Red Palm Oil Supplementation Ameliorates Oxidative Stress in Rats Injected with Tert-Butylhydroperoxide

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ABSTRACT
Potential benefits of dietary supplementation as quenching agent against oxidative stress-related conditions has been extensively investigated. Red palm oil (RPO), from the tropical plant Elaeis guineensis has captivated much interest in the health sector lately; hence the aim to assess the potential effects of RPO supplementation on the antioxidant status and protection against oxidative damage in experimentally-induced oxidative stress male Wistar rats. Male Wistar rats were randomly divided into four groups (n=5). Rats were fed 0.175g RPO (7g RPO/kg chow) supplementation for 6 weeks. Oxidative stress was induced by intraperitoneal injections of 0.5mL (20µM/100g of body weight) organic tert-butylhydroperoxide (t-BHP). All parameters were determined in plasma and erythrocytes by using appropriate methods. Data were expressed as mean ± SEM. Exposure to t-BHP caused a significant increase in malondialdehydes (MDA) levels in plasma of non-supplemented rats. MDA was significantly reduced by RPO supplementation. This proved that RPO supplementation reduced the increase in MDA level induced by t-BHP injection, thereby protecting cellular integrity against induced oxidative stress. Superoxide dismutase (SOD) activity increased significantly (p<0.05) in group supplemented with RPO not induced with oxidative stress. This suggests that RPO supplementation could also improve antioxidant status in a biological system. RPO supplementation offers a protective effect against lipid peroxidation in an oxidative stress-induced biological system and that RPO supplementation had potential benefits in improving antioxidant status.

Keywords; Palm oil, oxidative stress, antioxidant, antioxidative status, supplementation

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Received: July, 2016; Accepted: October, 2016

Abstracted by: Bioline International, African Journals online (AJOL), Index Copernicus, African Index Medicus (WHO), Excerpta medica (EMBASE), CAB Abstracts, SCOPUS, Global Health Abstracts, Asian Science Index, Index Veterinarius

INTRODUCTION
Oxidative stress is a metabolic state that occurs when there is a dysfunction in the overall balance between the production of reactive oxygen and nitrogen species (ROS/RNS) and the oxidative status shifts in favour of ROS/RNS production (Ceconi et al., 2003; Berk, 2007; Barbosa et al., 2008). Oxidative stress and ROS-mediated damage has been implicated in a variety of health disorders such as cancers, and other chronic diseases such as diabetes, cardiovascular diseases and infectious diseases (Salgo et al., 1995; Li et al., 2002; Park et al., 2002; Heistad et al., 2009). Lipid peroxidation is a reference marker of oxidative stress and a continuous process in living aerobic cells (Park et al., 2002). It is a breakdown chain of chemical reactions generated from the attacks of free radicals on polyunsaturated fatty acids (PUFAs) within the cell membrane (Fabbi et al., 2004). Peroxidation of unsaturated fatty acids leads to membrane disruption and release of highly reactive free radicals that alter the cellular function and contribute to tissue damage. Such breakdown leads to the generation of toxic secondary radicals, namely hydroperoxides, reactive aldehydes (malondialdehydes MDA, 4-hydroxynonenal 4-HNE) and other carbonyl compounds (alkoxyl radical RO•, peroxyl radical ROO•) (Ceconi et al., 2003; Park et al., 2002).

Previous studies have reported on the positive correlation between an increase in MDA levels and the occurrence of diverse types of oxidative stress-related conditions.
Experimental, clinical and epidemiological data suggest that dietary antioxidants can offer protection against oxidative stress-related health disorders and pro-oxidant mediated damages in vivo (Marnewick et al., 2000; Pantsi et al., 2011; Aboua et al., 2012; Awoniyi et al., 2012). Red palm oil, a dietary vegetable oil obtained from the tropical oil palm plant Elaeis guineensis contains fatty acids (saturated and unsaturated fatty acid ratio close to one) and is rich in phytonutrients such as α-, β-, γ-, and δ- carotene, lycopene, α-, β-, γ- and δ- tocotrienols and tocopherols, and ubiquinone (Akusu et al., 2000; Mathan et al., 2009). Red palm oil has been reported to have protective effects against the consequences of ischemic-reperfusion injury (Narang et al., 2004; Esterhuyse et al., 2005; Esterhuyse et al., 2006; Bester et al., 2006). The present study, for the first time, investigated the effects of dietary RPO supplementation in oxidative stress-induced state in an in vivo experimental animal model. Oxygen radical absorbance capacity and ferric reducing ability of plasma were measured as well as superoxide dismutase, glutathione peroxidase activities, total glutathione levels, lipid peroxidation status and MDA.

MATERIALS AND METHODS

Animal Care

All animals received humane care according to the Principle of the National Institutes of Health Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (National Institutes of Health publication no.80-23, revised 1978). Ethical approval was obtained from the Research Ethics Committee (REC), Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville, South Africa (NHREC: REC-230408-014). Adult male Wistar rats (120-150g) were obtained from the Department of Physiology, University of Stellenbosch, South Africa. In all experiment, animals were daily fed a standard rat chow (SRC) and/or supplementation with RPO (7g RPO/kg chow) and had free access to water. Animals were housed individually at 25˚± 3˚C, with 12h light/dark cycle and 50% ± 5% humidity.

Experimental Design

Rats were randomly divided into four groups (n=5 per group) and treated for six weeks as follows: Group I (Control): fed 25g of standard rat chow (SRC) daily; received no induction of oxidative stress. Group II (Control t-BHP): fed 25g of SRC daily; received induction of oxidative stress. Group III (6wks RPO): fed 25g of SRC and supplemented with 0.175g of RPO (7g RPO/kg chow) daily for 6 weeks; received no induction of oxidative stress. Group IV (6wks RPO + t-BHP): fed 25g of SRC and supplemented with 0.175g of RPO (7g RPO/kg chow) daily for six weeks; received induction of oxidative stress (Engelbrecht et al., 2006; Kruger et al., 2007).

Groups:
I: CTRL Normal: Control group I fed with SRC;
II: CTRL t-BHP: Control group II fed with SRC and injected with t-BHP
III: 6WK RPO: Group III supplemented with RPO for 6 weeks;
IV: 6WK RPO + t-BHP: Group IV supplemented with RPO for 6 weeks and injected with t-BHP.

Induction of Oxidative Stress

The animals in the experimental groups were subjected to oxidative stress induction. For oxidative stress induction, the animals received a chronic intra-peritoneal injection of 0.5ml (20μM/100g body weight) of organic tertiary-butyl hydroperoxide solution (t-BHP, 70% in water, Digma, South Africa (Aboua et al., 2009)). Animals in the control groups received a chronic intra-peritoneal injection of 0.05 ml placebo solution made of sterile phosphate buffer saline (10 mmol/L PBS) with 10-fold dilution. Injections of t-BHP or PBS were repeated every second day for the last two weeks of the experiment. Injections were performed using sterile 1ml disposable syringe and 26 G sterile hypodermic needles.

Chemicals

2,2’-azobis (2-methylpropionamide) dihydrochloride (AAPH), 6-hydroxydopamine (6-HD), diethylenetriaminepentaacetic acid (DTPAC), 5,5’-Dithiobis-(2-nitrobenzoic acid) reagent (DTNB), fluorescein sodium salt, glacial metaphosphoric acid (MPA), glutathione reduced (GSH), glutathione reductase (GR), L-ascorbic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), iron chloride hexahydrate and 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ), malondialdehyde (MDA) standard, orthophosphoric acid (O-PA), perchloric acid (PCA), potassium phosphate (KH2PO4), reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), sodium azide, sodium hydroxide (NaOH), sulphuric acid, superoxide dismutase standard, tert-Butylhydroperoxide (t-BHP), thiobarbituric acid (TBA) and trisodium citrate was purchased from Sigma-Aldrich (Johannesburg, SA). All solvents used were of analytical reagent grade. Acetic acid, chloroform, glacial acetic acid, hydrochloric acid (HCl), isopropanol, methanol, perchloric acid (PCA) 70%, sodium acetate and trifluoroacetic acid (TFA) were purchased from Merck (Johannesburg, SA). Ultrapure MilliQ water (Millipore) was used throughout the study. Atlas Animal Foods (Cape Town, SA) supplied the standard rat chow (SRC). Red palm oil was donated by Carotino SDN BDH (Malaysia).

Preparation of Plasma and Erythrocytes Lysate

At the end of each experiment, rats were anaesthetized using 1mL (± 60mg/kg) of sodium pentobarbitone. Once anaesthetized, blood samples were collected from the rat. Plasma samples were obtained after centrifugation at 4000rpm at 4˚C for 10min. To obtain erythrocytes lysate, theuffy coat was removed and the packed erythrocytes were washed three times with an equal volume of phosphate buffer saline (PBS, 1:10). Each centrifugation run was set at 4000rpm at 4˚C for 5min. Samples were aliquoted within 6 hours of collection and stored at -80˚C until further analysis.

Oxygen Radical Absorbance Capacity (ORAC) Assay

The oxygen radical absorbance capacity (ORAC) assay is used to measure the antioxidant scavenging activity of a substance (lipophilic, hydrophilic) by monitoring the fluorescence decay of the fluorescein probe. The assay used in the present study was determined according to a modified method (Rautenbach et al., 2010). All reagents and standards were prepared in phosphate buffer (75mmol/L, pH 7.4). Standard solutions were
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preparation of GSHt concentration was expressed as mmol/mL. Assay for plasma MDA was performed using a modern HPLC-based thiorbituric acid (TBA) method. The method is highly specific because it quantifies the genuine MDA-(TBA)2 adduct formed (Lykkefeldt, 2001). The quantitative analysis of the plasma content of MDA was performed using a modified method (Cunny et al., 2004) on Spectra system HPLC (Thermo Fischer Scientific, South Africa). The HPLC system consisted of Spectra system P2000 pump, equipped with HPLC column C18, 150 x 4.6mm, 5μm particle size (Agilent Zorbax, South Africa) and a Spectra system FL3000 fluorescence detector. The chromatographic conditions were: 1mL/min flow rate, 15min run time, 20μL sample injection volume. The mobile phases A and B were respectively made up of 50mM KH2PO4 and absolute methanol adjusted to pH 5.8. A gradient program was used as follows: from 60% A in 2min, from 60 to 30% A in 8min, then back to 60% B at 12min; from 40% B in 2min, from 40 to 70% B in 8min, then back to 40%B at 12min and 3min of reconditioning before the next injection. The column and detector array temperature was maintained at room temperature 25 ± 1°C. The analytical signals were monitored at 2-20mV potentials applied. Standard solutions were prepared in a range of 0.5 -10μmol/L MDA standards (in 0.1% sulphuric acid). High performance liquid chromatography mixture was made of 100μL of standards or plasma, 250μL TBA (40mmol/L in 0.1N NaOH) and 750μL O-PA (0.2mol/L), put in water-bath at 90°C for an hour, cooled on ice and centrifuged 14000g for a minute. The supernatant was injected into HPLC.

**Statistical Analysis**

Data are expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to test for significance between the groups. Bonferroni Multiple Comparison analysis was used to compare the differences between the groups. Statistical analysis of control and RPO groups at baseline level reading was performed by unpaired Student’s t-test. Differences were considered significant at P<0.05. GraphPad™ PRISM5 software package was used for all statistical evaluations and graphical representations.

**RESULTS**

**Effects of Dietary RPO Supplementation on FRAP and ORAC Levels**

Figures 1 and 2 showed the results of 6 weeks of RPO supplementation on FRAP and ORAC levels in the designated groups I, II, III and IV. They were no significant differences observed in plasma FRAP and ORAC levels among all groups.
Effects of Dietary RPO Supplementation on Antioxidant Enzyme activities
The effects of dietary RPO supplementation on SOD and GPx after the two-week injection period of t-BHP and placebo are presented in Figures 3 and 4. After 6 weeks of RPO consumption, a significant increase (p<0.05) in erythrocytes SOD activity was observed in group III (6WK RPO) when compared to its control group I (CTRL Normal) (Figure 3). However, no significant difference (p>0.05) was observed for GPx after 6 weeks of dietary supplementation with RPO among all the groups (Figures 4).

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Effects of Dietary RPO Supplementation on Antioxidant Glutathione Level
The effects of dietary RPO supplementation on erythrocytes total glutathione level after the two-week injection period of t-BHP and placebo are presented in Figures 5. After 6 weeks of RPO consumption, no significant difference (p>0.05) observed in group III (6WK RPO) and group IV (6WK RPO + t-BHP) when compared to the respective controls of group I (CTRL Normal) and II (CTRL t-BHP) (Figure 5).

Effects of Dietary RPO Supplementation on Lipid Peroxidation Biomarker MDA Level
The effect of dietary RPO supplementation on lipid peroxidation biomarker MDA after the two-week injection period of t-BHP and placebo are presented in Figure 6. Following 6 weeks of dietary RPO supplementation, MDA level in the oxidative stress control group II (CTRL t-BHP) was significantly higher (p<0.05) than the normal control group I (CTRL Normal). This increase in oxidative status proved that the model used to induce oxidative stress was effective. No differences (p>0.05) observed between the groups I (CTRL Normal) and IV (6wk RPO) for MDA levels. However, a significant decrease (p<0.05) in MDA level was noticed in group IV (4WK RPO + t-BHP) subjected to oxidative stress when compared to its corresponding control group II (CTRL t-BHP). Moreover, in the oxidative stress RPO supplemented group, MDA levels significantly reduced to values close to normal control. The current result proved that RPO supplementation reduced the increase in MDA level induced by t-BHP injection.
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**Figure 4:** Effects of dietary RPO-supplementation on erythrocytes GPx after 6 weeks consumption. Abbreviations: GPx: glutathione peroxidase, nmol/min/µl: nanomole per minute per microliter.

**Figure 5:** Effects of dietary RPO-supplementation on erythrocytes total glutathione level after 6 weeks consumption. Abbreviations: GSHt: total glutathione, mmol/mL: millimole per millilitre.

**Figure 6:** Effects of dietary RPO-supplementation on plasma MDA levels after 6 weeks consumption. Abbreviations: MDA: Malondialdehydes, µmol/L: micromole per litre. (a) Indicates significant difference when compared with control group I at p<0.05. (b) Indicates significant difference when compared with control group II at p<0.05.

**DISCUSSION**

Tert-butylhydroperoxide (t-BHP) is a prototypic initiator of oxidative stress (Garcia-Cohen et al., 2000; Kumar, 2007; Aboua et al., 2011). There are two pathways in which t-BHP can be metabolised. The first pathway leads to oxidative stress which involves a metabolism that leads to the formation of toxic peroxyl and alkoxyl radicals that initiate lipid peroxidation which negatively impart cellular integrity (Hogberg et al., 1975; Hwang et al., 2002). The second pathway does not lead to oxidative stress. It involves a cellular mechanism of detoxification against hydroperoxide-induced oxidative damage. During the detoxification reaction, t-BHP can be reduced to t-butyl alcohol which oxidised glutathione (GSSG) by GPx and ultimately produce GSH (Dringen et al., 1998; Kussmaul et al., 1999).

In the present investigation, where oxidative stress was induced with t-BHP, there was improvement in the lipid peroxidation status after RPO supplementation. This may be attributed to the antioxidant properties of RPO by means of scavenging the effects of hydroperoxide resulting from induced-oxidative stress. The finding agree with a recent study by Aboua and co-workers (Aboua et al., 2012), who reported that RPO reduced lipid peroxides accumulation in vivo and protected the epididymal sperm against the adverse effects of organic hydroperoxide. Red palm oil used in the present study is principally rich in fat-soluble antioxidants such as carotenes, tocopherols and tocotrienols which may help in the prevention of oxidative damage to cell membranes. Carotenes and vitamin E tocotrienols and tocopherols are considered among the most effective antioxidants (Van Stuijvenberg & Benade, 2000; Schroeder et al., 2006; Varoglu et al., 2010). It could therefore be argued that the carotenoids and vitamin E tocopherols and tocotrienols in RPO could have played an important role in protecting cellular membranes in vivo. Previously, it was reported that lipid peroxidation was inhibited through free radical scavenging activity resulting from the synergism between palm oil antioxidants especially α-tocopherol, α- and γ-tocotrienols and β-carotene (Schroeder et al., 2006). Red palm oil in contrast to other oil seem to possess a unique blend of vitamin E components. For example, Sundram and co-workers (2003) reported that RPO is not only rich in tocopherols but also unique because of its tocotrienols content. Although, the in vivo effect of RPO supplementation on the MDA status of oxidative stress-induced rat model had been scarcely investigated, administration of RPO fractions has been reported to show potential benefits (Budin et al., 2009; Narang et al., 2004; Mayne, 2003). For example, Budin and co-workers (2009) reported that tocotrienol rich fractions in diabetes-induced rat model significantly decreased plasma MDA and other oxidative stress markers.

There are reports of other studies which have used plant-rich antioxidant treatment and supplementation to counteract oxidative stress in animal models. For instance, Khakpour and co-workers reported significant decrease in MDA levels in mice treated with *Citrus aurantium* (Khakpour et al., 2012). In a study in ischemic stroke patients, *Ginkgo biloba* extract significantly reduced serum MDA levels (Thanoon et al., 2012). The bioactive extract of *Ginkgo biloba* is made of water-
soluble antioxidants (Thanoon et al., 2012). In our study, RPO consists of fat-soluble antioxidants. Our findings suggest that water-soluble and fat-soluble antioxidants may have similar effects on MDA levels. This argument is further supported by the fact that antioxidant supplementation in various other studies reduced the accumulation of MDA (Marnewick et al., 2000; Wilso et al., 2005; Shen et al., 2012).

The effect of RPO supplementation on TAC in the present study did not significantly affect the TAC, irrespective of oxidative stress induction. This could possibly due to the fact that majority of RPO antioxidants are located in the lipophilic compartment of rat plasma. There is still an on-going debate with regards to the assessment of total antioxidants in biological samples. For instance, Prior and co-workers (2005) are in support of the fact that the methodology of assessing the total antioxidant capacity should include both hydrophilic and lipophilic compartments of blood plasma. However, separating hydrophilic from lipophilic measurements may have limitations. Determination of lipophilic ORAC assay implies that lipophilic antioxidants should be extracted with hexane, after which plasma should be dried and finally suspended in a mixture of acetone, acetic acid and water (Prior et al., 2003). We can argue that such a method may not be suitable in the present in vivo model. In a true biological system both hydrophilic and lipophilic antioxidants communicate continuously. Therefore, by partitioning lipophilic from hydrophilic extracts, we could easily divert from our initial goal, which was to evaluate the effects of RPO on blood antioxidant capacity in vivo. The results of the current study support the previous finding of Ayeleso and co-workers who reported that dietary RPO did not influence TAC measured as ORAC and FRAP (Ayeleso et al., 2012). Similar report by Ajuwon and co-workers (2013) who reported that consumption of RPO and rooibos had no effects on TAC measured as FRAP. Our findings create opportunities for further investigations to elucidate the effects of RPO supplementation in modulating the total antioxidant capacity in a biological system.

Our study shows that RPO consumption for a period of 6 weeks significantly enhanced SOD activity when compared to normal control group. The results indicate that RPO supplementation, irrespective of oxidative stress induction, has the ability to increase endogenous antioxidant activities and enhance the antioxidant defence mechanism of the cell. It can be suggested that the potential mechanisms by which RPO enhance endogenous detoxification could involve one or more antioxidant defence mechanisms. Previous studies have shown that RPO antioxidant properties are adequate to potentially protect against oxidative stress induced-damages (Upritchard et al., 2003; Aboua et al., 2012).

Studies on dietary antioxidant supplementation have shown a positive correlation between natural dietary supplementation and increase in SOD activity and GSH levels (Oguntibeju et al., 2009; Oguntibeju et al., 2010). For example, Ananthan and co-workers (2004) investigated supplementation with Gymnema montanum leaf extract, an Indian medicinal plant. The authors reported that diabetic state-oxidative stress-related condition was associated with significant increase in SOD activity and GSH levels. In another study, Awoniyi and co-workers (2012) demonstrated that rooibos tea significantly increased the level of SOD after t-BHP induced-oxidative stress. Moreover, RPO supplementation improved SOD protein level expression in isolated hearts freezeclamped at the end of a working heart perfusion model of Wistar rats (Wergeland et al., 2011). Superoxide dismutase is a major scavenging enzyme which removes superoxide radical, a toxic radical, by converting it to more stable products such as hydrogen peroxide and water. An increase in SOD activity could therefore be seen as a possible response to reduce the risk of oxidative stress-induced damage.

In conclusion, this study demonstrated that RPO supplementation modulated MDA levels and SOD activity in an in vivo experimental Wistar rat model. Also, that RPO supplementation offers a protective effect against lipid peroxidation in an oxidative stress-induced biological system. Moreover, RPO supplementation had potential benefits in improving antioxidant status. Thus, this study proposed a possible mechanism of actions by which RPO-supplementation offer health benefits in the oxidative stress model. This explorative study has created opportunities for further investigations to explore additional RPO-related health benefits.

Acknowledgement
We acknowledged financial support from University Research Funding (URF), Cape Peninsula University of Technology (CPUT-RJ24) granted to Professor Oluwafemi O. Oguntibeju.

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