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Original Article

The *in vitro* effects of superoxide, some commercially available antioxidants and red palm oil on sperm motility

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Abstract

In this study, two commercially available superoxide scavengers, tetrakis (1-methyl-4-pyridyl) porphyrin (Mn[III]TMPyP) and superoxide dismutase (SOD), as well as red palm oil (RPO), a natural vegetable oil, had been used to investigate their possible *in vitro* effects against the toxic effects of superoxide ($O_2\cdot$) on human sperm motility. Semen samples were obtained from 12 normozoospermic healthy volunteer donors aged between 19 and 23 years. The $O_2\cdot$ donor 2,3-dimetoxyl-1,4-naphthoquinone (DMNQ) (2.5 μmol L^{-1}–100 μmol L^{-1}) was added to normozoospermic post-swim-up sperm in the presence or absence of Mn(III)TMPyP (50 μmol L^{-1}), SOD (50 IU) or RPO (0.1% or 0.5%). Computer-assisted semen analysis was used to analyze various motility parameters. The parameters of interest were percentage of motile cells, progressive motility, rapid cells and static cells. Concentrations of higher than 25 μmol L^{-1} DMNQ were detrimental to sperm motility. Mn(III)TMPyP was able to attenuate the effect of $O_2\cdot$ on the motility parameters. *In vitro* addition of SOD and RPO showed harmful effects on sperm motility.

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1 Introduction

Sperm motility has been shown to be a good predictor of human male fertility both *in vivo* and *in vitro* [1]. Motility parameters such as progressive motility, rapid cells and static cells determine the quality of human semen and are clinically important [2]. This implies that any natural or pharmaceutical compounds that enhance sperm motility might improve male fertility. However, sperm cells are under constant attack by reactive oxygen species (ROS), which had been associated with sub-fertility or even infertility [3–5]. The most common ROS that have potential implications in reproductive biology include superoxide anion ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), peroxyl radicals and the very reactive hydroxyl radical [3, 6, 7].

In sperm cells, the sources of ROS are broadly dispersed between external and internal sources. External production of ROS, particularly $O_2\cdot^-$ and H_2O_2, can be the result of leukocyte contamination within the semen. Another important source of ROS is immature and morphologically abnormal spermatozoa [8]. Recently, two mechanisms involved in ROS generation in spermatozoa, *per se*, have been characterized in rat epididymal spermatozoa [9]. One mechanism depends on the mitochondrial respiratory chain, whereas the other mechanism relies on an enzymatic system related to the

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reduced nicotinamide adenine dinucleotide phosphate oxidase family found bound to the sperm plasma membrane [9]. The mitochondrial electron transport chain is known to produce ROS not only during physiological, but also during pathological conditions. This production of ROS can be correlated to physical activity. The topology of O_2· production has been determined in the different complexes of the respiratory chain. Interestingly, complexes I, II and III were found to produce hydrogen peroxide, albeit at very low levels [10].

Excessive production of ROS is associated with peroxidative damage to the sperm plasma membrane and DNA. This can result in poor sperm quality and male factor infertility [11, 12]. Lipid peroxidation (LPO) of the sperm membrane is considered to be the key mechanism of ROS-induced sperm damage. Spermatozoa, unlike other cells, are unique in structure, function and susceptibility to damage by LPO [13]. A balance between the cellular production of ROS and their destruction by scavengers in sperm and in seminal plasma is vital for the cell survival. Oxidative stress (OS) arises as a consequence of excessive ROS production and/or impaired antioxidant defense mechanisms [8]. A variety of defense mechanisms including antioxidant enzymes (superoxide dismutase (SOD), catalase, glutathione peroxidase and reductase), vitamins (E, C and carotenoids) and biomolecules (ubiquinol) are involved [14, 15]. Studies have shown that some pharmaceutical [16] and nutritive substances [17, 18] improved sperm motility. Red palm oil (RPO), which is regarded as the only vegetable oil with a balanced composition of saturated and unsaturated fatty acids both in processed and unprocessed forms [19], contains carotenoids, phosphatides, sterols, tocopherols and trace metals [20, 21]. These agents are natural antioxidants and act as scavengers of oxygen-free radicals [22, 23].

In this study, two commercially available superoxide scavengers, Mn(III)TMPyP and SOD, as well as RPO, a natural cocktail of antioxidants, had been used to investigate their possible *in vitro* effects against the toxicity of superoxide on human sperm motility parameters.

2 Materials and methods

2.1 Sperm collection

Semen samples were obtained from 12 normozoospermic healthy donors aged between 19 and 23 years, after 2–3 days of abstinence, according to the World Health Organization criteria [24]. Semen samples were collected in sterile containers and allowed to liquefy for 30 min at 37ºC. Ethical approval from the Institutional Review Board was obtained and donors have provided consent to participate in this study.

2.2 Semen preparation

Motile sperm fractions were retrieved from the samples using a double wash in fresh Hams-F10 medium (400 × *g*, 5 min, Sigma Chemical Co., St. Louis, MO, USA) swim-up technique (Hams-F10 + bovine serum albumin, 3%, Sigma, 37ºC, 5% CO_2). After 1 h, the supernatant containing motile sperm was collected. Sperm concentrations were determined by means of computer-assisted semen analysis (CASA), and the retrieved cells were divided into aliquots and the concentration was adjusted to 2×10^6 cells per mL.

2.3 Sperm motility determination

Sperm cells were incubated at 37ºC with or without 50 μmol L^{-1} Mn(III)TMPyP (Sigma) or 50 IU mL^{-1} SOD (Sigma), or 0.1% or 0.5% RPO. RPO (Carotino SDN BHD Co: 69046-T, Johar-Bahru, Malaysia) was dissolved in propylene glycol as described previously [25] to administer the oil to an aqueous sperm suspension. After 30 min, 2,3-dimetoxyl-1,4-naphthoquinone (DMNQ), an O_2· generator (Calbiochem, San Diego, CA, USA, dissolve in DMSO) was added at concentrations of 0 μmol L^{-1}, 2.5 μmol L^{-1}, 5 μmol L^{-1}, 10 μmol L^{-1}, 25 μmol L^{-1}, 50 μmol L^{-1} and 100 μmol L^{-1} to the sperm samples and incubated for a further 60 min at 37ºC.

Several sperm motility parameters were determined by means of CASA on Hamilton-Thorne IVOS analyzers (Hamilton-Thorne Research, Beverly, MA, USA). The parameters of interest were percentage of motile cells, progressive motility, rapid cells and static cells, as they give a good indication of the general motility, DNA integrity and fertilizing ability status of spermatozoa [2]. The Hamilton-Thorne IVOS settings were as follows: Image capture—30 frames at 60 Hz; minimum contrast—80; minimum cell size—2; minimum static contrast—30; Progressive velocity: path velocity (VAP)—25 μ s^{-1} and Straightness (STR)—80%; Slow cells: static VAP cut-off, 5 μm s^{-1} and straight-line velocity (VSL) cut-off—11 μ s^{-1}; head size; STR = VSL/VAP × 100; Rapid cells = % of all cells moving with VAP > MVV; Medium = % of all cells moving with LVV < VAP > MVV; Slow = % of all cells moving with VAP < LVV or VSL < LVS; Static = % of cells that

are not moving at all; slow cells classified as not motile.

2.4 Statistical analyses

GaphPad™ PRISM 4 (GraphPad Software Inc., La Jolla, CA, USA) was used for all statistical evaluations. A one-way analysis of variance (ANOVA) test (with Bonferroni post test if $P < 0.05$) was used for statistical analyses. Data are expressed as mean ± SEM. Differences were regarded statistically significant if $P < 0.05$ and highly significant if $P < 0.001$.

3 Results

3.1 The effects of exogenous superoxide on sperm motility parameters

From Table 1, it can be seen that the *in vitro* addition of exogenous superoxide in the form of DMNQ considerably decreased the percentage of motile cells, progressive motility and rapid cells at a concentration of 50 μmol L^{-1} DMNQ. On the other hand static cells notably increased already from 25 μmol L^{-1} DMNQ when compared with the control (0 μmol L^{-1} DMNQ). Table 2 shows a decrease in the percentages of motile cells, progressively motile cells and rapid cells from 25 to 100 μmol L^{-1} DMNQ. However, the percentages of the static cells for all concentrations from 5 to 100 μmol L^{-1} DMNQ were significantly higher than control values. Table 3 shows the addition of 25–100 μmol L^{-1} DMNQ extensively decreased the percentage of motile cells while increasing the static cells. The percentages of progressive motility and rapid cells, was however significantly lower than control values for all concentrations from 2.5 to 100 μmol L^{-1} DMNQ.

3.2 The effects of superoxide on sperm motility parameters in the absence or presence of Mn(III)TMPyP

Table 1 shows that the addition of Mn(III)TMPyP reversed the negative effect of superoxide on motile cells, progressive motility and rapid cells at 50 μmol L^{-1} DMNQ and static cells at 25 μmol L^{-1} DMNQ. Moreover, Mn(III)TMPyP treatment considerably decreased the percentage of motile cells at 100 μmol L^{-1} DMNQ. However, Mn(III)TMPyP treatment notably increased the percentage of static cell at 100 μmol L^{-1} DMNQ.

3.3 The effects of superoxide on sperm motility parameters in the absence or presence of SOD

Table 2 shows that samples treated with DMNQ in combination with SOD showed considerably lower

Table 1. The effects of superoxide on sperm motility parameters in the presence or absence of Mn(III)TMPyP ($n = 12$).

Parameters	Treatment	DMNQ (μmol L^{-1})						
		0	2.5	5	10	25	50	100
Motile (%)	–	63.64 ± 6.20	58.50 ± 4.56	56.10 ± 4.43	59.00 ± 6.60	49.00 ± 4.69	39.70 ± 3.32*	28.10 ± 3.41*
	+ Mn(II)TMPy	59.82 ± 3.26	53.27 ± 5.06	49.64 ± 3.94	54.73 ± 3.08	54.27 ± 2.50	46.82 ± 3.74	16.36 ± 2.06*,$
Progressive motility (%)	–	37.00 ± 4.30	32.00 ± 3.95	26.60 ± 2.60	36.90 ± 4.48	27.50 ± 5.72	16.00 ± 2.37*	5.60 ± 1.12*
	+ Mn(II)TMPy	35.82 ± 2.55	31.64 ± 4.26	25.27 ± 3.23	32.00 ± 3.25	30.36 ± 2.20	23.36 ± 1.68	3.36 ± 0.52*
Rapid cells (%)	–	49.10 ± 6.23	47.40 ± 5.00	43.10 ± 4.25	50.90 ± 5.95	40.70 ± 4.55	29.70 ± 2.95*	13.50 ± 1.93*
	+ Mn(II)TMPy	49.45 ± 3.21	43.73 ± 5.45	38.18 ± 3.75	43.73 ± 3.78	43.73 ± 2.90	33.18 ± 3.17	9.27 ± 1.74*
Static cells (%)	–	24.60 ± 8.08	27.00 ± 4.15	28.90 ± 4.15	27.40 ± 7.11	34.60 ± 4.82*	38.50 ± 3.23*	42.00 ± 4.88*
	+ Mn(II)TMPy	24.09 ± 3.36	31.09 ± 5.16	31.36 ± 3.53	28.27 ± 3.33	30.91 ± 2.83	35.91 ± 5.44*	57.82 ± 5.54*,$

Abbreviation: DMNQ, 2,3-dimetoxyl-1,4-naphthoquinone.
Values are represented as means ± SEM. Differences were regarded statistically significant if $P < 0.05$ and highly significant if $P < 0.001$.
*$P < 0.001$, compared with corresponding controls (0 μmol L^{-1} DMNQ and no Mn[III]TMPyP); $P < 0.05$, compared with Mn(III)TMPyP untreated parameter of the same DMNQ.

Table 2. The effects of superoxide on sperm motility parameters in the presence or absence of SOD (n = 12).

Parameters	Treatment	DMNQ (μmol L^{-1})						
		0	2.5	5	10	25	50	100
Motile (%)	–	62.90 ± 3.80	51.00 ± 3.78	39.70 ± 3.44	47.30 ± 4.52	37.70 ± 3.51*	27.10 ± 3.91*	8.20 ± 1.97*
	+ SOD	56.10 ± 3.99	49.90 ± 3.97	46.60 ± 3.62	42.30 ± 3.01	38.60 ± 3.13	20.80 ± 4.22$^{*,\$}$	3.70 ± 0.96$^{*,\$}$
Progressive motility (%)	–	37.80 ± 3.68	23.40 ± 2.87	24.60 ± 1.82	25.50 ± 2.83	18.80 ± 2.43*	11.20 ± 1.99*	5.36 ± 1.03*
	+ SOD	30.30 ± 2.73	23.90 ± 1.82	22.70 ± 2.70	21.40 ± 2.16*	18.20 ± 2.02*	7.00 ± 1.80$^{*,\$}$	1.00 ± 0.42$^{*,\$}$
Rapid cells (%)	–	55.00 ± 4.23	41.30 ± 3.58	32.20 ± 4.02	39.60 ± 4.51	29.60 ± 3.18*	21.40 ± 3.49*	5.20 ± 1.48*
	+ SOD	47.10 ± 3.59	31.70 ± 1.64$^{\$}$	32.50 ± 1.77	33.10 ± 1.56$^{\$}$	30.70 ± 2.96*	14.50 ± 3.61$^{*,\$}$	2.60 ± 0.77*
Static cells (%)	–	24.60 ± 3.29	33.90 ± 4.55	42.90 ± 3.01*	38.50 ± 4.50*	48.20 ± 4.32*	61.20 ± 5.08*	82.70 ± 3.18*
	+ SOD	31.20 ± 4.66	44.90 ± 3.55$^{*,\$}$	46.90 ± 4.87*	41.10 ± 3.75*	44.90 ± 3.37*	60.10 ± 5.80*	90.56 ± 1.82$^{*,\$}$

Abbreviations: DMNQ, 2,3-dimetoxyl-1,4-naphthoquinone; SOD, superoxide dismutase.
Values are represented as means ± SEM. Differences were regarded statistically significant if $P < 0.05$ and highly significant if $P < 0.001$.
$^{*}P < 0.001$, compared with control (0 μmol L^{-1} DMNQ and no SOD); $^{\$}P < 0.05$, compared with SOD untreated parameter within the same DMNQ.

Table 3. The effects of superoxide on sperm motility parameters in the absence or presence of different concentrations of RPO (n = 12).

Parameters	Treatment	DMNQ (μmol L^{-1})						
		0	2.5	5	10	25	50	100
Motile (%)	–	76.42 ± 1.90	61.83 ± 5.02	60.33 ± 3.72	63.00 ± 2.33	55.33 ± 4.57*	49.75 ± 4.44*	30.42 ± 7.27*
	0.1% RPO	60.89 ± 5.32$^{\$}$	48.56 ± 6.66*	43.29 ± 6.48*	48.22 ± 5.91*	44.56 ± 5.97*	25.67 ± 6.11$^{*,\$}$	9.33 ± 3.62$^{*,\$}$
	0.5% RPO	60.62 ± 3.70$^{\$}$	49.46 ± 4.28*	49.80 ± 2.81*	56.31 ± 4.18*	48.23 ± 3.48*	33.23 ± 7.71$^{*,\$}$	13.46 ± 2.53$^{*,\$}$
Progressive motility (%)	–	47.42 ± 3.60	32.67 ± 4.08*	29.25 ± 3.65*	37.17 ± 2.86*	29.83 ± 4.22*	21.17 ± 3.01*	11.25 ± 5.36*
	0.1% RPO	43.00 ± 6.01	29.00 ± 7.65*	26.50 ± 6.23*	27.33 ± 5.42*	27.67 ± 5.81*	12.33 ± 4.40$^{*,\$}$	2.50 ± 1.14$^{*,\$}$
	0.5% RPO	40.46 ± 3.79	29.27 ± 3.87*	25.88 ± 2.81*	32.38 ± 3.22*	30.50 ± 3.71*	14.15 ± 3.90$^{*,\$}$	2.53 ± 0.63$^{*,\$}$
Rapid cells (%)	–	62.83 ± 3.24	47.50 ± 5.36*	46.17 ± 4.66*	51.25 ± 2.95*	43.00 ± 5.00*	34.17 ± 3.90*	17.92 ± 6.37*
	0.1% RPO	51.89 ± 5.40	40.11 ± 6.56*	34.78 ± 3.62*	38.56 ± 6.04*	35.11 ± 6.00*	18.22 ± 4.70$^{*,\$}$	5.22 ± 2.58$^{*,\$}$
	0.5% RPO	52.08 ± 3.93$^{\$}$	37.46 ± 3.88*	33.33 ± 3.35*	46.00 ± 4.03*	35.15 ± 3.85*	24.77 ± 6.68$^{*,\$}$	6.84 ± 1.63$^{*,\$}$
Static cell (%)	–	10.67 ± 1.70	22.00 ± 5.08	20.33 ± 1.73	19.08 ± 2.74	23.42 ± 4.86*	25.42 ± 4.69*	44.50 ± 6.96*
	0.1% RPO	20.89 ± 3.95	36.22 ± 6.24*	35.89 ± 7.28*	33.22 ± 6.30$^{*,\$}$	31.50 ± 5.10*	53.13 ± 8.73$^{*,\$}$	79.11 ± 7.31$^{*,\$}$
	0.5% RPO	23.92 ± 3.24	29.08 ± 3.96*	34.15 ± 4.68*	26.23 ± 3.56$^{*,\$}$	28.08 ± 3.38*	49.77 ± 7.79$^{*,\$}$	63.69 ± 6.64$^{*,\$}$

Abbreviations: DMNQ, 2,3-dimetoxyl-1,4-naphthoquinone; RPO, red palm oil.
Values are represented as means ± SEM. Differences were regarded statistically significant if $P < 0.05$ and highly significant if $P < 0.001$.
$^{*}P < 0.001$, compared with control (0 μmol L^{-1} DMNQ and no RPO); $^{\$}P < 0.001$, compared with RPO untreated parameter within the same DMNQ.

percentages of motile cells, progressive motility (from 50 to 100 μmol L^{-1} DMNQ) and rapid cells (at 2.5, 10 and 50 μmol L^{-1} DMNQ) compared with control (0 μmol L^{-1} DMNQ). Samples treated with DMNQ in combination with SOD have increased static cells (2.5 and 100 μmol L^{-1} DMNQ) compared with control (0 μmol L^{-1} DMNQ) values.

3.4 The effects of superoxide on sperm motility parameters in the absence or presence of RPO

The addition of 0.1% and 0.5% RPO (Table 3) significantly decreased the percentages of motile cells, progressive motility and rapid cells compared with the control from 50 to 100 μmol L^{-1} DMNQ. Moreover, 0.1% and 0.5% RPO considerably increased the number of static cells at 10, 50 and 100 μmol L^{-1} DMNQ.

4 Discussion

4.1 The effects of superoxide on sperm motility parameters

In this study, it was found that the addition of exogenous superoxide in the form of DMNQ at 100 μmol L^{-1} radically reduced the percentage of motile cells, progressive motility, and rapid cells, as well as led to an increase in the number of static cells. It is therefore clear that $O_2\cdot$, in the form of exogenously added DMNQ, is deleterious to sperm motility and sperm function at higher concentrations. These results are in agreement with De Iuliis *et al*. [26], who showed that human spermatozoa are capable of generating $O_2\cdot^-$ and that the production of this oxygen radical is inversely correlated with defective sperm function. Gil-Guzman *et al*. [27] showed that levels of ROS production were negatively correlated with teratozoospermia and spermatozoa developmental stages. ROS production was found to be highest in the immature fraction of ejaculated sperm, which also contained sperm with abnormal head morphology and cytoplasmic retention. It has been suggested that ROS induces membrane LPO in sperm and that the toxicity of the generated fatty acid peroxides are important causes of decreased sperm function, for example, motility [28, 29]. Previous studies [8, 28, 30, 31] had shown a correlation between high levels of ROS (superoxide, hydroxyl, hydrogen peroxide, nitric oxide and peroxynitrile) and decreased sperm motility. De Lamirande and Gagnon [32] also showed that ROS causes sperm immotility within 5–30 min, depending on the concentration.

4.2 The effects of superoxide on sperm motility parameters in the absence or presence of Mn(III)TMPyP

Manganese (Mn) is an essential ultra trace element similar to chromium, molybdenum and cobalt. It is needed for a wide variety of physiological processes ranging from the regulation of reproduction to normal brain function [33]. Mn can exist in various oxidation states ranging from −3 to +7, with +2 oxidation state being the most predominant in biological systems [33]. Although redox active metals such as Fe(II) can accelerate LPO, ionic Mn (10–100 μmol L^{-1}) has been shown to inhibit LPO in rat liver microsomes [34]. In addition, several known Mn complexes including the Mn salen and Mn bis(cyclohexylpyridine)-substituted macro cyclic ligand have shown promise as a possible SOD mimic [35–37]. Studies have shown that these complexes are as effective as SOD enzymes in detoxifying superoxide under some experimental conditions [38]. Metalloporphyrins such as Mn(III)TMPyP are a unique class of stable catalytic antioxidants possessing a broad range of antioxidant capacities that include the dismutation of superoxide [39–41], hydrogen peroxide [42] and scavenging of peroxynitrite [43, 44]. Metalloporphyrins exhibit other antioxidant capacities in addition to superoxide dismuting activity such as catalase-like activity [42], inhibition of LPO [45]. *In vitro* models of OS have been useful both in terms of confirming the antioxidant activities of metalloporhyrins obtained in cell-free systems and predicting their use as antioxidants in more complex *in vivo* models of human disease. Metalloporphyrins have been shown to be protective in a wide variety of *in vitro* OS models involving the generation of $O_2\cdot$, H_2O_2 and ONOO alone or in concert.

The addition of Mn(III)TMPyP (50 μmol L^{-1}) attenuated the effects of superoxide on the number of static cells (5 and 10 μmol L^{-1} DMNQ). As a result, it offered some form of protection to sperm motility against the harmful effects of superoxide. However, Mn(III)TMPyP decreased the percentages of motile cells and increased static cells at high concentrations of O_2. This finding is interesting or rather contradictive to the protective properties ascribed to Mn(III)TMPyP. Nevertheless, this finding is in agreement with Lin *et al*. [33] who found that Mn exhibits pro- and antioxidant characteristics in their study done on worms. Despite several reports suggesting the beneficial effects of Mn in unicellular organisms, it is well known that chronic exposure to high atmospheric levels of Mn is toxic [33].

Studies have shown that an overload of Mn causes the disease 'manganism', which has Parkinson's-like symptoms [46, 47].

4.3 The effects of superoxide on sperm motility parameters in the absence or presence of SOD

The addition of SOD (50 IU mL^{-1}) exacerbated the harmful effect of superoxide by drastically reducing motility, progressive motility (from 50 to 100 μmol L^{-1} DMNQ) and rapid cells (at 2.5, 10 and 50 μmol L^{-1} DMNQ).

Contradicting reports exists regarding the effect of antioxidant supplementation on sperm motility in both fresh liquefied and frozen-thawed semen from various species, including human [17, 48–51], the effect being dependent of the antioxidant employed and the dose used [52]. Johnson and Guilivic [53] argued that the conversion of superoxide anion to hydrogen peroxide by SOD may have anti- and pro-oxidant consequences. On the one hand, the dismutation of $O_2\cdot$ to H_2O_2 and oxygen facilitates both the distribution of ROS, that is, diluting their effects through diffusion between cellular compartments, and the removal of H_2O_2 by H_2O_2-consuming enzymes (antioxidant). On the other hand, if the actions of SOD and H_2O_2-consuming enzymes are not in concert, an increased production of H_2O_2 is expected from SOD activity.

Sikka [6] has shown that a relationship exists between an increase in ROS-induced OS, LPO, decreased levels of SOD and motility in spermatozoa. de Lamirande and Gagnon [32] argued that motility is impaired because of adenosine triphosphate depletion during LPO of the sperm plasma membrane. Peroxidation increased in proportion to the decrease in SOD effects [54]. Therefore, the time of complete motility loss is determined by the rate of peroxidation [54] and so would correlate with SOD activity [55]. The dismutation of $O_2\cdot^-$ by SOD converts it into a powerful oxidant, H_2O_2, which can readily penetrate sperm cells, lower their motility and cause irreparable damage to both DNA and membranes [54]. All these arguments might explain our findings.

4.4 The effects of superoxide on sperm motility parameters in the absence or presence of RPO

Crude palm oil is known to be the richest natural plant source of carotenoids in terms of provitamin A equivalents, such as α-carotene and β-carotene [20, 56]. The dietary intake of foods rich in carotenoids was associated with a reduced risk of some types of cancer [57, 58] and cardiovascular diseases [59], presumably affording antioxidant properties to reduce OS when tested in both endothelial and non-endothelial cells. Palm oil containing these antioxidants was shown to be effective against OS *in vitro* and *in vivo* [60]. Vitamin E and related compounds are also abundant in RPO. Chow and Hong [61] reported that dietary vitamin E is capable of reducing the production and/or availability of not only $O_2\cdot^-$, but also NO and $ONOO^-$. However, it is not clear whether the action of vitamin E to reduce the generation of $O_2\cdot^-$ and other ROS is independent of its antioxidant function.

However, the combination of RPO and DMNQ significantly decreased motile cells (50–100 μmol L^{-1}) and also increased the percentage of static cells (DMNQ 2.5–100 μmol L^{-1}). As RPO is a lipid and hydrophobic, it can possibly explain why no marked positive effects were seen with co-treatment, as it might not have been possible to deliver its antioxidant properties to the affected spermatozoa. Owing to its lipid nature, it could furthermore negatively affect the spermatozoa directly in solution.

In conclusion, this study has shown that Mn(III) TMPyP offered better protection against the harmful effects of $O_2\cdot^-$ than SOD or RPO when using sperm motility as an *in vitro* end point. We recommend that future studies should include the oral administration of different concentrations of RPO to animals for a period long enough to target complete spermatogenesis and epididymal maturation. This could give a much better appraisal of the possible phytotherapeutic and protective effects RPO can have on ejaculated sperm.

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