility improvement when the supplementation was performed after thawing or both during cryopreservation and post-thawing [59,67].

In our study, the increased percentage of sperm with dsDNA and the decreased F_2-IsoP levels indicated that the spermatozoa in the samples supplemented with the extract had a high level of cryotolerance. Whereas many papers reported that the use of antioxidants during semen handling is beneficial for DNA integrity [31], Meamar *et al*. [71] observed that the addition of *Opuntia ficus-indica* fig extract to cryopreservation medium had only slight benefits in preventing the DNA fragmentation.

In the frozen samples, the acrosome status was assessed since this sperm organelle is essential during fertilization process. Changes in the acrosome structure, a depletion in its content, or a loss of the ability to respond to stimuli may seriously impair sperm function. The most frequent alterations detected at ultrastructural level in frozen-thawed human spermatozoa are the wrinkling of the plasma membrane, the loss of acrosome content and the vesiculations [72]. OE protected acrosomes from insults of freezing-thawing processes; in particular, in the supplemented samples, the percentage of normal acrosome was increased and the percentage of sperm without an acrosome decreased.

Finally, we want to identify the shortcoming and limitations of this study. First of all, the obtained preliminary results should be confirmed by increasing the sample size, and, in particular, using semen samples of infertile patients that are usually processed in *in vitro* fertilization laboratories, to confirm that OE has the same effect than that observed in normozoospermic samples. In addition, other sperm characteristics should be tested, mainly those related to DNA fragmentation, an important endpoint that affects fertilization [73]. Moreover, in the future research prospect the expression of genes related to oxidative stress should be investigated in order to hypothesize the mechanism of action of the extract. In addition, the research could consider the characterization of extracts from other *O. europaea* cultivars to be tested in human sperm cryopreservation protocols.

Conclusions

OE is characterized by high concentration of polyphenols, in particular OLE, and exhibits scavenging properties against oxidative damage induced by H_2O_2 in human ejaculated sperm. After cryopreservation, the samples supplemented with the extract showed increased sperm quality; therefore, OE could be considered as a safe option during semen handling procedures.

Another important aspect to be considered concerns the origin of the preparation from waste products, which are

a rich source of compounds with strong antioxidant activity. This particular research area is worth implementing because the by-products can be used in the industrial, cosmetic and, nutraceutical fields, promoting recycling, and converting a linear economy to a circular and sustainable green economy [74].

Availability of Data and Materials

The data generated and analysed during this study are included on this published article and are available from the corresponding author.

Author Contributions

EMo, GCo, MB, EMi designed the research study. RC, FV, CS, GB, GCa, RP performed the research. LM analyzed the data. EMo, RC, MB, FV wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee: ID CEAVSE 18370; Ethic Committee Siena University Hospital. Written informed consent has been obtained from the participants to the research.

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Conflict of Interest

The authors declare no conflict of interest.

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