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Original Article

Sperm quality improvement after date seed oil *in vitro* supplementation in spontaneous and induced oxidative stress

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Abstract

In vitro supplementation with date seed oil (DSO) can protect spermatozoa against hydrogen peroxide (H_2O_2)-mediated damage and can improve sperm function, possibly owing to antioxidant properties. We tested the antioxidant effects of DSO on human sperm motility, sperm viability, reacted acrosome and lipid peroxidation assessed *in vitro* after H_2O_2-mediated oxidative damage in spermatozoa. Sixteen patients (mean age: 35 years; range: 25–45 years) referred to the Histology–Embryology Laboratory of the Medicine Faculty of Sfax for semen analysis after 12–24 months of sexual intercourse without conception were selected. After spermiogram, sperm selection by two-interface discontinuous Sill Select gradient was performed, and selected spermatozoa were used in four experimental assays: control; incubation with 100 μm H_2O_2; incubation with 0.1% DSO; and co-incubation with 0.1% DSO and 100 μm H_2O_2. Motility and viability were determined using World Health Organization criteria. Acrosome reaction and lipid peroxidation were assessed by staining with fluorescein isothiocyanate-Pisum sativum and spectrophotometric measurement of malondialdehyde, respectively. Results showed that incubation with H_2O_2 alone led to a significant increase in lipid peroxidation (57.83%, $P < 0.05$) associated with a significant decrease in sperm motility, sperm viability (after 30 min and 24 h) and percentage of reacted acrosome ($P < 0.05$). Date seed oil improved sperm motility after 24 h of incubation ($P < 0.05$) and protected spermatozoa against the deleterious effects of H_2O_2 on motility, viability, acrosome reaction and lipid peroxidation. We conclude that supplementation with DSO may have a function in antioxidant protection against male infertility.

1   Introduction

Although many studies have shown improvements

in sperm quality with antioxidant treatment, recent attention has been directed toward extracts of plants rich in natural antioxidant compounds. In fact, there are many medicinal plants known to control human fertility; a large number of such plants have been used by indigenous people as part of folk medicine, especially in India [1]. In recent years, extracts of plants have been the source of many new drug therapies for pathologies such as infertility [2]. Fruits of the date palm (*Phoenix dactylifera L. Arecaceae*, also called 'deglet nour') are popular food in many countries and the date palm is a vital component of diets in arid and

semi-arid regions of the world. It has been found that date seed oil (DSO) has better oxidative stability than most vegetables and also has a high antioxidant capacity owing to its richness in polyphenol and tocopherol compounds [3, 4]. Recently, Dammak *et al.* [5] showed that DSO has a protective effect against hydrogen peroxide (H_2O_2)-induced oxidative stress (OS) in human skin organ culture and suggested that the use of DSO as a dietary supplement may have beneficial effects in protecting against skin disorders in humans. Many pharmacological compounds have been shown to enhance sperm motility and, potentially, sperm-fertilizing capacity [6, 7]. Spermatozoa are particularly susceptible to OS because their plasma membranes contain large amounts of polyunsaturated fatty acids [8]. OS stress induces deleterious effects on sperm viability and function and may lead to persistent infertility [9, 10]. Vitamin E, a major lipid-soluble antioxidant belonging to the tocopherols and the most effective antioxidant for chain-breaking within the cell membrane, is one of the vitamins that contribute to the detoxification process from reactive oxygen species (ROS). Vitamin E is able to repair oxidizing radicals directly, preventing the chain propagation step during lipid peroxidation [11].

In addition, many studies have shown that protective effects against ROS damage and an improvement of the overall functional parameters of spermatozoa can be provided by supplementing sperm preparation media with antioxidants such as vitamin E, catalase and ethylene diamine tetraacetic acid (EDTA) [12].

This study was carried out to test the antioxidant effects of DSO on human sperm motility, sperm viability, reacted acrosome and lipid peroxidation assessed *in vitro* after H_2O_2-mediated oxidative damage in spermatozoa.

2 Materials and methods

2.1 Date seed oil extract, preservation and emulsification

The seeds of the date palm were separated from 50 kg of waste date fruit that was collected from the National Institute of Arid Zone (Degach, South of Tunisia) at the 'Tamr stage' (full ripeness). The seeds were soaked in water, washed to free them of any adhering date flesh, air-dried and then dried (12 h) at about 50ºC. Date pits were separately milled in a heavy-duty grinder to pass 1–2 mm screens and then kept at –20ºC. Lipid extraction was carried out with an SER 148 Solvent Extractor (Velp Scientifica, Milan, Italy) equipped with

six Soxhlet posts. The extraction was carried out over a 30-min period with thimbles immersed in boiling petroleum ether followed by 60 min of reflux washing. The solvents from seed oils were removed under a stream of nitrogen and then stored in a freezer (–20ºC) until use [13]. This oil was emulsified with 10% 'gum arabic' (10 g of gum arabic/100 mL of physiological water: extemporarily prepared by dissolving 9 g of sodium chloride in 1 L of distilled water). Gum arabic did not show any negative effect on sperm parameters (motility, viability) at different times of incubation.

2.2 Collection and analysis of semen samples

Semen specimens were provided by 16 men aged from 25 to 45 years (mean age 35 years) and attending the Laboratory of Histology–Embryology for routine semen analysis after 12–24 months of sexual intercourse without conception. Azoospermic men and patients having a history of endocrine or anatomical disorders were excluded from this study. All semen samples were collected by masturbation after 3–5 days of abstinence. After liquefaction, semen volume, sperm concentration, motility, viability and morphology were determined according to World Health Organization (WHO) guidelines [14]. Motility was assessed at room temperature; sperm count was determined by diluting (1/20) a semen sample in immobilizing solution (9 g NaCl and 100 mL formol 40% made up to 1 L with distilled water, pH 7.4). After that, some of the diluted sample was transferred to a hemocytometer counting chamber, and the spermatozoa were counted under a microscope. Sperm viability was assessed by eosine–nigrosine test. Only specimens with sperm concentrations $\geq 20 \times 10^6$ mL^{-1} and total motility < 50% were included in the study. We excluded samples with high leukocyte concentrations (leukocytes > 1×10^6 cell mL^{-1}).

2.3 Preparation of samples and experimental design

After semen analysis, samples were prepared using a two-interface discontinuous Sill Select gradient (95%–45%) (FertiPro NV, Beernem, Belgium) and Ferticult culture medium (FertiPro NV) [15]. Immediately after semen selection, sperm concentration, motility and viability were determined and, to optimize the experimental groups, the final sperm concentration was adjusted to 10^7 mL^{-1} by addition of an adequate volume of Ferticult medium to each preparation.

Four experimental samples were prepared: (1) spermatozoa without H_2O_2 or DSO (control); (2) sper-

matozoa with 100 μmol L^{-1} H$_2$O$_2$ (H$_2$O$_2$); (3) spermatozoa with 0.1% DSO (DSO); and (4) spermatozoa incubated successively with 0.1% DSO for 30 min, then with 100 μmol L^{-1} H$_2$O$_2$ (H$_2$O$_2$ + DSO). In each experimental situation, sperm motility and viability were determined 30 min and 24 h after addition of H$_2$O$_2$ and/or DSO.

To choose the optimal concentration of H$_2$O$_2$ to give a significant reduction of semen parameters, we tested a range of concentrations (20, 40, 60 and 100 μmol L^{-1}). We found that significant changes in sperm motility and viability were observed only at 100 μmol L^{-1} and after 30 min of incubation. We also tested two concentrations of DSO (0.01% and 0.1%) and found that semen parameters were improved only at the highest concentration.

2.4 Measurement of lipid peroxidation

Lipid peroxidation was assessed by a specific spectrophotometry method that measures thiobarbituric acid reacted substances, such as malondialdehyde (MDA), according to Yagi [16]. After 30 min of incubation, 2 mL of TBA reagent (15% [v/v] trichloroacetic acid and 0.25 μmol L^{-1} HCl) was added to 1 mL of each experimental sample. The mixture was treated in a boiling water bath for 15 min. After cooling, it was centrifuged at 1 000 × *g* for 10 min. The supernatant was then removed and absorbance of the pellet was measured by a spectrophotometer at 535 nm. The MDA concentration was determined from a calibration curve, and the results were expressed as nmol MDA 10^{-7} cells.

2.5 Evaluation of the acrosome status with fluorescein isothiocyanate-Pisum sativum (FITC-PSA)

After 30 min of incubation, samples were kept in the incubator with 5% CO$_2$ in air at 37ºC for spontaneous acrosome reaction. Staining with FITC-PSA was performed following Mendoza *et al.* [17]. Briefly, aliquots (100–150 μL) of the sperm suspensions were smeared onto glass slides and air-dried. To permeabilize the sperm membranes, the slides were dipped in 100% methanol for 15 min at room temperature. Thereafter, the smears were mounted with 150 μL FITC-PSA (50 mg mL^{-1} phosphate-buffered saline, pH 7.4) and incubated for 30 min at room temperature in a humid chamber. The FITC-PSA solution was washed by dipping the slides 20 times in purified water. After air-drying, labelled spermatozoa were covered by 30 μL of an anti-fading medium (50% v/v glycerol, 50% v/v distilled water, 25 mg mL^{-1} 1',4-diazabicyclo[2, 2, 2]octane) to
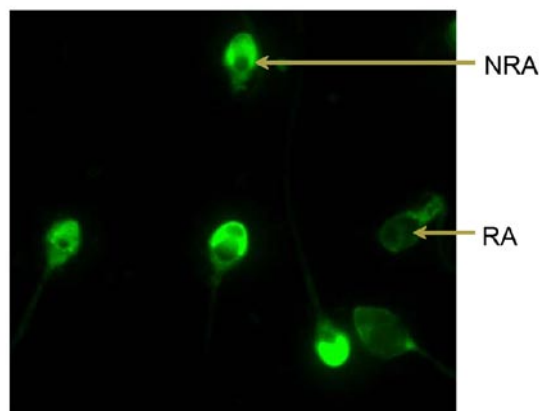

Figure 1. Evaluation of the acrosome status with fluorescein isothiocyanate-*Pisum sativum* (FITC-PSA) after 30 min of incubation. No reacted acrosome (NRA): bright fluorescence, acrosome intact; reacted acrosome (RA): no fluorescence or only fluorescence of the equatorial segment, loss of acrosomal membranes.

prevent photobleaching. By means of a fluorescence microscope (Nikon B 2A filter, excitation filter 450–490 nm, dichroic mirror 510 nm, barrier filter 520 nm), at least 200 spermatozoa were differentiated blindly according to the fluorescence pattern of their acrosomes (bright fluorescence: non-reacted acrosome; no fluorescence or only fluorescence of the equatorial segment: reacted acrosome) (Figure 1).

2.6 Statistics

Data were expressed as mean ± SD. The one-way analysis of variance and the Student–Newman–Keuls *post hoc* test were performed on the data for inter-group comparisons. Database management and statistical analysis were performed using the SPSS (Chicago, IL, USA) statistical software package.

3 Results

3.1 Sperm motility and viability

Data concerning sperm motility and viability are presented in Table 1. We found that after 30 min of incubation with 100 μmol L^{-1} H$_2$O$_2$, sperm motility and viability significantly decreased ($P < 0.05$) compared with the control. However, co-incubation with DSO and H$_2$O$_2$ improved motility and vitality after 30 min and 24 h compared with H$_2$O$_2$ alone (Table 1). Sperm motility was also significantly increased after 24h of incubation with DSO alone, in comparison with the control.

Table 1. Sperm motility and viability of the different experimental groups ($n = 16$) after 30 min and 24 h of incubation.

	Control	Experimental groups		
		H_2O_2	DSO	DSO + H_2O_2
After 30 min				
Motility (%)	42.19 ± 2.56	$21.56 \pm 3.96^*$	43.21 ± 5.12	$30.72 \pm 4.16^{*,**}$
Viability (%)	65.38 ± 6.13	$34.94 \pm 11.54^*$	60.00 ± 6.83	$39.38 \pm 8.92^*$
After 24 h				
Motility (%)	33.75 ± 7.63	0^*	$42.19 \pm 8.36^*$	$27.81 \pm 5.76^{**}$
Viability (%)	38.75 ± 9.33	0^*	45.31 ± 7.29	$31.75 \pm 5.96^{*,**}$

Abbreviation: DSO, date seed oil.
$^*P < 0.05$, all groups are compared with control (spermatozoa without H_2O_2 and DSO).
$^{**}P < 0.05$, comparison between group H_2O_2 (spermatozoa with 100 µmol L^{-1} H_2O_2) and group DSO + H_2O_2 (spermatozoa combined successively with 0.1% DSO and 100 µmol L^{-1} H_2O_2).
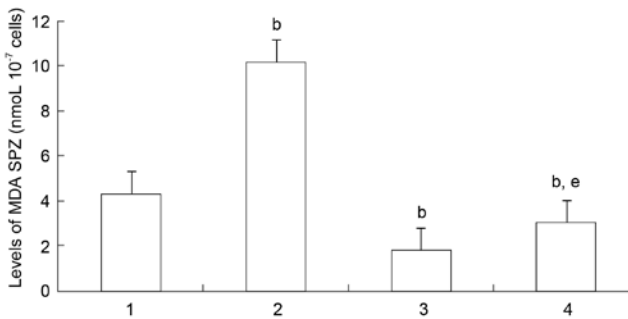

Figure 2. Malondialdehyde (MDA) levels in spermatozoa in each experimental group ($n = 16$) after 30 min of incubation. Values are presented as mean ± SD. Experimental groups include: (1) spermatozoa without H_2O_2 and date seed oil (DSO) (control); (2) spermatozoa with 100 µmol L^{-1} H_2O_2 (H_2O_2); (3) spermatozoa with 0.1% DSO (DSO); and (4) spermatozoa incubated successively with 0.1% DSO for 30 min, then with 100 µmol L^{-1} H_2O_2 (H_2O_2+ DSO). $^bP < 0.05$, compared with control. $^eP < 0.05$, compared with group 2.
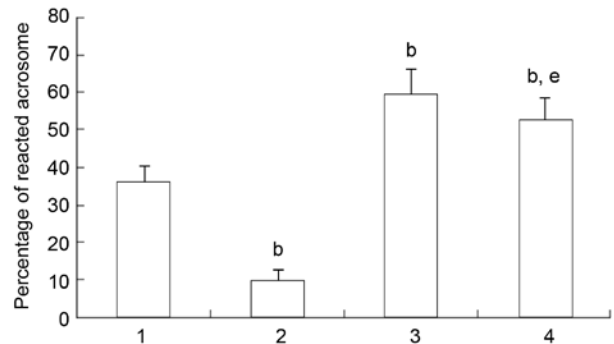

Figure 3. Evaluation of the acrosome reaction in different experimental groups ($n = 16$) after 30 min of incubation. Values are presented as mean ± SD. Experimental groups include: (1) spermatozoa without H_2O_2 and date seed oil (DSO) (control); (2) spermatozoa with 100 µmol L^{-1} H_2O_2 (H_2O_2); (3) spermatozoa with 0.1% DSO (DSO); and (4) spermatozoa incubated successively with 0.1% DSO for 30 min, then with 100 µmol L^{-1} H_2O_2 (H_2O_2 + DSO). $^bP < 0.05$, compared with control. $^eP < 0.05$, compared with group 2.

3.2 Lipid peroxidation

Compared with the control, the levels of lipid peroxide contents, as determined by MDA assay, were significantly increased after the exposure of spermatozoa to H_2O_2, and significantly decreased after incubation with DSO alone and with both DSO and H_2O_2 ($P < 0.05$) (Figure 2). In addition, a significant decrease in sperm MDA contents was observed after addition of DSO and H_2O_2 compared with incubation in H_2O_2 alone.

3.3 Acrosome reaction

Figure 3 reveals that the percentage of spermatozoa with reacted acrosome was significantly decreased after incubation with H_2O_2 in comparison with the control; it was significantly increased after incubation with DSO alone and with both DSO and H_2O_2. We also observed that the percentage of reacted acrosome was higher after incubation with DSO and H_2O_2 than with H_2O_2 alone ($P < 0.05$).

4 Discussion

The fruits of the date palm (*Phoenix dactylifera L. Arecaceae*) are popular in many countries. Recently, Dammak *et al.* [5] showed that DSO prevents H_2O_2-induced OS in human skin organ culture. Their data

about DSO composition and physico-chemical properties have encouraged us to test it in human fertility and sperm quality. To our knowledge, this study is the first to test the effects of DSO on human sperm motility, sperm viability, acrosome reaction and lipid peroxidation after *in vitro* H_2O_2-induced oxidative damage. We found that DSO has a protective effect on sperm motility and viability against *in vitro* induced OS and can improve sperm function. This oil has been analyzed in terms of phenol and tocopherol profiles, and it was found to be a rich source of natural phenolic compounds in the Mediterranean diet (in DSO, total phenol content is 520.81 mg kg^{-1}), which was one of the main reasons for the better oxidative stability of this oil. Alpha-tocopherol constituted about 24.97% of the total tocopherols for DSO [3].

Recently, treatment of infertility by natural products has been a topic of interest to several research teams. In fact, Piomboni *et al.* [7] reported that treatment with natural supplements containing high concentrations of vitamins C and E can be considered a valid alternative to anti-inflammatory drugs in the treatment of asthenospermia with leukocytosis. Similarly, a positive effect of a natural potent antioxidant, astaxanthin, as complementary treatment was found to improve sperm parameters and fertility [18]. In other studies, it has been reported that the benzene chromatographic fraction of *Carica papaya* seed extract showed contraceptive efficacy without adverse toxicity, mediated through inhibition of sperm motility [19].

Some reports have questioned the usefulness of antioxidants in the treatment of male infertility. Oral antioxidant treatment appears to improve intracytoplasmic sperm injection outcomes in patients with sperm DNA damage, and reduces the percentage of damaged spermatozoa [20]. Conversely, Ménézo *et al.* [21] reported that oral administration of antioxidant vitamins associated with zinc and selenium induced a decrease in DNA fragmentation but an unexpected increase in sperm decondensation, probably owing to the ability of antioxidant vitamins to interfere with interchain disulfide bridges of sperm protamines.

Male infertility has been linked with the excessive generation of ROS by defective spermatozoa. When produced in excess, H_2O_2 activates the lipid peroxidation cascade, which in turn causes loss of membrane fluidity and decreases sperm quality. In this investigation, we studied the direct effect of lipid peroxidation induced by H_2O_2 on sperm quality. We showed that H_2O_2 increased the formation of lipid peroxidation products (MDA) in sperm, and we confirmed published reports regarding the deleterious effects of H_2O_2 on sperm motility and viability [10]. In addition, we found that H_2O_2 decreased the percentage of reacted acrosomes. Furthermore, we showed for the first time a protective effect of DSO against *in vitro* H_2O_2-induced OS and its beneficial effect on sperm motility in patients with poor semen quality. Date seed oil improved the ability of sperms to perform the acrosome reaction and therefore their ability to fertilize oocytes. It is known that hyperactivation and acrosome reaction can be induced when spermatozoa are incubated in culture medium [22]. In our view, the improvement in the percentage of spermatozoa with reacted acrosome seen in this study is not related to the influence of the culture medium but could be attributed to the beneficial effect of DSO, as the same culture medium was used in all experimental groups with and without DSO. Therefore, we suggest that natural antioxidants present in DSO protect against H_2O_2-induced oxidative damage.

Various *in vitro* and *in vivo* studies have indicated that supplementation by antioxidants attenuated the negative effects of ROS and improved sperm function, capacity for fertilization and sperm membrane fluidity [12, 23, 24]. Chi *et al.* [12] reported that *in vitro* supplementation by antioxidants (EDTA, catalase) in sperm suspensions significantly reduced the ROS levels and DNA fragmentation rate of spermatozoa. Moreover, incubation with catalase increased the acrosome reaction ability of spermatozoa. In the same way, in a recent study testing the *in vitro* antioxidant effects of the isoflavones genistein, equol (plant compound), ascorbic acid and alpha-tocopherol on sperm DNA integrity after H_2O_2-induced damage, Sierens *et al.* [23] found that genistein was the most potent antioxidant, followed by equol, ascorbic acid and alpha-tocopherol. Genistein and equol added in combination were more protective than either added singly. Furthermore, Comhaire *et al.* [24]. evaluated the effects of oral antioxidants (*N*-acetyl-cysteine or vitamins A plus E) and essential fatty acids on sperm biology and showed that treatment reduced ROS production and increased both acrosome reaction and sperm membrane fluidity. In contrast, another study indicates that live and acrosome-reacted spermatozoa, kinetic parameters and fertility rate were not modified by alpha-tocopheryl acetate and selenium *in vitro* supplementation of rabbit spermatozoa [25].

On the basis of the promising reports here and in the

literature, it seems that treatment with natural products containing high concentrations of alpha-tocopherol and phenols can be considered as a valid alternative to improve sperm parameters in the treatment of male infertility.

This original study proved that supplementation with DSO may have a function in antioxidant protection against male infertility. Further investigations testing the effects of DSO extract *in vivo* should be performed to confirm the beneficial effects on semen parameters and on the fertilization capacity of spermatozoa.

Acknowledgment

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