ORIGINAL ARTICLE

A red palm oil diet can reduce the effects of oxidative stress on rat spermatozoa

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Summary

Male Wistar rats (n = 54) received daily supplementation of red palm oil (RPO: 0, 2, 4 ml). Subgroups were subsequently injected with saline, cumene hydroperoxide (cHP, 10 *l*m) or t-butyl hydroperoxide (tbHP, 20 *l*m) over a 60-day period after which animals were sacrificed. Epididymal sperm motility, concentration, reactive oxygen species (ROS), lipid peroxidation and enzymes were measured. Sperm concentration, motility, superoxide dismutase (SOD) concentration, glutathione (GSH) and catalase (CAT) activities were significantly lower, while dichlorofluorescein (DCF) and malondialdehyde (MDA) were higher in sperm of hydroperoxide-treated animals compared to controls ($P < 0.05$). DCF and MDA levels were significantly lower, while SOD, CAT and GSH were significantly higher in the sperm of rats supplemented with RPO in combination with hydroperoxide treatment when compared to those receiving hydroperoxide and no RPO supplementation ($P < 0.05$). Moreover, the DCF, SOD, CAT and GSH levels in the RPO hydroperoxide groups did not differ from control values ($P > 0.05$). RPO supplementation can successfully attenuate the oxidative stress-induced sperm damage due to organic hydroperoxide exposure. We therefore propose that a daily intake of RPO supplement to the diet might be helpful in protecting males against the adverse effects of high ROS in sperm function and help preserve fertility.

Introduction

Oxidative stress (OS) is associated with an imbalance between the production of free radicals, mainly reactive oxygen species (ROS), and scavenging capacity of antioxidants. When ROS production exceeds the available antioxidant defence, significant OS-induced damage occurs to many cellular organelles due to damage of lipids, proteins and DNA molecules, ultimately leading to cell death (Irvine *et al.*, 2000; Moustafa *et al.*, 2004). Two mechanisms involved in ROS generation in sperm, *per se,* have been characterised in rat epididymal sperm. One mechanism depends on the mitochondrial respiratory chain (Gavella & Lipovac, 1992), while the other relies on an enzymatic system related to reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase located near the sperm plasma membrane (Aitken *et al.*, 1992; Vernet *et al.*, 2001).

Reactive oxygen species production is critical for spermatozoa function in physiological as well as pathological conditions (Agarwal *et al.*, 2003, 2006; Holstein *et al.*, 2003). However, mammalian cells are equipped with antioxidant mechanisms capable of restoring the balance between ROS production and their metabolism. In addition, the body possesses defence mechanisms to reduce the OS-induced damage, and such mechanisms use both enzymes and antioxidant nutrients or medicine to arrest the damaging properties of excited oxygen species (Hwang *et al.*, 2002; Weisburger, 1991). Irreversible OS-induced sperm damage may occur only when its protective mechanisms break down, or when the effectiveness of antioxidant sources is reduced.

A large number of studies have suggested that antioxidant nutrients and/or medicines play a protective role in human health (Aruoma, 1999; Fang *et al.*, 2002; Devasagayam *et al.*, 2004). Crude red palm oil (RPO) is known to be the richest natural plant source of carotenoids in terms of provitamin A equivalents, such as *a*-carotene and *b*-carotene (Sundram *et al.*, 2003). Isong *et al.* (1997) had shown that RPO exerted effects on reproductive capacity through improving the efficiency of protein biosynthesis or utilisation in a way that was favourable to sex hormone function in rats fed with RPO-supplemented diet. It is also likely that RPO provided vitamin A, which is known to play a part in reproduction through the synthesis of sexual steroids (Alais & Linden, 1991), embryogenesis and spermatogenesis (McArdle & Ashworth, 1999).

Studies have shown that exposure to organic hydroperoxides such as t-butyl hydroperoxide (tbHP) and cumene hydroperoxide (cHP) dramatically increases the level of lipid peroxidation (LPO) and enhances ROS generation in rats. Organic hydroperoxides have been extensively used as model pro-oxidants to induce OS in various *in vivo* systems (Sestili *et al.*, 1998; Chen *et al.*, 2000; Kumar *et al.*, 2002; Kaur *et al.*, 2006a,b; Kumar & Muralidhara, 2007).

The aim of this study was two-fold, (i) To create an *in vivo* animal model to study the effects of OS on rat sperm caused by organic hydroperoxides and (ii) To study the effect of RPO supplementation on this induced OS model.

Materials and methods

Animal care and experimental protocol

Ethical approval was obtained from the Institutional Review Board at Cape Peninsular University of Technology. Male Wistar rats aged 10 – 12 weeks were randomly placed in three groups and fed daily 25 g standard rat chow (SRC) (Epol Ltd., Johannesburg, South Africa) with *ad lib* access to water. Group 1 received no supplement while the food of groups 2 and 3 was supplemented with 2 and 4 ml RPO (Carotino SDN BHD Co: 69046-T, Johar-Bahru, Malaysia) in 25 g SRC per day respectively. Each group was further divided into three subgroups. These subgroups were injected intra-peritoneally with either saline (0.5 ml), 10 /m cHP (0.5 ml, 80% aqueous, Sigma Chemical Co., Johannesburg, South Africa) or 20 /m tbHP (0.5 ml, 70% aqueous, Sigma Chemical Co.) respectively. The injections were performed daily for five consecutive days per week over a 60-day period in order to target at least one complete cycle of spermatogenesis. Body weights were recorded at onset and completion of the experimental period. Immediately after sacrificing the animals, the testes and epididymes were excised and weighed. The caudal epididymis was subsequently rinsed, and gently minced in 1.5 ml of phosphate buffered saline (PBS, Sigma Chemical Co.) containing no calcium and magnesium. The fragments were allowed to sediment, and the supernatant containing epididymal sperm retrieved for further analysis. Rats fed with SRC and injected with saline are referred to as control.

Sperm count and motility

Epididymal sperm count was determined by the method as described in the WHO Manual (WHO, 1999). Briefly a 50 /l aliquot of epididymal sperm was diluted with 95 /l diluent (50 g sodium bicarbonate, 10 ml 35% formalin, and 0.25 g trypan blue were added and made up to a final volume of 1000 ml with distilled water). A cover slip was secured to the counting chambers of a Neubauer type haemocytometer (Marienfeld, Germany). Approximately 10 /l of the thoroughly mixed diluted specimen was transferred to each of the counting chambers of the haemocytometer, which was allowed to stand for 5 min in a humid chamber to prevent drying. The cells sediment during this time and were counted with a light microscope at 40· magnification.

For the manual determination of sperm motility, one drop of sperm suspended in PBS, was placed on a glass microscope slide. Ten random fields were scored for the number of motile and non-motile sperm. Motility was expressed as a percentage of motile sperm compared to the total number of cells observed.

Assessment of ROS

Both ROS and lipid peroxidation (LPO) levels were assessed in epididymal sperm using a microplate reader (GloMax Multi Detection System, Promega, UK).

Reactive oxygen species were measured using 2¢7¢-dichlorofluorescin (DCFH, Sigma Chemical Co.) as the probe (Driver *et al.*, 2000). The nonfluorescent DCFH oxidises rapidly in presence of ROS to the highly fluorescent dichlorofluorescein (DCF). DCFH (10 /mol) was added to 100 /l of medium or samples and incubated for 45 min at room temperature (in dark) in a 96 well microplate. Fluorescence intensity was measured at 530 nm emission (485 nm excitation). DCF production was expressed as relative fluorescence units (RFU).

Assessment of lipid peroxidation

Malondialdehyde (MDA) levels indicate the amount of cellular damage secondary to LPO and have been widely adopted as a measure of free radical formation. In this

study, LPO was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) and expressed as nmol MDA formed (Draper *et al.*, 1993). In short, 50 µl of epididymal sperm ($0.1 \cdot 10^6$) was added to 6.25 µl cold butulated hydroxyl toluene/ethanol (4 nm) and 50 µl of ortho-phosphoric acid (0.2 m) in an Eppendorf tube. After mixing for 10 s, 6.25 µl of thiobarbituric acid reagent (0.11 m) was added and then heated to 90 C (45 min). Samples were subsequently cooled on ice (2 min) and then at room temperature (5 min) before addition of n-butanol (500 µl) and saturated NaCl (50 µl). The Eppendorf tubes were centrifuged (16 500 g, 2 min, 4 C) and 300 µl supernatant (top butanol) was transferred to 96 well plates and absorbance was measured (532 and 572 nm).

Assessment of antioxidant activities

Superoxide dismutase (SOD), catalase (CAT) and glutathione (GHS) activities were determined in epididymal sperm by ready made kits/reagents and assessed using a microplate reader.

Superoxide dismutase activity was determined from the conversion of xanthine and oxygen to uric acid and hydrogen peroxide (H_2O_2) by xanthine oxidase to form superoxide anion (O_2). The O_2 then converts WST-1 to WST-1 formazan, a coloured product that absorbs light at 450 nm. The relative SOD activity of the experimental sample is determined from percentage inhibition of the rate of formation of WST-1 formazan. The assay was conducted according to the protocol provided by the manufacturer (Assay Designs, Ann Arbor, MI, USA) using $0.1 \cdot 10^6$ cells.

Catalase fluorometric detection Kit is a sensitive assay which utilises a non fluorescent detection reagent that is converted to resorufin (excitation 530 – 571 nm, and emission 590 – 600 nm) in the presence of perioxidase and H_20_2 substrate left over from the CAT reaction (Zhou *et al.*, 1997). In brief, 50 µl of H_2O_2 (40 µm) solution was added to 50 µl of standard or samples in a 96 well plate and incubated at room temperature for 45 min. Subsequently, 100 µl of the reaction cocktail (freshly prepared) was added to each well according to the manufacturer's protocol (Assay Designs). CAT activity was expressed as RFU.

The GSH assay is based on the conversion of a luciferin derivative into luciferin in the presence of GSH, catalysed by glutathione S-transferase (GST). The signal generated in a coupled reaction with firefly luciferase is proportional to the amount of GSH present in the sample. In short, 50 µl of epididymal sperm (50 000 cells) was added to 50 µl of prepared GSH-Glo Reagent 2X and incubated at room temperature for 30 min. Subsequently, 100 µl of prepared Luciferin Detection Reagent was added and incubated for 15 min before reading luminescence (Promega, UK). The activity of GSH was expressed as relative luminescence units (RLU).

Statistical analyses

GaphPad prism version 4 for Windows (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com) was used for all statistical evaluations and graphical representations. Data are expressed as mean ± SEM. For comparative studies, Student's *t*-test (two groups' comparisons) or one-way analysis of variance (anova, >2 groups' comparisons) test with Bonferroni correction as a *post-hoc test* for base pair comparisons (if $P < 0.05$) were used for statistical analyses. Differences were regarded as statistically significant if $P < 0.05$ and highly significant if $P < 0.001$.

Results

Comparison of animal related parameters

Body, testicular and epididymal weights were comparable in all study groups ($P > 0.05$ for all, Table 1). However, the epididymal sperm concentration in animals injected with 10 µm cHP ($13.22 \pm 0.74 \cdot 10^7$ ml^{-1}, $P < 0.01$) and 20 µm tbHP ($14.22 \pm 1.10 \cdot 10^7$ ml^{-1}, $P < 0.05$) was significantly reduced compared to control ($18.85 \pm 1.34 \cdot 10^7$ ml^{-1}). Similarly, the sperm motility in animals injected with 10 µm cHP (65.00 ± 3.65%) and 20 µm tbHP (65.83 ± 5.06%) showed a significant decrease ($P < 0.01$) compared to the control (86.67 ± 3.33%). Interestingly, the sperm concentration and sperm motility of animals supplemented with (2 and 4 ml) RPO were in the normal range when compared to the control group despite the hydroperoxide treatment.

Intracellular sperm ROS production

Animals injected with 10 µm of cHP (31 585 ± 1851 RFU) showed a significant ($P < 0.001$) increase in DCF levels in their sperm compared to those injected with saline (19 308 ± 682 RLU) (Fig. 1). The amounts of DCF were significantly lower in rats injected with cHP and receiving the 2 ml (22 434 ± 1774 RFU, $P < 0.01$) and 4 ml (19 329 ± 2313 RFU, $P < 0.001$) RPO supplementation with their diet versus the group injected with cHP and fed only with SRC (31 585 ± 1851 RFU) (Fig. 1). The production of DFC in sperm of rats injected with 20 µm tbHP (30 500 ± 1630 RFU, $P < 0.001$) was significantly increased compared to those injected with saline (19 308 ± 682 RFU) (Fig. 1). Interestingly, the amount of DCF produced in the sperm of rats fed with 2 ml

Table 1 Body weights, testicular weights, epididymal weights and sperm concentrations of rats treated with organic hydroperoxides (n = 6)

	Group 1			Group 2			Group 3		
	SRC			SRC + 2 ml RPO			SRC + 4 ml RPO		
	Saline	cHP	tbHP	Saline	cHP	tbHP	Saline	cHP	tbHP
Body weight (g)	404.7 ± 20.13	397.0 ± 20.69	413.8 ± 16.41	411.0 ± 14.23	370.5 ± 12.99	404.7 ± 20.64	393.8 ± 9.65	374.3 ± 10.61	394.0 ± 8.74
Testicular weight (g)	3.88 ± 0.06	3.94 ± 0.06	3.77 ± 0.12	3.92 ± 0.12	3.84 ± 0.13	3.53 ± 0.26	3.82 ± 0.12	3.87 ± 0.12	3.75 ± 0.11
Epididymal weight (g)	0.53 ± 0.01	0.49 ± 0.01	0.51 ± 0.01	0.49 ± 0.02	0.47 ± 0.01	0.49 ± 0.01	0.48 ± 0.03	0.48 ± 0.01	0.49 ± 0.02
Sperm concentration (10^7 ml^{-1})	18.85 ± 1.34	13.22 ± 0.74**	14.22 ± 1.10*	17.27 ± 2.04	15.00 ± 1.67	15.70 ± 1.76	14.33 ± 1.81	16.40 ± 2.31	15.43 ± 1.84
Motility (%)	86.67 ± 3.33	65.00 ± 3.65**	65.83 ± 5.06**	82.50 ± 3.81	82.50 ± 3.81	79.17 ± 3.51	79.17 ± 7.00	75.00 ± 4.835	75.00 ± 5.00

cHP, cumene hydroperoxide; RPO, red palm oil; SRC, standard rat chow; tbHP, t-butyl hydroperoxide.
Values are expressed as mean ± SEM. *P < 0.05 versus saline, **P < 0.01 versus saline.

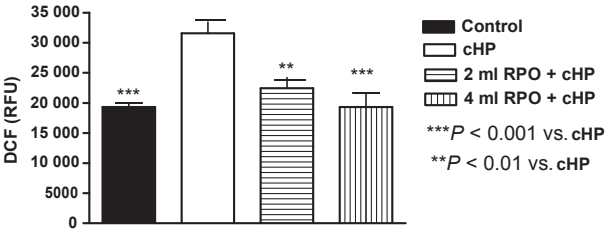

Fig. 1 Effects of cumene hydroperoxide (cHP) and red palm oil (RPO) on dichlorofluorescein (DCF) fluorescence (reactive oxygen species levels) in rat sperm (n = 6).

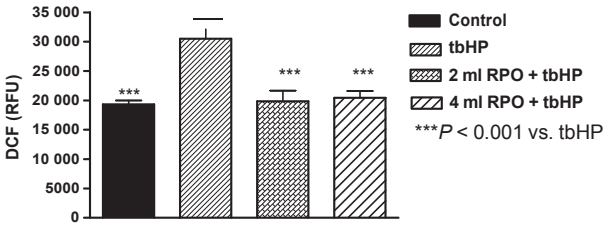

Fig. 2 Effects of t-butyl hydroperoxide (tbHP) and red palm oil (RPO) on dichlorofluorescein (DCF) fluorescence (reactive oxygen species levels) in rat sperm (n = 6).

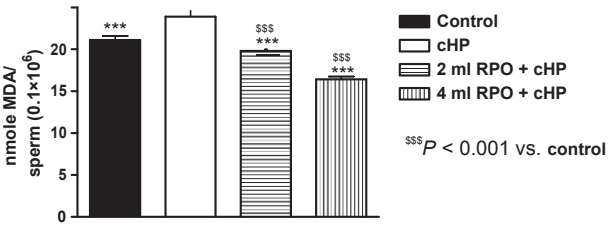

Fig. 3 Effects of cumene hydroperoxide (cHP) and red palm oil (RPO) on malondialdehyde (MDA) formation and thus lipid peroxidation in rat sperm (n = 6).

(19 849 ± 1798 RFU, P < 0.001) or 4 ml (20 424 ± 1184 RFU, P < 0.001) supplementation of RPO in addition to 20 μm tbHP injection showed a significant decrease when compared to rats receiving SRC only and injected with tbHP (30 500 ± 1630 RFU). RPO supplementation alone did not affect DCF production (data not shown) (Fig. 2).

Lipid peroxidation of spermatozoa

Rats injected with 10 μm cHP and receiving SRC only, showed a significant increase in MDA in their sperm (23.91 ± 0.70 nmol, P < 0.001) versus those injected with saline (21.12 ± 0.48 nmol) (Fig. 3). On the other hand, the amount of MDA produced in the sperm of rats fed

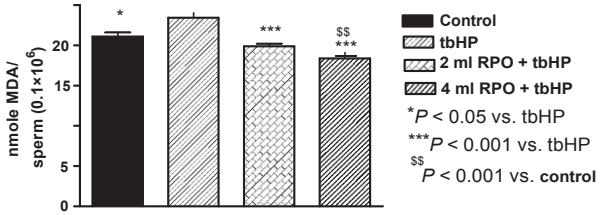

Fig. 4 Effects of t-butyl hydroperoxide (tbHP) and red palm oil (RPO) on malondialdehyde (MDA) formation and thus lipid peroxidation in rat sperm (n = 6).

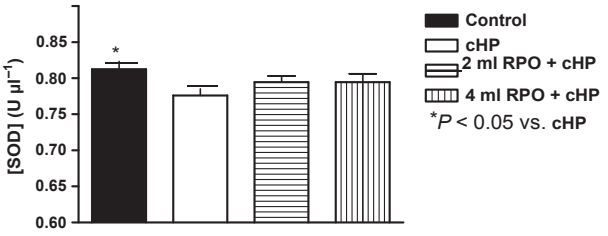

Fig. 5 Effects of cumene hydroperoxide (cHP) and red palm oil (RPO) on superoxide dismutase (SOD) production in rat sperm (n = 6).

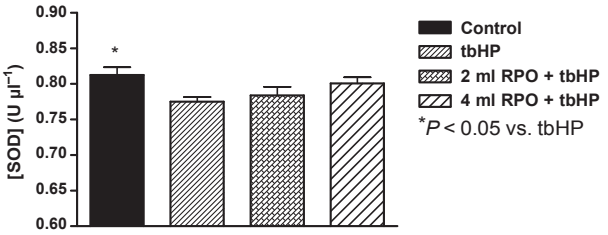

Fig. 6 Effects of t-butyl hydroperoxide (tbHP) and red palm oil (RPO) on superoxide dismutase (SOD) production in rat sperm (n = 6).

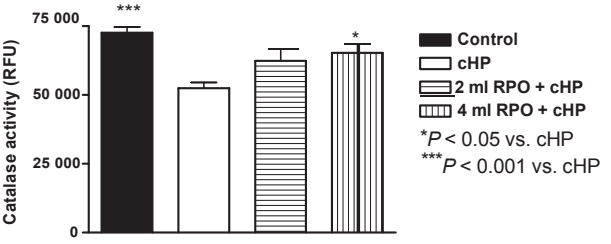

Fig. 7 Effects of cumene hydroperoxide (cHP) and red palm oil (RPO) on catalase production in rat sperm (n = 6).

with 2 ml (19.82 ± 0.31 nmol, $P < 0.001$) or 4 ml (16.42 ± 0.32 nmol, $P < 0.001$) supplemented with RPO and 10 µm cHP injection also decreased significantly when compared to rats receiving SRC only and injected with 10 µm cHP (23.91 ± 0.70 nmol) as well as those fed with only SRC and injected with saline (Fig. 3). However, there were no significant differences in the amount of MDA in the sperm of rats injected with saline and receiving 2 or 4 ml RPO supplementation when compared to those receiving only SRC and injected with saline (data not shown). Figure 4 shows that rats injected with 20 µm tbHP and receiving SRC only, had a significant increase in MDA in their sperm (23.45 ± 0.58 nmol, $P < 0.05$) compared to those injected with saline (21.12 ± 0.48 nmol). The amount of MDA produced in the sperm of rats supplemented with 2 ml (19.89 ± 0.32 nmol, $P < 0.001$) or 4 ml (16.42 ± 0.32 nmol, $P < 0.001$) RPO in addition to the 20 µm tbHP injection also decreased significantly when compared to rats receiving SRC only and injected with 20 µm tbHP. Moreover, the levels of MDA in the sperm of rats injected with 20 µm tbHP and receiving 4 ml RPO supplementation in SRC also significantly decreased (16.42 ± 0.32 nmol, $P < 0.01$) compared to those fed with only SRC and injected with saline (21.12 ± 0.48 nmol) (Fig. 4). The supplementation of RPO alone did not affect the amount of MDA in rats injected with saline (data not shown).

Antioxidant activities of spermatozoa

Superoxide dismutase

Figures 5 and 6 show that the concentrations of SOD in the sperm of rats injected with cHP (0.7762 ± 0.0091 U µl^{-1}, $P < 0.05$) and tbHP (0.7750 ± 0.0066 U µl^{-1}, $P < 0.05$) had decreased significantly compared to the control (0.8126 ± 0.0108 U µl^{-1}). However, the concentrations of SOD in the epididymal sperm of rats injected with cHP or tbHP and fed with RPO did not differ from the control. The supplementation of RPO alone did not affect the production of SOD in rats (data not shown).

Catalase

The fluorescence in the sperm of rats injected with cHP (52 499 ± 2002 RFU, $P < 0.001$) decreased significantly compared to those injected with saline (72 605 ± 2080 RFU). However, the sperm of rats fed with 4 ml RPO and injected with cHP (65 310 ± 3140 RFU, $P < 0.05$) had a significant increase in fluorescence compared to those injected with cHP and fed with SRC only (52 499 ± 2002 RFU) (Fig. 7). The sperm of rats fed with 4 ml RPO and injected with cHP did not show a significant difference in fluorescence when compared to the control. Similarly, from Fig. 8 it can be seen that rats injected with tbHP (49 218 ± 6647 RFU, $P < 0.05$) had a significant decrease in their sperm fluorescence compared to those injected with saline (72 605 ± 2080 RFU).

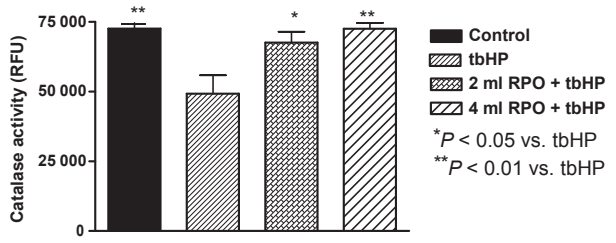

Fig. 8 Effects of t-butyl hydroperoxide (tbHP) and red palm oil (RPO) on catalase production in rat sperm (n = 6).

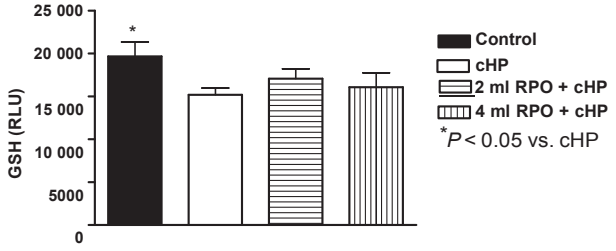

Fig. 9 Effects of cumene hydroperoxide (cHP) and red palm oil (RPO) on glutathione (GSH) production in rat sperm (n = 6).

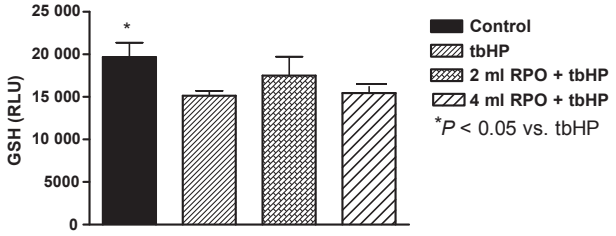

Fig. 10 Effects of t-butyl hydroperoxide (tbHP) and red palm oil (RPO) on glutathione (GSH) production in rat sperm (n = 6).

However, the sperm of rats fed with 2 ml (67 533 ± 3905 RFU, $P < 0.05$) or 4 ml (72 559 ± 2094 RFU, $P < 0.01$) RPO and injected with cHP showed a significant increase in fluorescence when compared to those of animals injected with cHP and fed with SRC only (49 218 ± 6647 RFU). The sperm of rats receiving 2 or 4 ml RPO supplementation and simultaneously injected with cHP had no increase in fluorescence compared to the control. The supplementation of RPO did not affect the CAT activity fluorescence (data not shown).

Glutathione

From Figs 9 and 10, it can be seen that the formation of luciferase in the sperm of animals injected with cHP (15 192 ± 806.6 RLU, $P < 0.05$) and tbHP (15 126 ± 598.6 RLU, $P < 0.001$) ,respectively, was significantly decreased compared to the control group (19 669 ± 1697 RLU). The sperm of rats receiving 2 or 4 ml RPO supplementation and simultaneously injected with either cHP or tbHP did not differ in luminescence from the control. The supplementation of RPO did not affect the GSH production expressed as RLU in the sperm (data not shown).

Table 2 Correlations between sperm concentration, motility, reactive oxygen species production, lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) activities after cumene hydroperoxide (cHP), t-butyl hydroperoxide (tbHP) and red palm oil (RPO) exposure

		DCF	TBARS	SOD	CAT	GSH
Sperm	r	)0.312	)0.175	)0.095	0.6107	0.1456
	P	0.0474	0.2731	0.5544	<0.0001	0.3766
Motility	r	)0.371	)0.311	0.1476	0.2079	0.0851
	P	0.0155	0.0452	0.3508	0.1864	0.6014
DCFH	r		0.3799	)0.186	)0.535	)0.18
	P		0.0131	0.2387	0.0003	0.2668
TBARS	r			)0.15	)0.326	0.0517
	P			0.3421	0.0353	0.7512

TBARS, thiobarbituric acid reactive substances.

Correlation studies

The correlations performed between various parameters are reported in Table 2. DCF fluorescence i.e. ROS production correlated negatively to sperm concentration (r =)0.312), sperm motility (r =)0.371) as well as catalase activity (r =)0.535), while a positive correlation was observed with TBARS i.e. LPO (r = 0.3799). Catalase on the other hand showed a strong positive correlation with sperm concentration (r = 0.6107) and negative correlation with LPO (r =)0.0353). Sperm motility also correlated negatively with LPO (r =)0.311).

Discussion

Organic hydroperoxides have been used in various short term studies as inducer of OS in *in vivo* systems (Kaur *et al.*, 2006a,b; Kumar & Muralidhara, 2007; Younes & Strubelt, 1990). In this study, longer exposure of animals to hydroperoxides (60 days) via intraperitoneal injection of cHP or tbHP clearly shows that sperm parameters were negatively influenced as indicated by low epididymal sperm concentration and low sperm motility. It is evident from the correlations done in this study that both sperm concentration and motility correlated negatively to intracellular ROS levels. These results are in support of findings previously reported from our group (Aboua *et al.*, 2009). Our data are also confirmed by reports from other studies (Agarwal *et al.*, 2003; Armstrong *et al.*, 1999; Bilodeau *et al.*, 2002; Lenzi *et al.*, 1993) which showed a

correlation between high levels of ROS (superoxide, hydroxyl, hydrogen peroxide, nitric oxide, peroxynitrile) and decreased sperm motility. We speculate that the injection of organic hydroperoxides might have led to H_2O_2 diffusing across the membranes into the sperm cells thereby inhibiting the activity of some enzymes such as glucose-6-phosphate dehydrogenase (G6PD). This can lead to a decrease in the availability of reduced nicotinamide adenine dinucleotide phosphate (NADPH). In turn, this subsequently decreased the formation of ATP, which is an important metabolite for sperm motility. In the process, cHP and tbHP created an imbalance between ROS and the scavenging capacity of the enzymes in the sperm leading to OS (Aboua *et al.*, 2009). ROS-mediated damage of sperm membranes has also been reported to be responsible for impaired sperm motility (de Lamirande & Gagnon, 1992). This can be attributed to H_2O_2 attacking the lipids in the sperm plasma membrane, thereby initiating an LPO cascade and subsequently, leading to the sperm losing their capability for movement.

The correlation found between LPO and DCFH (positive) can be ascribed to the peroxidative process initiated by cHP and tbHP ultimately leading to ROS-mediated protein oxidation (Ong *et al.*, 2002). The reduced sperm motility observed can also be attributed to LPO as seen from the negative correlation. It was previously reported that LPO can lead to reduced sperm counts (Agarwal *et al.*, 2003). This was possibly due to cell death attributed to OS. H_2O_2 can penetrate the plasma membrane, cause protein oxidation and increase LPO production. Griveau *et al.* (1995) have shown that ROS cause a decrease in sperm motility, an increase in LPO and a loss of membrane polyunsaturated fatty acids. Our data further confirm the results from those studies.

Under physiological situations, adequate levels of antioxidants, SOD, CAT, GSH peroxidase and reductase maintain the ROS scavenging potential in the male reproductive tract and seminal fluid. When monitored more objectively, these scavengers can be used as a good indicator of sperm damage and infertility caused by oxidative stress (Sikka *et al.*, 1995). SOD is involved in dismutation of the O_2^- to H_2O_2 and oxygen. In this study, a significant decrease in the SOD concentration in the epididymal sperm of rats treated with 10 *l*m cHP or 20 *l*m tbHP indicated that the synthesis of the enzyme was reduced. However, the treatment with RPO increased SOD concentration to levels comparable to the control. Catalase is known to neutralise H_2O_2 to water and superoxide. There is a significant decline in the activity of catalase in the epididymal sperm of rats treated with 10 *l*m cHP or 20 *l*m tbHP. From the correlations performed in this study it is evident that increased ROS levels are accompanied by decreased catalase, more than likely due to the depleting effect caused by catalase's scavenging activity. LPO also correlated negatively with catalase, yet again confirming that in the absence of catalase or the depletion thereof it can lead to an imbalance in pro-oxidants and antioxidants, allowing ROS to cause OS with accompanying LPO. The positive correlation between catalase and sperm concentration furthermore confirms these findings. However, the regain in the catalase activity in animals fed with RPO showed that RPO was actively involved in the free radical scavenging mechanism caused by the H_2O_2. This is evident from the reduced amount of DCF in the sperm of rats injected with cHP or tbHP and receiving RPO supplementation compared to those injected with cHP or tbHP and receiving only SRC. Our data showed that GSH content was significantly decreased in the sperm of rats treated with 10 *l*m cHP or 20 *l*m tbHP. The lower levels of GSH content in the rat sperm indicated a greater participation of reduced glutathione in H_2O_2 detoxification (Krishnamoorthy *et al.*, 2007). However, the supplementation of RPO diet increased the level of GSH in sperm. This increased intracellular transport of GSH is essential for maintaining the redox state during OS.

Due to its content in (*a* and *b*) carotene (*a* and *b*) tocopherol and phenolic-flavonoid-rich antioxidant complex, RPO was used in this study, to investigate its scavenging and antioxidant capacity on hydroperoxide-induced OS in rat epididymal sperm *in vivo*. Tocopherol can transfer a hydrogen atom with a single electron to a free radical, thus removing the radical, before it can interact with the cell membrane. Besides playing a beneficial biological role as radical quenchers *in vivo*, tocopherols and tocotrienols are also antioxidants, which contribute to the stability of RPO. Tocopherols can interrupt lipid oxidation by inhibiting hydroperoxide formation in the chain-propagation step, or the decomposition process by inhibiting aldehyde formation (van Rooyen *et al.*, 2008). The main function of *a*-tocopherol is to prevent the peroxidation of membrane phospholipids and prevent cell membrane damage through its antioxidant action (Sundram *et al.*, 2003). The antioxidant properties of carotenoids have been suggested to reflect not only the rates of free radical scavenging, but also the reactivity of the resultant carotenoid-derived radicals. Carotenoid radical-cation or adduct radicals have been shown to be highly resonance stabilised and predicted to be relatively unreactive. They may further undergo bimolecular decay to generate non-radical products or in the case of carotene radical-adducts, may terminate radical reactions by binding to the attacking free radical (Everett *et al.*, 1996). Both *b* carotene and lycopene (found in tomatoes) have shown membrane protection against

LPO and form components of antioxidant defences (Gupta & Kumar, 2002).

Several studies have suggested that tocopherols at higher levels accelerate the formation of hydroperoxides especially at the early stage of oxidation. Jung & Min (1990) showed that tocopherols, above their optimum level, had a pro-oxidant effect on hydroperoxide formation. At higher tocopherol concentrations and higher oxidation rates, dihydroperoxides will be formed at the expense of hydroperoxy epidioxides. Kamal-Eldin & Appelqvist (1996) argued that the best antioxidant should be the one that is active so that the concentration of the antioxidant radical will be at a minimum (Kamal-Eldin & Appelqvist, 1996). High concentrations of antioxidant radicals will certainly be problematic at some stage of the oxidation reaction. Our results did not show any sign of toxicity regarding the amount of RPO (2 or 4 ml) used in this study.

Increasing intake of dietary antioxidants may help maintain an adequate antioxidant status and, therefore, the normal physiological function of a living system (Record *et al.*, 2001). Some functional foods and vegetables are the important sources of exogenous antioxidants. RPO was able to reverse the negative effects of the organic hydroperoxides in this animal model. Moreover, the sperm of animals receiving RPO had increased SOD, CAT and GSH activities and sperm motility while the amount of MDA and LPO was reduced.

Conclusion

Recently, much attention has been focused on the protective biochemical function of naturally occurring antioxidants in biological systems, and on the mechanisms of their action. Many natural antioxidant products are capable of preventing or inhibiting the process of OS. RPO supplementation protected the epididymal sperm *in vivo* against the adverse effects of organic hydroperoxide i.e. loss of sperm motility by preventing LPO, scavenging the formation of ROS, increasing SOD synthesis and the activities of catalase and GSH via antioxidant potential. We therefore propose that a daily intake of RPO supplement to the diet might be helpful to protect males against the adverse effects of ROS in sperm function and help preserve fertility.

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