EFFECTS OF RED PALM OIL-SUPPLEMENTATION ON OXIDATIVE STRESS BIOMARKERS IN AN EXPERIMENTAL RAT MODEL

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By

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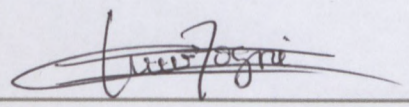
DECLARATION

I, Olatogni Berenice Lidwine Alinde, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.



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ABSTRACT

Oxidative stress, in recent times appears to be a major underlying risk factor in the occurrence of various diseases such as cardiovascular disease (CVD) and ischemic heart disease (IHD). During oxidative stress, there is an imbalance between the production of reactive oxygen species (ROS) and antioxidant defence mechanisms in favour of ROS. This results in severe cellular damages in the heart, vascular membranes and other organs. Potential benefits of dietary supplements as one of the major quenching elements against oxidative stress have been highlighted. Thus, a growing interest has been stimulated in finding natural alternatives for the treatment and/ or prevention of oxidative stress-mediated diseases. Red palm oil (RPO), refined from the tropical plant *Elaeis guineensis* was used in this study since it has captivated much attention in the health sector lately.

The effects of RPO-supplementation on oxidative stress biomarkers as well as homocysteine, a cardiovascular disease risk factor in an oxidative stress-induced rat model were investigated in this *in vivo* study. All experiments were conducted for a period of six weeks. Male Wistar rats (120-150g) were randomly divided into six groups (n=5) where all the rats received a standard diet. Two groups (groups C, D) were supplemented with 0.175g RPO (7g RPO/kg chow) for four weeks whereas groups (groups E, F) were given 0.175g RPO (7g RPO/kg chow) supplementation for six weeks. Rats in control groups (groups A, B) were not given any RPO-supplementation. Groups B, D, F were induced with oxidative stress by injection of 0.5ml (20μM/100g of body weight) organic tertiary-butyl hydroperoxide. All parameters were determined using appropriate methods in plasma, serum and erythrocytes. Data were expressed as mean ± SEM. No significant differences were obtained between groups for total antioxidant capacity and glutathione peroxidase activity. Red palm oil supplementation significantly increased superoxide dismutase activity after 6 weeks consumption, total glutathione levels after 4 weeks consumption and homocysteine levels after four and six weeks consumption in rats not subjected to oxidative stress. Under oxidative stress conditions, malondialdehyde (MDA) level, a marker of oxidative stress related damage, significantly increased in rats receiving a standard diet. However, when RPO diet was supplemented for 4 and 6 weeks, MDA levels significantly decreased towards the value of normal controls.

In conclusion, our findings suggest that RPO-supplementation could ameliorate antioxidant status in the body through its potential ability to increase some antioxidant enzymes activity.

Similarly, it is suggested that RPO-supplementation could protect the rat against oxidative stress induced damage in diseased state.

Key words: Antioxidants, Blood, Cardiovascular disease, Homocysteine, Malondialdehyde, Oxidative stress, Rats, Red palm oil, tertiary-butyl hydroperoxide.

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DEDICATION

This thesis is dedicated to the glory of God.

LIST OF OPERATIONAL TERMS AND CONCEPTS

Antioxidants	Compounds that act as scavengers of free radicals. Molecules that prevent oxidation of other molecules
Endogenous	Originating from within an organism
Exogenous	Taken up from external environment
Free radical	Any molecule capable of independent existence that contains one or more unpaired electrons
In vivo	A measurement or a process taking place in the living body
Lipid peroxidation	Oxidative degradation of lipids and propagating lipid chain breaking reaction initiated by the attack of free radicals
Oxidation	The combination of a substance with oxygen with subsequent loss of electrons
Oxidative Stress	Metabolic imbalance between the production of free radicals and their scavenging counteract antioxidants in favour of free radical overload and subsequent cellular damages
Reactive oxygen species	Any compound derived from oxygen which contains one or more unpaired electrons

LIST OF ABBREVIATIONS

A

ATP	Adenosine triphosphate
AUC	Area under curve, Fluorescence decay curve
ATx	Antioxidant

B

BH4	Tetrahydrobiopterin

C

°C	Degrees Celsius
CAD	Coronary artery disease
CAT	Catalase
CD	Conjugated dienes
CHD	Chronic heart defect
CHF	Chronic heart failure
cm	Centimetre
CVD	Cardiovascular disease

D

DNA	Deoxyribonucleic acid

E

ETFQOR	Electron transferring flavoprotein ubiquinone oxidoreductase

F

FMN	Flavin mononucleotide
FRAP	Ferric ion reducing antioxidant power

G

G	Gram
GPDH	Glycerol 3-phosphate dehydrogenase
GR	Glutathione reductase

GSH	Reduced glutathione
GSHt	Total glutathione
GSSG	Oxidised glutathione

H

4-HNE	4- Hydroxynonenal
Hcy	Homocysteine
HPLC	High performance liquid chromatography

I

IHD	Ischemic heart disease

L

LDL	Low density lipoprotein
LP	Lipid peroxidation

M

mg	Milligram
min	Minute
ml	Millilitre
MDA	Malondialdehyde
MUFA, MUFAs	Monounsaturated fatty acids

N

NAD(P)H	Nicotine adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
nm	Nanometer

O

OGDH	2-oxoglutarate dehydrogenase
ORAC	Oxygen radical absorbance capacity
OxLDL	Oxidised LDL
OXs	Oxidative stress

P

ppm	parts per million
PBS	Phosphate buffer saline

PDH	Pyruvate dehydrogenase
PhGpx	Phospholipids glutathione peroxidase
PKO	Palm kernel oil
PUFA, PUFAs	Polyunsaturated fatty acids

R

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPO	Red palm oil

S

Sec	Second
SFA	Saturated fatty acids
SOD	Superoxide dismutase
SRC	Standard rat chow

T

TAC	Total antioxidant capacity
t-BHP	tertiary-butyl hydroperoxide
TNF	Tumour necrosis factor
TE	Trolox equivalents

U

µmol	micromole

V

VCAM-1	Vascular adhesion molecule
VSMCs	Vascular smooth muscles cells

X

XOR	Xanthine oxidoreductase

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CHAPTER 1

INTRODUCTION

1.1 Statement of the research problem

Cardiovascular disease (CVD) is a non-communicable disease affecting the heart and the vascular system. Non-communicable diseases account for six deaths out of 10 and CVD remain a major threat to the public health of every nation (Mackay *et al.,* 2004; Sacco, 2011). In a global report of the World Health Organisation in 2004, CVD was regarded as the leading cause of mortality worldwide with an estimate of 31.5% total deaths in the female population and 26.8% total deaths in male population (Mackay *et al.,* 2004). Moreover, more than 80% of CVD-related deaths were reported to occur in low- and middle-income countries (Fuster and Kelly, 2011). There is a growing interest in finding natural alternatives for the treatment and prevention of CVD and ischemic heart disease (IHD) such as advances in dietary strategies. In this study, the focus was on Red Palm Oil (RPO) supplementation and its potential effects on oxidative stress (OXs) which is an underlying risk factor of CVD. We used the male Wistar rat model.

1.2 Background of the research problem

Recent research has identified what could be seen as novel risk factors that may assist to identify individuals or populations at risk of developing CVD and IHD (Kadiri, 2005). One such risk factor is free and hydroxyperoxide radicals that promote OXs. Although the pathogenesis of CVD is multifactorial, it is believed that a common underlying factor could be identified in oxidative stress metabolic condition (Ceriello and Motz, 2004). Oxidative stress and ROS-mediated damage have been associated with the aetiology and pathogenesis of a variety of human disorders such as cardiovascular disease. It has been reported that oxidative stress, through free and hydroxyperoxide radical attacks, has the potential to damage biological compounds and structures such as proteins, membrane lipids, deoxyribonucleic acid (DNA) and carbohydrates in the cardiovascular system (Kadiri, 2005; Thorogood *et al.*, 2007).

With the current dietary shift characterized by more fat-intake, more added caloric sweeteners, more animal product consumption, chronic alcohol intake, lack of fitness and weight maintenance, the correlation between dietary antioxidants and oxidative stress-related diseases have become more pronounced (Popkin, 2003, 2006). Experimental, clinical and epidemiological data suggest that antioxidants can offer protection against oxidative

stress-induced health disorders and pro-oxidant mediated damage *in vivo*. As health and nutrition are highly correlated, awareness of the relationship between diet and diseases is on the rise. Thus, renewed attention is given to the specific role of antioxidants in health. On-going investigations and archiving reports emphasized a favourable link between RPO-supplementation and improved oxidative status.

Red palm oil, obtained from the tropical plant *Elaeis guineensis* is a dietary vegetable oil with a large fraction of fatty acids (saturated and unsaturated fatty acid ratio close to one) and a minor fraction rich in phytonutrients (α- and β-carotene, lycopenes, α-, β-, δ-tocotrienols and tocopherols, and ubiquinone) (Akusu *et al.*, 2000; Matthan *et al.*, 2009). Red palm oil, a refined deacidified and deodorised palm oil has been reported to have protective effects against the consequences of ischemic-reperfusion injury associated with oxidative stress (Narang *et al.,* 2004; Esterhuyse *et al.,* 2005, 2006; Bester *et al.,* 2006). However, these studies did not investigate the biochemical effects of RPO-supplementation in an *in vivo* oxidative stress-induced animal model. The measurements of biochemical biomarkers in one single *in vivo* study following RPO-supplementation may provide further evidence to support the oxidative stress-protective effects of RPO.

1.3 Research aim and objectives

The overall aim of this experimental study was to evaluate the effects of RPO-supplementation on oxidative stress biomarkers using an *in vivo* oxidative stress-induced rat model.

The objectives of the study were as follows:

- To evaluate whether dietary RPO-supplementation could increase the total anti-oxidant capacity in experimental animals;
- To assess the potential effects of RPO-supplementation on antioxidant enzymes and total glutathione;
- To evaluate whether RPO-supplementation could reverse/reduce induced oxidative stress *in vivo*.
- To determine the effects of RPO-supplementation on homocysteine levels;
- To determine the time period of RPO-supplementation that would give optimal results.

CHAPTER 2

LITERATURE REVIEW

The intake of oxygen (O_2) is fundamental to sustain aerobic life. During cellular respiration in most mitochondria, oxygen is being reduced into water to stimulate adenosine triphosphate (ATP) generation, the core energy source of cellular metabolism (Figure 2.1). However, it is believed that in the course of oxygen metabolism, 1-5% of all inhaled molecular oxygen (O_2) highly reacts with other molecules to generate downstream oxygen derivatives called reactive oxygen species/ reactive nitrogen species (ROS/ RNS) (Cann and Hughes, 2002; Berk, 2007).

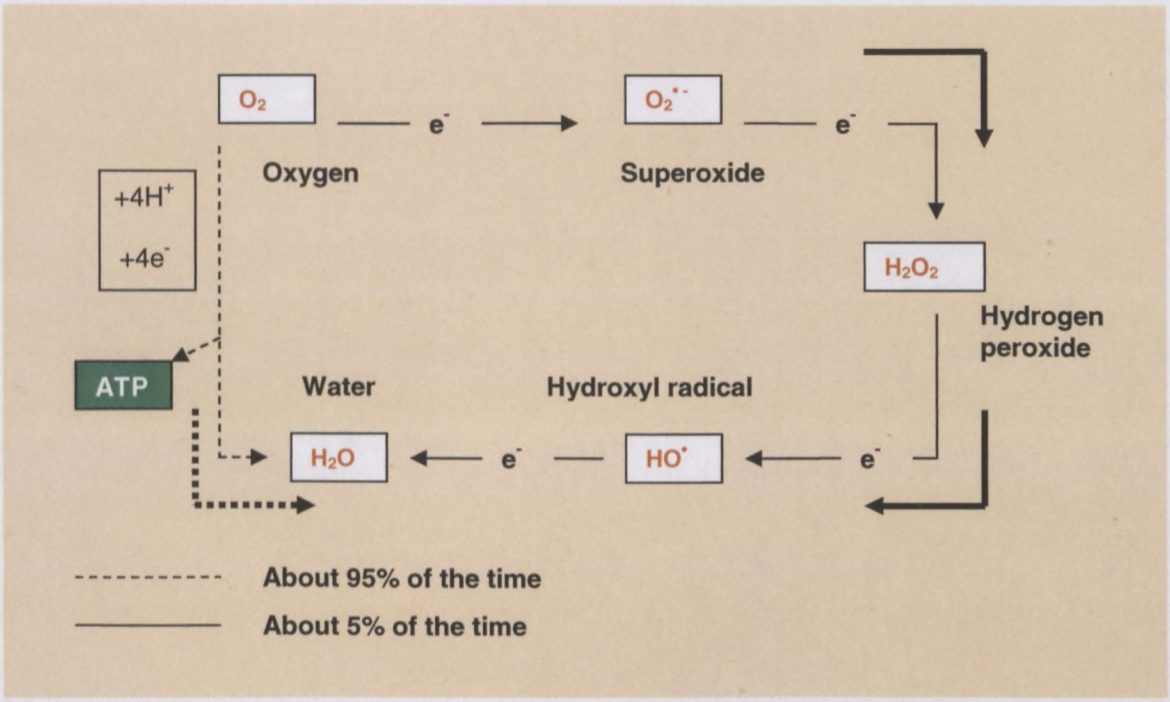

Figure 2.1: Production of ROS during normal cellular respiration (Adapted from Nindl, 2004)

Reactive oxygen species / reactive nitrogen species are constantly being formed in living organisms (Ceconi *et al.*, 2003). Reactive oxygen species have oxidation ability and are classified either as free radicals (superoxide anion $O_2^{\bullet-}$, hydroxyl radical $OH^{\bullet}$ and nitric oxide

NO) or as non-free radicals (hydrogen peroxide H_2O_2 and peroxynitrite $ONOO^-$) (Higashi *et al.*, 2006).

They have been described to show both beneficial and deleterious effects (Valko *et al.*, 2007).

With homeostasis, the overall production of ROS/RNS is normally maintained at low concentration where ROS are involved in many cellular signalling pathways, gene expression, cell growth and apoptosis through reduction/oxidation (redox) metabolism (Lavrovsky *et al.*, 2000). However, this balance is determined by the rate of production of ROS and the rate of degradation by antioxidants (Lavrovsky *et al.*, 2000; Covarrubias *et al.*, 2008). See Figure 2.2. Antioxidants constitute effective and powerful defence mechanisms against a rise in oxygen by-products (ROS/RNS).

If pro-oxidants overwhelm antioxidant capacity, the excessive generation of ROS leads to deleterious redox state called oxidative stress. Under oxidative stress, most cells enter toxic states and are damaged. Hence, oxidative stress metabolic condition is associated with the pathogenesis of various disorders (Lin and Beal, 2006; George and Ojegbemi 2010; Galley, 2011; Pirinccioglu *et al.*, 2012).

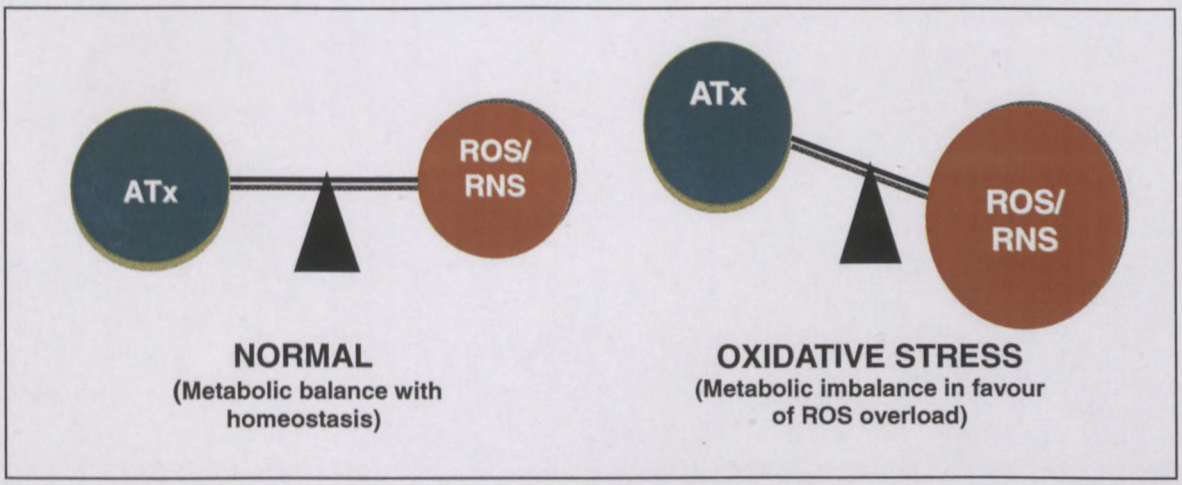

Figure 2.2: Schematic drawing of metabolic balance and imbalance related to oxidative stress

Abbreviations: ATx: antioxidants, ROS/RNS: reactive oxygen species/ reactive nitrogen species

2.1. Reactive oxygen species

2.1.1 Superoxide anion

In the late sixties, McCord and Fridovich (1969) first suggested the production of superoxide free radical anion by the mammalian tissue and is believed to be the first ROS generated free radical anion referred to as "primary ROS". Superoxide anion is produced *in vivo* through enzymatic pathways such as NAD(P)H oxidase, xanthine oxidase, myeloperoxidase and nitric oxide synthase. ROS production can be enhanced by a range of external cofactors such as exposure to physical irradiation and/or physico-chemical agents. Superoxide anion is formed by the addition of one extra electron to molecular dioxygen as indicated in Equation (1).

$$O_2 + e^- \rightarrow O_2^{\bullet-} \qquad (1)$$

Superoxide anion is an unstable, highly reactive water-soluble molecule that generates a large number of downstream oxidants referred to as "secondary" ROS (Chapple, 1996; Touyz and Schiffrin, 2004). Superoxide anion has the ability to act either as a reducing agent or as an oxidizing agent. When superoxide acts as a reducing agent, it transfers its extra electron to other molecules such as nitric oxide ($NO^{\bullet}$) with which it forms a toxic compound called peroxynitrite $ONOO^-$.

Peroxynitrite is a toxic compound with detrimental actions such as oxidation of lipids and thiols, inhibition of mitochondrial respiration, stimulation of iron release and nitration of protein tyrosine residue (Brown and Borutaite, 2004). When superoxide anion behaves as an oxidizing agent (that is reduced), it produces for instance hydrogen peroxide (H_2O_2) which leads to a range of downstream oxidants such as: hydroxyl radical ($HO^{\cdot}$). Under physiological conditions, superoxide anion favoured the production of hydrogen peroxide (H_2O_2); however, under pathophysiological conditions, overproduction of superoxide favoured the reaction with nitric oxide and the subsequent production of peroxynitrite (Darley-Usmar *et al.*, 1995).

2.1.2 Hydrogen peroxide

Superoxide anions ($O_2^{\bullet-}$) spontaneously undergo dismutation to form hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) as shown in Equation (2).

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \longrightarrow {}^1O_2 + H_2O_2 \qquad (2)$$

Superoxide anions also undergo dismutation to form hydrogen peroxide by the action of superoxide dismutase (intracellular Cu/Zn SOD, Mn SOD or extracellular Cu/Zn SOD) (Chapple, 1996; Hamilton *et al.*, 2004). See Equation (3).

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (3)$$

Hydrogen peroxide is not a free radical, but is a stable and lipid permeable molecule that can easily cross cellular membranes. Hydrogen peroxide (H_2O_2) is scavenged into oxygen and water by catalase enzymes and glutathione peroxidase (GPx) (in the presence of reduced glutathione GSH). Glutathione peroxidase (GPx) is a selenium dependent enzyme that reduces H_2O_2 and oxidizes reduced glutathione (GSH) to its oxidized form (GSSG) (Chapple, 1996; Hamilton *et al.*, 2004; Zalba *et al.*, 2006). See Equations 4 and 5.

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2 \qquad (4)$$

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O \qquad (5)$$

2.1.3 Hydroxyl radical

In biological systems hydrogen peroxide is reduced to form the highly reactive hydroxyl radical ($HO^{\bullet}$) in the presence of metal-containing molecules such as iron Fe^{2+} in a biochemical reaction called Fenton reaction (Chapple, 1996; Fridovich, 1997; Valko *et al.*, 2007). See Equation 6.

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{\bullet} + HO^- \qquad (6)$$

Hydroxyl radical is a highly reactive molecule known to interact with the majority of biological molecules (DNA, lipids and proteins). Its interaction with membrane lipids leads to the initiation of a highly damaging chain breaking reaction called lipid peroxidation (LP). Hydroxyl radical provokes oxidative damage to DNA and proteins altering their functions (Wiseman and Halliwell, 1996; Wagner and Cadet, 2010).

2.2 Sources of reactive oxygen species

Reactive oxygen species (ROS) including oxygen free radical and non-radicals are generated endogenously and exogenously from enzymatic and non-enzymatic systems (Wiseman and Halliwell, 1996). Endogenously, ROS are mostly derived from the incomplete reaction of oxygen during aerobic metabolism *in vivo.* Reactive oxygen species are produced from various sources such as mitochondrial electron transport chain, nicotine adenine

dinucleotide phosphate NAD(P)H oxidases, arachidonic acid pathway enzymes, namely cyclooxygenase and lipoxygenase, NO synthase, peroxidases, xanthine oxidases and phagocytes-derived myeloperoxidase (Cai and Harrison, 2000; Szeto, 2006; Zalba *et al.,* 2006). Exogenously, ROS are derived from exposure to environmental agents such as UV radiation and redox-cycling agents (Park *et al.,* 2003).

2.2.1 Nicotine adenine dinucleotide phosphate (NAD(P)H) oxidases

Nicotine adenine dinucleotide phosphate NAD(P)H oxidase has been identified as a major source of production of superoxide in the vasculature of living organisms (Equation 7).

$$NAD(P)H + O_2 \xrightarrow[\text{shunt}]{\text{NAD(P)H Oxidase}} NADP^+ + H^+ + O_2^{\bullet -} \quad (7)$$

Superoxide anion is produced *in vivo* because of most aerobic mechanisms. There is strong evidence showing that the principal source of superoxide anion in the vascular system prevails in the NAD(P)H oxidase metabolism (Valko *et al.,* 2007). See Figure 2.3.

Two types of NAD(P)H oxidase have been identified, namely the phagocytic NAD(P)H oxidase produced by migrating inflammatory cells such as activated macrophages and leucocytes (Babior *et al.,* 2002), the non-phagocytic NAD(P)H oxidase produced by the vascular cells such as endothelial, adventitial and vascular smooth muscles cells (VSMCs) (Lassegue and Clempus, 2003; Taniyama and Griendling, 2003). The functional enzyme produces predominantly superoxide radicals after an electron transfer to molecular oxygen In this reaction, NAD(P)H is considered as the electron donor (Babior *et al.,* 2002; Vignais, 2002; Touyz *et al.*, 2003; Lassegue and Clempus, 2003; Wassmann *et al.,* 2004). See Equation 8.

$$2O_2 + NAD(P)H \longrightarrow 2O_2^{\bullet -} + NADP + H^+ \quad (8)$$

2.2.2 Uncoupled Nitric Oxide Synthase (NOS)

Nitric oxide synthase is the enzyme primarily responsible for the production of nitric oxide (NO) through the well-known nitric oxide and guanosine 3', 5'-cyclic monophosphate (NO/cGMP) signalling cascade. Besides, NOS has been reported to generate superoxide anion in condition of substrate (arginine) or cofactor tetrahydrobiopterin (BH4) depletion. This condition has introduced the concept of "NOS uncoupling" whereby NOS favoured superoxide anion formation and decrease nitric oxide production. This new compound, uncoupled NOS, is a dysfunctional endothelium NOS which promote an excessive release of

superoxide and favours peroxynitrite-mediated cellular injury (Xia *et al.*, 1996; Touyz and Schiffrin, 2004). See Figure 2.3.

2.2.3 Xanthine Oxidoreductase

Xanthine oxidoreductase (XOR) is a complex molybdoflavoenzyme that is readily available from mammalian or human milk. Xanthine oxidoreductase is also present both in the cytoplasm and on the outer surface of endothelial cells. Vascular XOR is generally recognized as the terminal enzyme of purine catabolism in man and has been established in recent times as a source of ROS in the vasculature. XOR catalyses the hydroxylation of hypoxanthine to xanthine and the hydroxylation of xanthine to urate (Harrison, 2002). This enzyme is present in two forms in mammals. It is synthesized as xanthine-dehydrogenase, which uses NAD^+ as an electron acceptor. XOR can be converted to xanthine oxidase under certain stimuli such as exposure of endothelial cells to cytokines (TNFα) (Friedl *et al.*, 1989) or such as chronic activation of the renin-angiotensin system (Mervaala *et al.*, 2001). Several studies reported that inhibition of XOR has been found to partially normalize the overproduction of superoxide anion, which confirms XOR implication in ROS surplus in the vasculature (Mervaala *et al.*, 2001; McNally *et al.*, 2003). See Figure 2.3.

2.2.4 Mitochondrial respiratory chain

The mitochondrial respiratory chain reaction represents the main energy source for cellular metabolism via oxidative ATP generation (Szasz *et al.*, 2007). During cellular respiration in the membranes of mitochondria, the activity of the electron chain transport is responsible for most ROS generation (Brand, 2010). It has been shown that the electron transfer chain, situated in the inner mitochondrial membrane, contains several redox centres generally grouped into four complexes and leads to the generation of ATP by the fifth complex after been shuttled via coenzyme Q and cytochrome C. Electron transfer may leak electrons to molecular oxygen and such reduction mostly account for superoxide production (Turrens, 2003; Szasz *et al.*, 2007). See Figure 2.3.

Brand (2010) reported the existence of seven identified sites of superoxide production within mitochondria in physiological condition, namely pyruvate dehydrogenase (PDH); 2-oxoglutarate dehydrogenase (OGDH); 2 sites in complex I such as site IF (flavin mononucleotide FMN - containing NADH binding site) and site IQ (ubiquinone reduction site); electron transferring flavoprotein ubiquinone oxidoreductase (ETFQOR; glycerol 3-phosphate dehydrogenase (GPDH); and site $IIIQ_0$ (the outer quinine binding site of the Q-cycle) in complex III. Complex II (succinate dehydrogenase) and complex IV produced insignificant amount of superoxide radical, while complex I (site IQ) and complex III (site $IIIQ_0$) are

reported to have the maximum rate of superoxide production. It is believed that PDH and OGDH could also directly produce hydrogen peroxide (Brand, 2010).

It has been recognised in recent times that the mitochondrial dysfunction plays a pivotal role in the pathogenesis of diseases such as neurodegenerative disorders, sepsis, diabetes mellitus and cardiovascular disease through onset of oxidative stress in the mitochondrion (Lin and Beal, 2006; Galley, 2011; Tsutsui *et al.*, 2011).

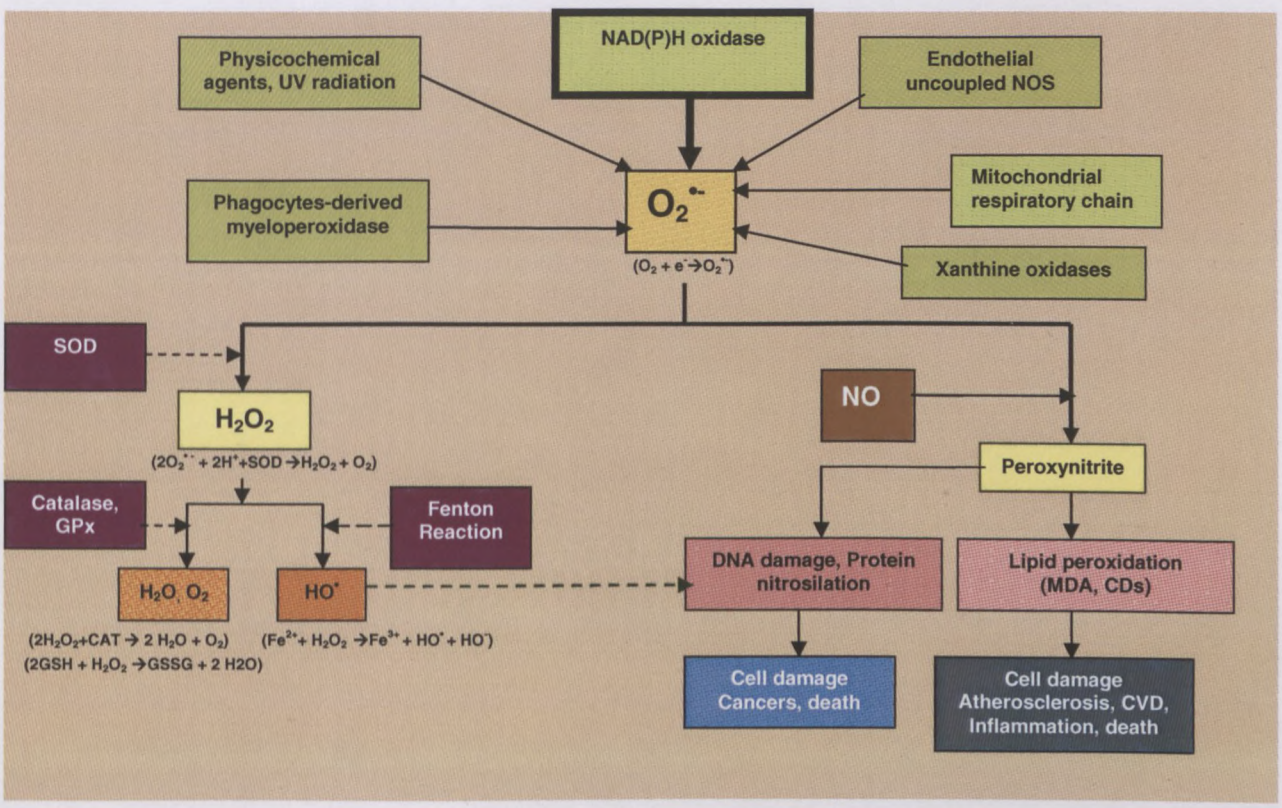

Figure 2.3: Schematic drawing of reactive oxygen species pathways described in the current study

Abbreviations: CAT: catalase, CVD: cardiovascular disease, CDs: conjugated dienes, e^-: free electron, GPx: glutathione peroxidase, H_2O_2: hydrogen peroxide, $HO^{\bullet}$: hydroxyl radical, MDA: malondialdehyde, O_2: molecular oxygen, NO: nitric oxide, NOS: nitric oxide synthase GSSG: oxidised glutathione, GSH: reduced glutathione, SOD: superoxide dismutase, $O_2^{\bullet-}$: superoxide radical, UV: ultraviolet, H_2O: water.

2.3 Antioxidants

Antioxidants is a general term used to characterize the following:

- antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase);
- antioxidant proteins (iron-binding protein transferrin, copper-binding proteins, caeruloplasmin and albumin);
- low molecular weight molecules.

These low molecular weight molecules can be divided into two groups, respectively:

- water-soluble antioxidants such as vitamin C, urate, polyphenol (flavonoids) and thiol groups (glutathione);
- lipid-soluble antioxidants such as vitamin E (tocopherols, tocotrienols), ubiquinol-10 (reduced coenzyme Q10) and beta-carotene.

Antioxidants play a major role in biological systems. They constitute effective and powerful defence mechanisms against the toxic damage caused by oxygen by-products *in vivo*. In physiological conditions, antioxidants help to equilibrate the oxidative stress-status balance and protect against the onset of CVD, carcinogenesis and other oxidative stress-related health disorders. Antioxidants can be either enzymatic or non-enzymatic, each antioxidant having a specific function. Antioxidants in the human being can be derived from endogenous and exogenous sources. However, endogenous antioxidants alone (superoxide dismutase, catalase) do not totally prevent total reactive species damage *in vivo*; thus, most antioxidants are acquired from our daily diet intake (Pokorny *et al.,* 2001). Adequate intake of natural antioxidants has been strongly associated with a reduced risk of chronic diseases such as cardiovascular disease, cancers, diabetes, Alzheimer disease, cataracts and age-related degenerative disease (Marnewick *et al.,* 2000; Knekt *et al.,* 2002; Sweeney *et al.,* 2002; Esterhuyse *et al.,* 2006; Liu *et al.,* 2010).

2.3.1 Endogenous antioxidants

2.3.1.1 Superoxide dismutase and catalase

Superoxide dismutase (SOD) and catalase are major antioxidant metalloenzymes involved in the elimination of superoxide radicals and hydrogen peroxide, respectively.

- **Superoxide dismutase**

Discovered by McCord and Fridovich (1969), SOD is a key antioxidant enzyme against superoxide radical mediated toxicity. Superoxide dismutase families are characterized according to the metal ion content and the location in organisms. There are three families of SOD, namely copper and zinc SOD (Cu/Zn-SOD) found in eukaryotes, manganese or iron SOD (Mn-SOD or Fe-SOD) present in prokaryotes, protists and in mitochondria and nickel SOD (Ni-SOD) restricted to the prokaryotic cells. In mammals and humans, three types of SOD are found namely dimeric cytosolic Cu/Zn SOD (SOD-1), tetrameric mitochondrial Mn SOD (SOD-2) and tetrameric extracellular SOD (SOD-3) (Chan, 1996; Brand, 2010). Despite the diversity, any SOD enzyme always catalyses the dismutation of superoxide radical into hydrogen peroxide, a more stable ROS, as in the Equation (9):

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} 2H_2O_2 + O_2 \quad (9)$$

- **Catalase**

Discovered by Louis-Jacques Thenard in 1818 (Faraci, 2006), and named in 1900 by Oscar Loew (Loew, 1900), catalase is identified as a homotetrameric heme-containing enzyme, a ubiquitous enzyme which can be found in different organs (brain, kidney and heart) in animals, with highest concentrations recorded in the liver. Subcellularly, catalases are found in mitochondria and peroxisomes (Rodriguez *et al.,* 2000; Devasagayam *et al.,* 2004). Catalase enzymes exert two types of activities, namely catalytic and peroxidic. Catalase favours the decomposition of hydrogen peroxide into water and oxygen molecules (Equation 10) and also favours the oxidation of hydrogen donors such as alcohols and phenol formic acids (Equation 11).

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2 \quad (10)$$

$$ROOH + AH_2 \xrightarrow{Catalase} H_2O + ROH + A \quad (11)$$

2.3.1.2 Glutathione redox cycle

- **Glutathione**

Glutathione (GSH) is a non-enzymatic antioxidant composed of a tripeptidic chain. L-γ-glutamyl-L-cysteinylglycine. GSH represents reduced monomeric glutathione and GSSG represents the oxidized glutathione. The oxidised glutathione disulphide is reverted to reduced glutathione by the action of glutathione reductase (Szasz *et al.,* 2007) and the ratio between oxidised and reduced glutathione is important to evaluate the toxicity in the cells. GSH is recycled to its initial reduced form by the enzyme glutathione reductase (GR) as shown in Equation 12. Glutathione plays an important role in detoxification of peroxide, hydrogen peroxide and other free radicals.

$$GSSG + NAD(P)H + H^+ \xrightarrow{GR} 2GSH + NADP^+ \quad (12)$$

- **Glutathione peroxidases**

Glutathione peroxidase (GPx) is a group of selenoproteins composed of tetramer of four identical subunits (monomer 22-23KDa). This enzyme requires selenium as a cofactor and contains a selenocysteine amino acid residue in the active site of each monomer. In mammals, 4 different subgroups are identified, respectively GPx 1 found in erythrocytes, kidneys, liver and lungs; GPx 2 found in the gastrointestinal tract, GPx 3 found in plasma and the membrane associated GPx 4 called phospholipids GPx (PHGPx) (Margis *et al.,* 2008).

Glutathione peroxidase specifically plays an important role in the detoxification of peroxides in the cell. It prevents destruction of cell membranes since peroxides decompose in high reactive free radicals. Therefore, they generally help in preventing lipid peroxidation of cell membranes (Szasz *et al.,* 2007, Covarrubias *et al.,* 2008). Glutathione peroxidases (GPx) transfer electrons from the reduced form of glutathione to hydrogen peroxide yielding the formation of water and oxygen molecules. They also catalyse the reduction of organic peroxide into equivalent stable alcohols (Equations 13, 14).

$$2H_2O_2 \xrightarrow{GPx} 2H_2O + O_2 \qquad (13)$$

$$ROOH + 2GSH \xrightarrow{GPx} ROH + GSSG + H_2O \qquad (14)$$

2.3.2 Exogenous antioxidants

Endogenous antioxidants are insufficient to prevent all kinds of oxidative damages *in vivo.* Therefore, medical experts and nutritionists have agreed on the necessity of an adequate supplementation of antioxidant-rich products which could help to reinforce the overall antioxidant defence. Exogenous antioxidants occur in diverse representations namely vitamins, enzymes, trace element and proteins. They derived from various sources including natural and synthetic products. However, natural antioxidants are preferred sources as they are readily available, safer and more efficient (Pokorny *et al.*, 2001).

Natural dietary supplementation of antioxidants is achieved through the dietary intake of animal products, fruits and vegetables (cereals, oil seeds, legumes, herbs, spices, tomato, spinach and carrots), vegetable oils (RPO, olive oil and sunflower oil) and natural beverages (rooibos and honey bush teas). Some of them are summarized in Table 2.1. These antioxidants may inhibit the formation of reactive oxygen species by:

- sequestration of metal ions (Hu and Kitts, 2000),
- reduction of hydroperoxides and hydrogen peroxide (Hu and Kitts, 2000),
- direct combination with superoxide and singlet oxygen (Hu and Kitts, 2000),
- scavenging radicals and inhibition of lipid chain breaking reaction, initiation and propagation (Nicolle *et al.,* 2003),
- repair of damaged molecules (DNA, proteins and lipids) and reconstitution of enzymes (Pokorny *et al.*, 2001).

Table 2.1: Natural antioxidants identified in various food products

Sources	*Antioxidants*	*References*
Animal product (muscle)	Glutathione peroxidase, catalase and superoxide dismutase	Chan and Decker,1994
Nut (peanuts)	Polyphenols, resveratrol and tocopherols	Tosun and Inkaya, 2010
Cereals:		
▪ Buckwheat	Purified flavonoid fractions	Watanabe, 1998
▪ Corn	Carotenoids (α-, β-carotene), tocopherols and sterol	Chen and Yang 1992, Kurilich and Juvik,1999,
▪ Rice bran	Tocopherols, tocotrienols and oryzanol	Hu *et al.*, 1996; Bergman and Xu, 2003
▪ Wheat	Tocopherol	
Root (*Echinacea*)	Phenolic acids and echinacoside	Hu and Kitts, 2000
Tuberous crop:		
▪ Carrots	Carotenoids (α-, β-carotene and lutein)	Nicolle *et al.*, 2003
▪ Potatoes	Carotenoids(zeaxanthin and violaxanthin), phenolic compound and flavonoids	Brown, 2005
Cruciferous vegetables (broccoli)	Phenolics, carotenoids and ascorbic acid	Zhang and Hamauzu, 2004
Green leafy vegetables (lettuce and spinach)	Ascorbic acid and vitamin A	Ogunlesi *et al.*, 2010

2.4 Reactive oxygen species: functions and dysfunctions

Reactive oxygen species can affect tissues positively and negatively depending on whether or not they are well eliminated by their counteract antioxidants.

2.4.1 Reactive oxygen species function

Reactive oxygen species, as unavoidable product of aerobic respiration, are reported at low levels to be excellent second messengers in cellular signalling (Valko *et al.*, 2007). Cellular signalling or signal transduction can be defined as biological mechanisms by which "cells communicate with each other" (Valko *et al.*, 2007). Consequently, Covarrubias and co-workers (2008) reported ROS as:

- signalling molecules that regulate cellular processes such as proliferation, differentiation, migration and death;
- regulators of signalling molecules;
- regulators of developmental process such as embryogenesis, haematopoiesis, spermatogenesis, oogenesis and growth.

In the cardiovascular system, under physiological conditions, superoxide and hydroxyl radicals can be produced in response to platelet-derived growth factor and granulocyte macrophage-colony stimulating factor. These ROS act as potent second messengers and trigger signal transduction pathways that regulate cells growth, aging and transformation (Fujii *et al.,* 2006). For example, in the coronary endothelium and in the cardiac myocytes nitric oxide (NO), a ROS/RNS product, exerts a biphasic effect on endothelial cell proliferation, migration and survival (Schulz and Triggle, 1994). At low levels, NO is seen as an upstream signal in the promotion of cell survival and neovascularisation such as vascular endothelial growth (Parenti *et al.*, 1997).

On the other hand, with high concentrations of NO in the vasculature, as observed by activated macrophages, NO production intervenes with the promotion of cytostasis and cell death (Roberts *et al.,* 2007). In addition, other studies have reported the importance of NO in the regulation of angiogenesis (Ziche *et al.,* 1994; Murohara and Asahara, 2002; Kondo *et al.*, 2005; Roberts *et al.,* 2007). Nitric oxide can promote or inhibit neovascular formation in angiogenesis. Angiogenesis is a tightly regulated process that leads to the induction, proliferation and differentiation of new capillaries from pre-existing microvasculature beds (Murohara and Asahara, 2002; Kondo *et al.,* 2005; Roberts *et al.,* 2007).

2.4.2 Reactive oxygen species dysfunction: Oxidative stress

With the wide variety of functions and large distribution of ROS throughout the body, any dysfunction of the well-maintained balance between ROS and antioxidants can easily threaten the integrity of the whole health system. In normal cellular signalling, ROS production plays a very important physiological role as a secondary messenger (Valko *et al.*, 2007), but with loss of regulation of ROS production, the normal cell transduction is shifted to generate pathological conditions through oxidative stress.

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 and the antioxidant defence mechanisms in favour of ROS production (Ceconi *et al.,* 2003; Berk, 2007; Barbosa *et al.,* 2008). Oxidative stress and ROS-mediated damage have been implicated in a variety of human disorders such as cancers, lung diseases, and UV-mediated skin diseases and also in aging (Park *et al.*, 2003). Oxidative stress is involved in apoptosis, genotoxicity, mitochondrial damage and carcinogenesis (Salgo *et al.*, 1995; Li *et al.,* 2002). Likewise, oxidative stress is believed to play a critical role in the pathophysiology of cardiovascular disease such as atherosclerosis, stroke and hypertension (Heistad *et al.,* 2009).

2.5 Implication of oxidative stress in cardiovascular disease

2.5.1 Cardiovascular disease

Cardiovascular disease (CVD) is a non-communicable disease that remains a major public health problem and constitutes one of the leading global threats to human health. According to the World Health Report (2003), CVD accounted for 16.7 million of total global deaths. Moreover, CVD is believed to be the leading cause of death in developing countries (WHO, 2003). Cardiovascular diseases are classified into many disorders such as coronary or ischemic heart disease IHD (heart attacks), cerebrovascular disease (stroke), hypertension (raised blood pressure), congenital heart disease, rheumatic heart disease, heart failure, peripheral vascular disease and cardiomyopathies (WHO, 2009). CVD affects both young and old people and the risk increases with age and is similar for men and women (Mackay *et al.*, 2004).

On a global scale, high blood pressure, tobacco use, high blood cholesterol level (hypercholesterolemia), diabetes mellitus, physical inactivity, low fruit and vegetable intake, obesity, aging and alcohol consumption have been reported as leading risk factors of CVD (Mackay *et al.*, 2004). Nevertheless, these traditional known risk factors do not provide a full explanation for all cases of CVD (Keaney and Vita, 1995; Sliwa *et al.*, 2008). Recent research identified what could be seen as novel risk factors that may assist in identifying individuals or populations at risk of developing CVD (Kadiri, 2005). One such novel risk factor is free and hydroxyperoxide radicals which promote oxidative stress. It has been reported that cardiovascular disease is typically characterized by elevated levels of reactive oxygen species (ROS) which induce endothelial dysfunction and proinflammatory states such as atherosclerosis (Swei *et al.*,1997; Fabbi *et al.*, 2004; Fujii *et al.*, 2006).

2.5.2 Lipid peroxidation in the pathophysiology of CVD

2.5.2.1 Lipid peroxidation

Lipid peroxidation is a chain of chemical reactions generated from the attacks of free radicals on the polyunsaturated fatty acids (PUFAs) in the cell membrane (Figure 2.4). It is accepted as a reference marker of oxidative stress (Park *et al.*, 2003). Lipid peroxidation is a continuous process in living aerobic cells and it has been reported that 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 damages. Such toxic breakdown radicals are hydroperoxides (hydroxyl radicals •OH), reactive aldehydes

(malondialdehyde MDA, 4- hydroxynonenal 4-HNE) and other carbonyl compounds (alkoxyl radical RO•, peroxyl radical ROO•) (Ceconi *et al.,* 2003, Park *et al.,* 2003). See Figure 2.4.

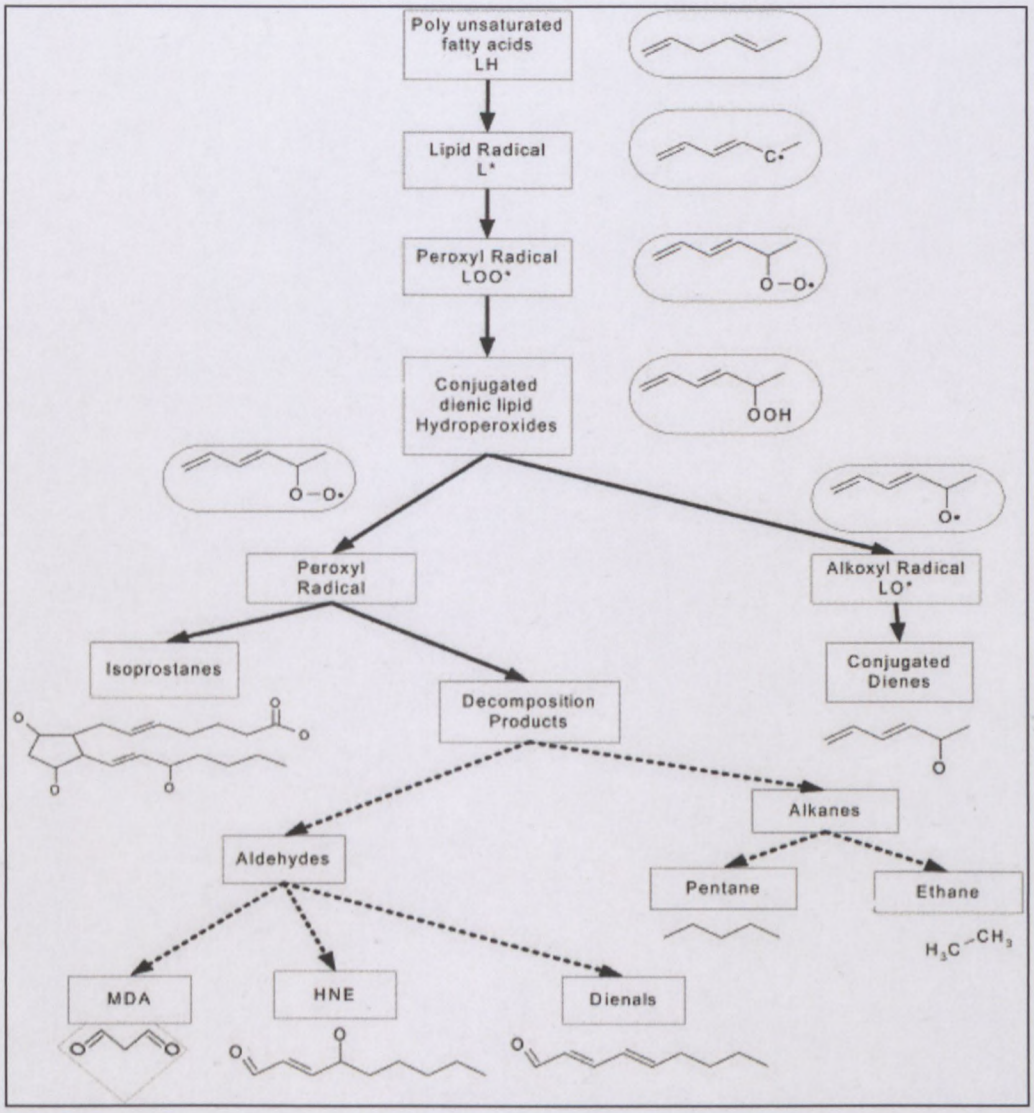

Figure 2.4: By-products and pathways relating to lipid peroxidation (Adapted from Dotan *et al.,* 2004)

Abbreviations: Lipid radical ($L^{\bullet}$), hydroxyl radical ($OH^{\bullet}$), peroxyl radical ($LOO^{\bullet}$), conjugated dienic hydroperoxides (LOOH), hydroxynonenal (HNE), malondialdehyde (MDA), alkoxyl radical ($LO^{\bullet}$), polyunsaturated fatty acids (LH).

The mechanisms by which lipid peroxidation occurs have been summarised in three principal phases:

- The initiation phase (Equations 15, 16) is characterised by a free or hydroxyl radical ($OH^{\bullet}$) attack on a PUFA (LH). This reaction yields the formation of a lipid radical ($L^{\bullet}$) which under aerobic condition reacts with oxygen and form a peroxyl radical ($LOO^{\bullet}$).

- The propagation phase (Equation 17): The newly formed peroxyl radical is a potent oxidant which in turn attacks more PUFAs. Such attacks result in the formation of conjugated dienic hydroperoxides (LOOH) and lipid radical ($L^{\bullet}$). Those newly formed radicals promote and perpetuate the propagation of the chain breaking reaction.

- The termination phase is characterised by the decomposition of hydroperoxides into intermediate by-products or end-products such as conjugated diene (CD), malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), dienals and alkanes (Figure 2.4). Such by-products are toxic compounds which are potent oxidants of more cellular membrane damages (Gaté *et al.,* 1999; Dotan *et al.,* 2004).

$$LH + OH^{\bullet} \longrightarrow L^{\bullet} + H_2O \quad (15)$$

$$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet} \quad (16)$$

$$LOO^{\bullet} + LH \longrightarrow LOOH + L^{\bullet} \quad (17)$$

2.5.2.2 Lipid peroxidation in the aetiology of CVD events

In the vascular vessel, free radicals and other ROS participate in the oxidation of fatty materials (mainly low-density lipoprotein cholesterol-LDL cholesterol) which are deposited into the inner endothelium layer of the vascular wall of the cardiovascular system (Maxwell and Lip, 1997; Rudijanto 2007; Adam *et al.,* 2008; Heistad *et al.*, 2009). The oxidation of LDL-c into oxidised LDL (oxLDL) generates the formation of oxidative by-products of cholesterol (oxysterols and remnant lipoproteins), which plays an important role in the pathogenesis of atherosclerosis and CVD (Yasunobu *et al.,* 2001, Hiki *et al.*, 2009). Atherosclerosis is a pathological condition characterized by the formation of atherosclerotic plaques due to lipid peroxidation (oxidation of fatty acids) and is a critical factor in the pathogenesis of most cardiovascular diseases (Chancharme *et al.,* 1999; Frostegård, 2002).

The oxLDL generated are thought to be particularly atherogenic. It has been demonstrated that oxLDL and oxysterols play important role in atherogenesis (Olkkonen and Letho, 2004). Oxidized-LDL induces morphological changes and increase stiffness of endothelial cells

(Chouinard *et al.*, 2008). Khan and co-workers (1995) demonstrated that oxLDL present at the site of lesions selectively augments redox-sensitive vascular adhesion molecule (VCAM-1) gene expression in vascular endothelium and therefore induce chemotactically the recruitment of monocytes and macrophages on the surface of endothelial cells. This allows monocytes and macrophages at the site of lesions to enter the subendothelial space and initiate the so-called early atherosclerotic lesion (Yasunobu *et al.,* 2001). Furthermore, OxLDL is more avidly taken up by macrophages via their scavenger receptors than unoxidized LDL (Adam *et al.,* 2008). The OxLDL receptors on macrophage cells allow internalization of oxLDL cholesterol molecules. Macrophages embedded into the vascular tissue and enriched with oxLDL become large lipid laden cells called foam cells. Accumulation of foam cells in the vascular wall cause the formation of fatty streaks. The growth of fatty acids causes the formation of a fibrous capsule that surrounds the fatty streaks, which develop into an atheromatous plaque which is the atherosclerotic plaque. The plaque expansion is a threat to blood flow. As it starts growing into the inner vessels, it narrows the opening of the artery and the plaque becomes rigid due to the deposition of calcium. When the vessel affected is the coronary artery or any cardiac vessels, this obstruction may be fatal (Chien and Braunwald, 1999). Therefore, an increase in lipid peroxides (by-products of oxygen free radicals attacks) has been associated with aetiologies of heart failure and various CVD.

2.5.2.3 Correlation between malondialdehyde and CVD events

Malondialdehyde (MDA) is a secondary aldehyde radical which is derived from the chain-breaking peroxidation of polyunsaturated fatty acids of biological membranes as previously described. Previous animal and human studies have reported a positive correlation between an increase in MDA levels and the occurrence of diverse types of CVD. For instance, a study of Prasad and co-worker (1996) revealed that MDA content in myocardial tissue of dogs induced with chronic volume overload was considerably higher than those of normal controls. A study of Jian-xun and co-workers (2006) showed that in an ischemic rat model, blood MDA levels were significantly increased. Similar findings have been reported in patients suffering from coronary artery disease (CAD) (Movahed *et al.,* 2012); in children suffering from chronic heart defect (CHD) (Pirinccioglu *et al.,* 2012), in chronic murine cardiomyopathy model (Rashikh *et al.,* 2012) and many more diseases (Prasad and Sihna 2010; Arab *et al.,* 2011; Hadi *et al.,* 2012).

2.5.2.4 Correlation between conjugated dienes and CVD events

Lipid hydroperoxides are formed prior to MDA in the lipid peroxidation pathway and LOOH contain conjugated dienic structures. Consequently, conjugated dienes (CD) are also measured as biomarkers of lipid peroxidation (Figure 2.4). Investigators have reported the measurement of CDs as an assessment tool for LDL oxidation *in vitro* (McEneny *et al.,* 2012; Söderholm *et al.,* 2012). Söderholm and co-workers (2012) has reported that conjugated diene production might be a marker to express the atherogenic potential of a lipoprotein compound since it is able to provide an estimate of the oxidation level within lipoproteins. However, with regards to the onset of CVD related events, the effects of CDs are not well reported.

2.5.3 Involvement of glutathione and endogenous antioxidant enzymes in the pathophysiology of CVD

Decreased activities and inactivation of free radical scavenging enzymes such as reduced glutathione (GSH), SOD, catalase and GPx have been associated with oxidative stress induction and increased lipid peroxidation products (Park *et al.,* 2003, Hadi *et al.,* 2012 Prahalathan *et al.,* 2012). Such correlations have been portrayed in many cardiovascular studies. Landmesser and co-workers (2000) have reported a substantial reduction in vascular SOD activity in patients suffering from coronary artery disease. A report from Prasad and Sinha (2010) indicated a significant decrease in SOD activity in type 2 diabetic patients who had coexistent hypertension. In a recent study, Pirinccioglu and co-workers (2012) reported a decline in total antioxidant capacity (TAC) of children with CHD. A similar finding was reported in patients with CAD (Movahed *et al.,* 2012). In a study on ischemia-reperfusion injury in Wistar rats, Kalenikova and co-workers (2004) reported a depletion in antioxidant defence, especially in SOD, associated with the increase of pro-oxidant mechanisms.

2.6 Homocysteine in the pathophysiology of CVD

Homocysteine (Hcy) is a sulphur containing amino acid which is mainly derived from the dietary intake of methionine (an essential amino acid presents in protein). Under normal physiological conditions, human plasma/serum homocysteine concentrations are maintained at a level < 15 µmol/L through a regulated methionine-homocysteine metabolism (Malinow *et al.,* 1999; Falk *et al.,* 2001). Any excess in methionine results in the degradation of homocysteine into taurine or cysteine through a vitamin B_6-dependent transulfuration pathway, while insufficient methionine concentration triggers the conversion of homocysteine back to methionine through a vitamin B_{12}-dependent remethylation pathway. It has been reported that any impairment of this metabolism especially a lack in any of the co-factors (vitamin B_6 and B_{12}) results in hyperhomocysteinemia (elevated plasma/serum homocysteine concentration) (Falk *et al.,* 2001).

Hyperhomocysteinemia has first been recognised in children presenting rare genetic defects in methionine metabolism by the pathologist Kilmer McCully in 1969 (Falk *et al.,* 2001). Hyperhomocysteinemia has been associated with known cardiovascular pathologies such as CAD, IHD, stroke, venous thrombosis, peripheral artery disease and myocardial infarction (Wald *et al.,* 2002; Wang *et al.,* 2012). Hence, over a period 40 years, it has been reported as an independent risk factor for CVD in many studies.

The mechanism by which hyperhomocysteinemia induces CVD are still under investigations. However, proposed mechanisms of action by which moderate or mild hyperhomocysteinemia is associated with cases of CVD include:

- promotion of endothelial dysfunction,
- cytotoxic effects,
- increase of ROS production,
- potentiation of LDL oxidation, (Adam *et al.*, 2008).

In addition, Wang and co-workers (2012) reported that hyperhomocysteinemia not only promote CVD through induction of endothelial dysfunction (through decrease in NO bioavailability), but also through induction of cardiomyocyte dysfunction in an experimental mouse model.

2.7 Oil Palm plant (*Elaeis guineensis)*

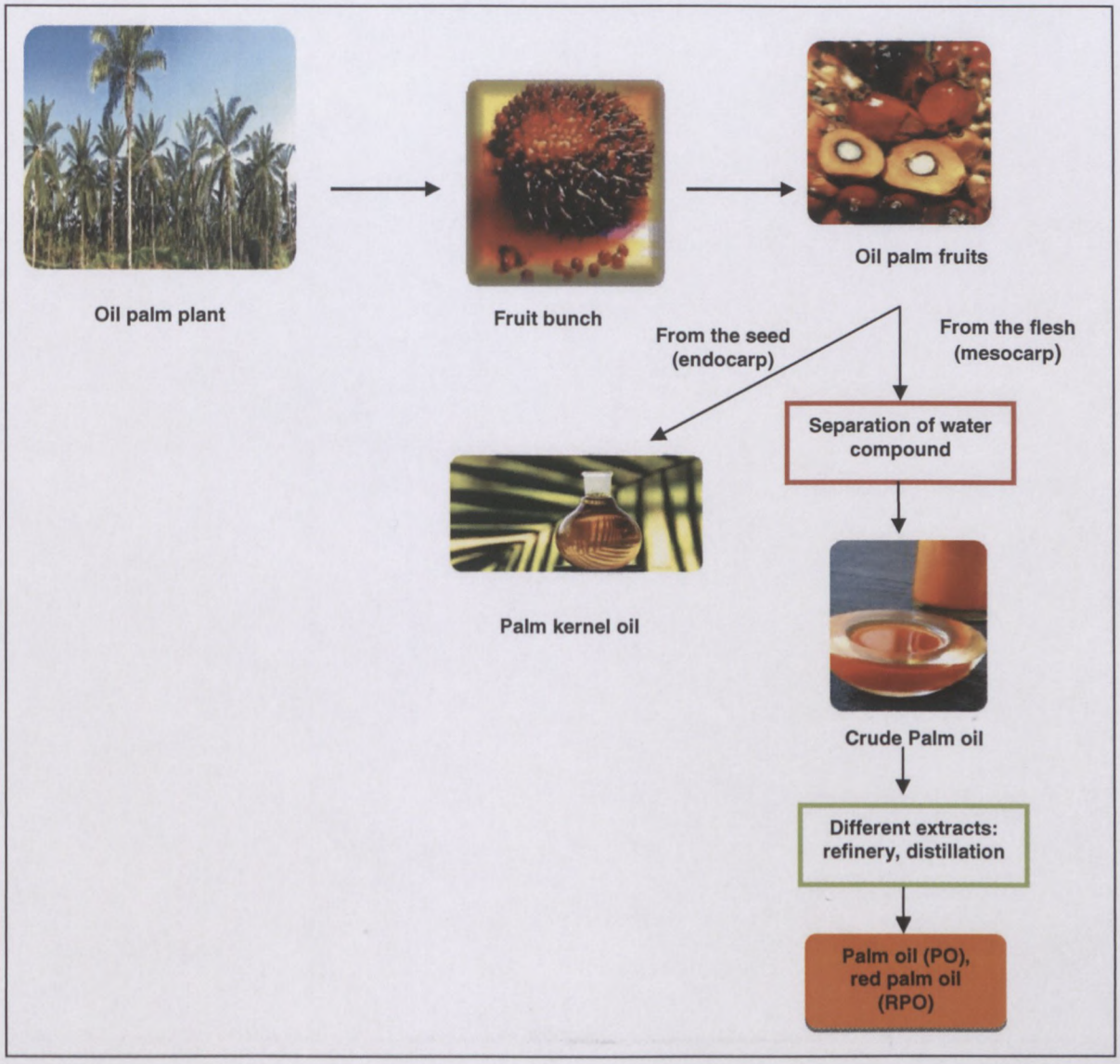

Figure 2.5: Schematic drawing of Oil Palm plant oil products described in the current study

The oil palm is a plant of the Palmae (Arecaceae) family, Arecoideae subfamily, Cocoeae tribe, Elaeidinae subtribe, *Elaeis* genus and *guineensis* species (Price *et al.*, 2007). This plant was first illustrated by Nicholas Jacquin in 1763. E*laeis* derives etymologically from the Greek *elaia* which means olive and refers to a fruit rich in oil and *guineensis* refers to the site of origin which is the guinea's rain forest of West Africa (Henderson and Osborne, 2000). Oil palm has been well distributed in the tropical belt of Africa (Angola, Benin, Burundi, Cameroon and Nigeria) and Madagascar (Henderson and Osborne, 2000; Chopra and Peter, 2005). At present, this tropical crop is reported as one of the most rapidly expanding plants in

the world and from its origin in Africa, oil palm has crossed the oceans to the tropical South-eastern Asia particularly in Indonesia and Malaysia (Jalani *et al.,* 2002).

Two different types of oils are extracted from palm crops, namely palm kernel oil (PKO) and crude palm oil. Crude palm oil can be processed into different oil by-products such as palm oil or red palm oil (Figure 2.5). Both kernel oil and crude palm oil derivatives have gained significant interest in the world market.

Palm kernel oil (PKO), the minor oil component (32%), is obtained from the oil-rich seed of the palm fruit (Bora *et al.,* 2003). Palm kernel oil is characterized by high levels of saturated fatty acid (SFA: fatty acids with no double bonds between carbon atoms), about 92%, and a low melting point. Palm kernel oil is similar to coconut oil both in colour (white to slightly yellow) and in containing a high level of saturated lauric acid (Bora *et al.,* 2003). Palm kernel oil is mostly used for non-edible products for the manufacturing of detergent and soaps, or biodiesel (Edem, 2002; Jitputti *et al.*, 2006). PKO can also be used in confectioneries (margarine, creamy fill of biscuit and chocolate coating), high-energy sports drinks and infant formulations (BerGer 2007, Murphy, 2007).

Crude palm oil, the principal oil component (73%) is extracted from the mesocarp of the mature fruit from which it acquires its unusual orange-red colour (Bora *et al.,* 2003; Murphy, 2007). Palm oil has been described as the second largest oil consumed and as one of the major vegetable oil sources (Bayorh *et al.,* 2005). Palm oil can be used for home cooking (baking, frying and cooking) and for industrial frying (potato chips, instant noodles and snacks) (Ong and Goh, 2002). Al-Saqer and co-workers (2004) demonstrated that functional foods in bakery products can be developed using red palm olein and red palm shortening, both, fractions of red palm oil obtained after refining. Shyu and co-workers, (1998) emphasized palm oil admirable frying properties. They demonstrated that palm oil has a superior thermal stability compared to that of other oil (soybean oil) in vacuum frying which is a deep frying method (180°C) that protect frying oil from oxidative degradation (Aladedunye and Przybylski, 2009).

The findings of Seppanen and co-workers (2010) are indicative of the benefits of natural dietary supplementation in ameliorating oxidative stress especially in chronic diseases such as CVD. Red palm oil (RPO) is very rich in tocopherols and tocotrienols and thus some researchers are currently debating and reviewing the role of RPO as a useful supplement in preventive medicine (Boateng *et al.,* 2006, Oguntibeju *et al.,* 2009). Thus, the interest of the current work is to review the effect of dietary RPO-supplementation on the onset of oxidative stress-related conditions such as cardiovascular disease in an experimental rat model.

2.7.1 Red palm oil fatty acids composition

Crude palm oil refers to the fresh pressed oil that has not been processed completely and of which the water soluble phase has been separated (Tan and Chu, 1991). It can be processed into palm oil, a more complete heat processed oil such as the African palm oil (Owu *et al.,* 1998). The main difference between palm oil and red palm oil is mostly in the carotene fraction concentrations. Red palm oil is a refined palm oil which does not contain water soluble compounds and is enriched in carotene. During the refining of crude palm oil into red palm oil, a large fraction of carotenes is preserved and represent up to 80% of the original carotenoids as described in a previous review of Sundram and colleagues (2003).

Bayorh and co-workers (2005) showed that palm oil has an unsaturated–to-saturated fatty acid ratio close to one being composed of saturated palmitic acids (40-45%), mono-unsaturated omega-9 oleic acids (35-40%), polyunsaturated omega-6 linoleic acid (10%) and trace of polyunsaturated omega-3 linolenic acid. Palm oil may contain 2-5% of free fatty acids (Akusu *et al.*, 2000; Matthan *et al.*, 2009). Although dietary palm oil has a similar content of unsaturated and saturated fatty acids, the position sn -2 of the triglycerides of the total fatty acids has been reported essentially occupied by monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) with a high proportion of mono-unsaturated oleic acid (Ong and Goh, 2002). With a semi solid texture and an attractive property, red palm oil does not undergo hydrogenation process and is therefore a trans-fat free oil (Chiu *et al.*, 2009).

2.7.2 Red palm oil, a cocktail of antioxidants

Red palm oil is a refined dietary vegetable oil with a large fraction of fatty acids and a minor fraction known for excellent phytonutrients, which provides oxidative stability. Many studies investigated the beneficial effects of antioxidant-rich supplements against oxidative stress-related conditions (Marnewick *et al.,* 2000; Pantsi *et al.*, 2011; Aboua *et al.*, 2012; Awoniyi *et al.*, 2012). Natural antioxidant intake has strongly been associated with a reduced risk of chronic diseases such as cardiovascular disease, cancers, diabetes, Alzheimer disease, cataracts and age related degenerative disease. Each antioxidant has a specific pathway of defence against oxidative stress and acts at a different level to quench oxidative stress related-events. (Cao *et al.,* 1996; Wang *et al.,* 1996; Velioglu *et al.,* 1998; Knekt *et al.,* 2002; Sweeney *et al.,* 2002; Liu *et al.,* 2010).

Research on the minor components of red palm oil highlighted promising results in phytonutrients performance. Red palm oil is a carotene-enriched palm oil. The carotene-rich

refined palm oil retains up to 80% of the carotene and vitamin E originally present in the crude palm oil. Red palm oil has been described as a rich cocktail of lipid-soluble antioxidants such as carotenoids (α- and β-carotenes, lycopenes), vitamin E (α- , β-, δ-tocotrienols and tocopherols) and ubiquinone (Sundram *et al.,* 2003; Tan *et al.,* 2007). Other minor components are sterols, phospholipids, squalene, tripternic and aliphatic hydrocarbons, ubiquinone (co- enzymes Q_{10}) and phytosterols (Nagendran *et al.,* 2000). Through the refining process of RPO, sterols decrease gradually and account for 0.02-0.06% of original crude palm oil. However, the main sterols present are β-sitosterol (63%), campesterol (21%) and stigmasterol (21%) (Boateng *et al.*, 2006). The minor components (sterols and ubiquinols) are gaining positive repute with β-sitosterol found to be cholesterol-lowering agent (Miettinen *et al.,* 1995).

2.7.3 Red palm oil in health

The oil extracted from the mesocarp of the palm fruit has a rich-orange colour due to its high carotene content. Carotenoids were first isolated from carrots in 1831 by Wakenroder (Krinsky, 1993). Carotenoids are derived from animal sources (retinol) and from vegetable sources (mostly as β-carotenoids) and are the direct precursors of the essential micronutrient vitamin A. Vitamin A deficiency causes a general impairment of the immune system, which renders children impotent to fight common childhood infections and therefore causes subsequent associated-mortality. In addition, the lack of vitamin A daily intake result in the impairment of eye function and vision (Faber and Van Jaarsveld, 2007).

Diverse strategies to improve vitamin A status of children in developing countries led to increase use of RPO as a readily available, cost effective and natural dietary supplement (Van Stuijvenberg and Benade, 2000). The carotenoid content of RPO (α- β- γ-) has long been investigated (Hunter and Krakenberger, 1946) and edible red palm oil is established as one of the richest natural sources of pro-vitamin A (Lietz *et al.,* 2001). An animal study on Wistar rats conducted by Manomara and Rukmini in the early 1990s reported that RPO had potential to combat vitamin A deficiency in developing countries. This assumption was later confirmed by many nutritional studies conducted in Tanzania, India, and South Africa (Manorama *et al.,* 1996; Rao, 2000; Van Stuijvenberg and Benade 2000; Lietz *et al.,* 2001).

In South Africa, a national intervention programme of the Medical Research Council in deficient school children showed that fortified biscuits baked with RPO shortening of approximately 400ppm of total carotenoids were effective against the deficiency of vitamin A replacing synthetic β- carotene with RPO (Van Stuijvenberg and Benade, 2000). Therefore, RPO has been adopted to fight vitamin A deficiency in children and infants of pregnant and lactating women (Canfield and Kaminsky, 2000). A study by Lietz and co-workers (2001)

revealed that supplementation with RPO increases α- and β-carotene concentrations both in plasma and breast milk of pregnant women and recommend increased consumption of the vegetable oil with mild cooking procedures. Other studies in lactating women report similar findings such as the study by Canfield and Kaminsky (2000) which demonstrated that RPO in the maternal diet increases provitamin A carotenoids in breast milk and improves the vitamin A status of lactating mothers and their infants in many developing countries. The urge for preventive action against vitamin A deficiency, a major public health condition of developing countries has mobilised researchers throughout the world that RPO has the potential to combat vitamin A deficiency (Scrimshaw, 2000). Moreover, RPO carotenoids have also been reported as a potent inhibitor of carcinogenesis (Manorama *et al.,* 1993).

In 1922, Evans and Bishop reported the existence of vitamin E as an unknown factor "factor X" from their reproductive studies on pregnant rats and in 1937, the antioxidant activity of vitamin E was first revealed. Vitamin E is a generic term that refers to a family of eight stereoisomeric compounds. It is subdivided into tocopherols and tocotrienols that possesses similar chemical structure containing a chromanol ring with a 16-carbon hydrophobic side chain (phytyl in the case of tocopherols, isoprenyl in the case of tocotrienols) (Schwartz *et al.,* 2008; Banks *et al.,* 2010). Diverse representations, respectively α- β- γ- δ- tocopherols and α- β- γ- δ- tocotrienols have been identified and isolated. Both tocopherols and tocotrienols display antioxidant activity due to the hydroxyl group on the chromanol ring (Banks *et al.,* 2010).

In the human body, α-tocopherol has been identified as the more abundant vitamin isomer in plasma and tissues (Schwartz *et al.,* 2008; Feng *et al.,* 2010). Vitamin E, especially α-tocopherol isomer, is referred to as a superior lipid soluble radical scavenging and chain-breaking antioxidant (Samhan-Arias *et al.,* 2011). Hence, the vitamin E protective effect against lipid peroxidation of cell membranes and against oxidative stress damage is strongly associated with CVD. Many studies have reported the importance of a diet rich in vitamin E towards oxidative-related diseases and strong evidence showed their important anti-oxidant activities. For instance, Witt and co-workers (1991) showed that supplementation with tocopherols and tocotrienols have a protective effect against oxidative protein damage induced during physical exercise. Allard and co-workers (1998) revealed that supplementation with vitamin E and C could significantly decrease oxidative stress in HIV infected individuals and even showed a trend towards reduction of the viral load. Gumustekin and co-workers (2010) indicated that both vitamin E and HRe-1 (a hexane extract of *H. rhamnoides*, a plant rich in vitamin E and vitamin C) protected the heart against nicotine-induced oxidative stress; a finding which support vitamin E as an excellent dietary supplement to prevent nicotine-induced oxidative stress in heart tissue of smokers.

Moreover, Vitamin E has been shown to attenuate oxidative stress induced by intravenous iron in patients on hemodialysis (Roob *et al.,* 2000) and therefore appears, as first suggested by Cristol and collaborators (1997), as an appropriate agent to decrease hemodialysis-induced oxidative stress. Another study demonstrated that vitamin E and selenium (an essential micronutrient) promoted wound healing activity (Varoglu *et al.,* 2010). A recent study of Seppanen and co-workers (2010) reported the important role of tocopherols and tocotrienols in the shelf life and stability of most vegetables oils, fats and food systems.

Contrary to most edible oils, RPO vitamin E composition is unique. Red palm oil is not only composed of tocopherols, but also very rich in tocotrienols, a unique feature which provides its beneficial effect. Today, RPO appears as one of the unique tocotrienol enriched oils. Recent research reveals that tocotrienols have potent anti-carcinogenic properties (Banks *et al.,* 2010; Samant *et al.,* 2010). Recently, Samant and co-workers (2010) explored the molecular mechanisms involved in anti-proliferative effects of γ- tocotrienol in highly malignant mammary cells. They suggested that γ- tocotrienol could be used as a therapeutic in treatment of breast cancer because of its tumour cells -stopping activity. A study done by Goh and colleagues (1994) on inhibition of tumour promotion revealed that γ- and δ-tocotrienols derived from red palm oil exhibit a strong activity against tumour promotion.

Tappeiner and colleagues (2010) demonstrated that all tocotrienols may also have antifibrotic effects and could serve in postoperative fibrosis. In a study, Muharis and co-workers (2010) provided evidence of RPO tocotrienol benefits against cardiovascular dysfunction. They showed that RPO tocotrienol fractions could restore endothelium dependent relaxation in aortic rings of diabetic-induced and spontaneously hypertensive rats. A research group led by Uto-Kondo (2009) investigated the effects of tocotrienols on adipocyte accumulation which is an obesity and CVD risk factor. They concluded that tocotrienols (α- and γ-) can be seen as anti-adipogenic vitamins. Kooyenga and co-workers (1997) investigated red palm oil antioxidant effects in patients with hyperlipidaemia and carotid stenosis and reported that both red palm oil γ- tocotrienol and α- tocopherol antioxidants have the potential to prevent and possibly reverse the course of carotid atherosclerosis.

2.7.4 Red palm oil in oxidative stress and cardiovascular disease

Many investigations in nutritional research have highlighted the benefits of RPO-supplementation in health. For instance, Boateng and co-workers (2006) showed how red palm oil displays an increased protection against various cancers. Hence, potential health benefits of dietary supplementation with red palm oil in CVD, vitamin A deficiency, cancers, and diabetes mellitus are under current investigation.

Wilson and colleagues (2005) reported that RPO preparations reduce plasma cholesterol concentrations and aortic cholesterol accumulation in hypercholesterolemic hamsters. Karaji-Bani and co-workers (2006) suggested that red palm oil could reduce CVD risk factor. Red palm oil has been reported to have beneficial effects on arterial thrombosis and hypertension associated with oxidative stress (Narang *et al.,* 2004). Research by Bayorh and co-workers (2005) investigated the effect of red palm oil on blood pressure, endothelial function and oxidative stress. The outcome of the research reveals that supplementation with red palm oil attenuates endothelial dysfunction, oxidative stress and reduces the mortality associated with hypertensive rats. Thus, red palm oil could be seen as a natural supplement against vascular resistance and remodelling in hypertension, but also as a natural food product in improving endothelium dependent relaxation.

Studies in various animal models have shown that RPO is beneficial to health in reducing oxidative stress-related conditions (Ebong *et al.*, 1999, Bester *et al.*, 2006; Engelbrecht *et al.,* 2006; Kruger *et al.*, 2007; Szucs *et al.,* 2011; Aboua *et al.,* 2012). Many studies highlighted potential protective effects of RPO in ischemia/reperfusion in the animal model (Esterhuyse *et al.*, 2005; Bester *et al.*, 2006; Engelbrecht *et al.,* 2006; Kruger *et al.*, 2007). Another study has described the modulatory effect of RPO on antioxidant enzymes profile in rats (Oguntibeju *et al.*, 2010). Other studies have also highlighted the cardioprotective effects of supplementation with red palm oil in hyperlipidemic rats (Esterhuyse *et al.,* 2006; Szucs *et al.,* 2011). Moreover, a previous study on RPO-supplementation indicated its potential protective effect in hypercholesterolemia (Hariharan *et al.,* 1996). This study suggested that RPO protection in hypercholesterolemic rats could be due mainly to the presence of tocopherols and tocotrienols (Hariharan *et al.,* 1996).

Consumption of diet high in hydrogenated fat (*trans*-fat) is reported to increase the production of inflammatory cytokines which have been associated with the pathophysiology of atherosclerosis, an underlying condition of most cardiovascular events (Han *et al.,* 2002).

Red palm oil is *trans*-fat free oil; hence, it does not carry *trans*-fat -associated risk factor for CVD (Chiu *et al.*, 2009). Red palm oil is recognised to have 50% of saturated fatty acids (SFA). It is also recognised to possess 50% of both polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids (MUFAs). However, the position sn-2 of the triglycerides of its total fatty acids has been reported to be essentially occupied (87%) by MUFAs and PUFAs, with a high proportion of mono-unsaturated oleic acid (Ong and Goh, 2002). Fatty acids of the sn-2 position being the most absorbed, dietary RPO may behave mostly as MUFA, instead of PUFA. This might also explain RPO's potential cholesterol-lowering properties in nutritional studies in CVD cases which could add more credit to its potential preventive performance against CVD events (Ong and Goh, 2002; Bayorh *et al.*, 2005).

CHAPTER 3

RESEARCH DESIGN AND METHODOLOGY

3.1 Animal care and ethical clearance

Adult male, 2-3 month-old Wistar rats obtained from the Department of Physiological Sciences, (University of Stellenbosch, Stellenbosch, South Africa) were used throughout the study. In all experiments, animals were fed on a daily basis (standardized food pellets with or without supplementation of RPO shortening (7g RPO/kg chow) for 4 and 6 weeks and had free access to water. Animals were housed individually in order to monitor their diet intake at 25°± 3°C, with 12h light/dark cycle and 50% ± 5% humidity. The cages were stainless steel roof, plastic bottom and supplied with a combination of water bottle holder and feeder. The body weights of the rats were measured at the beginning and end of each experiment which was conducted over a period of 6 weeks.

All the experimental protocols were conducted according to the guidelines of the care and use of experimental animals. 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). Ethics approval was obtained from the Faculty of Health and Wellness Sciences Ethical Committee at the Cape Peninsula University of Technology (Cape Town, South Africa) (NHREC: REC-230408-014).

3.2 Experimental treatment

3.2.1 Oxidative stress induction

The experimental groups were subjected to OXs induction. They received a chronic intraperitoneal injection of 0.5 ml (20 µM/100 g of body weight) of organic tertiary-butyl hydroxyperoxide solution (t-BHP, 70% in water, Sigma, South Africa) (Lazze *et al.*, 2003; Kumar, 2007). The control groups received a chronic intraperitonial injection of 0.5 ml placebo solution. The placebo solution is made of sterile phosphate buffer saline with 10-fold dilution (PBS 1X). Injections (t-BHP or PBS) were repeated every second day for the last two weeks of the experiment. Injections were done using sterile 1 ml disposable syringes and 26 G sterile hypodermic needles - modified method of Kumar (2007).

3.2.2 Dietary RPO-supplementation

The intake of food was monitored and analysed by giving each group 25 g of standard rat chow (SRC) with or without 0.175g of red palm oil shortening (7g RPO/kg chow) per day (Engelbrecht *et al.*, 2006; Kruger *et al.*, 2007). Red palm oil shortening was given daily in a petri dish to rats of the experimental diet group. The approximate energy and macronutrient content of rat diets is described in Table 3.1 and RPO composition is described in Table 3.2.

Table 3.1: Composition of the rat diets

Nutrients	SRC	SRC diet supplemented with RPO
Energy (kJ)	272.5	277.6
Total non-structural carbohydrates (g)	8.375	8.375
Total protein (g)	4.5	4.5
Total fat (g)	0.625	0.758
Total SFA (g)	0.139	0.206
Total MUFA (g)	0.168	0.219
Total PUFA (g)	0.297	0.312
Total n-6 PUFA (g)	0.247	0.248
Total n-3 PUFA (g)	0.049	0.061

Table adapted from Engelbrecht *et al.*, 2006.

Abbreviations: *SFA (g): Saturated fatty acids, MUFA (g): monounsaturated fatty acids. PUFA (g): polyunsaturated fatty acids.*

Table 3.2: Description of RPO (Carotino shortening - CS35HV)

Parameters	Results
Free Fatty Acids, %	0.052
Iodine Value, min	50.5
Moisture and Impurities, %	0.05
Slip Melting point, °C	36.6
Carotenes, ppm	500
Tocopherols and tocotrienols, ppm	>400

Table adapted from Carotino SDN BHD Ref 0072/ RC99567

3.2.3 Experimental design

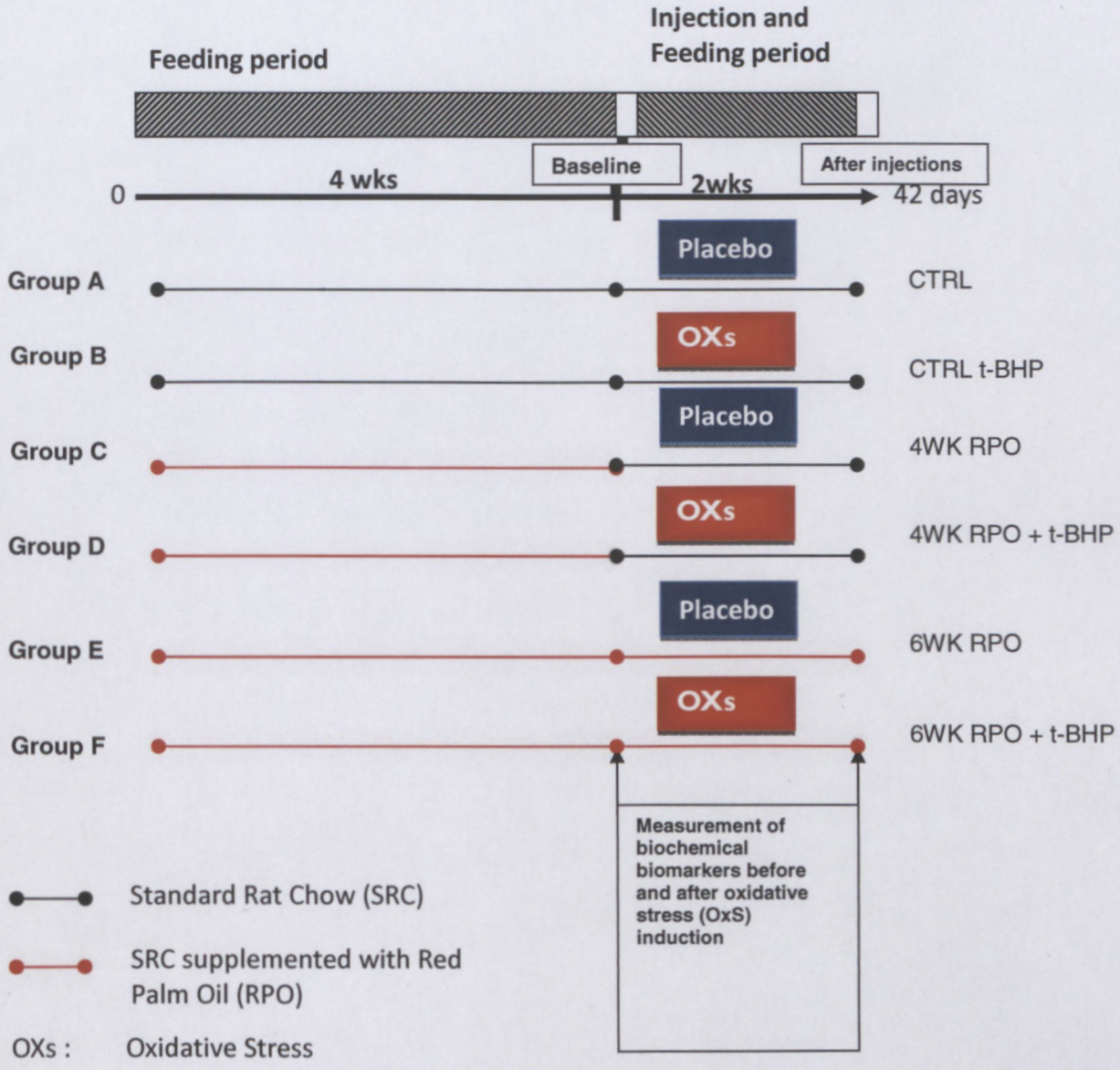

Figure 3.1: Schematic representation of the experimental design

The overall time period for the current study was 6 weeks (wks). Rats were randomly divided in six groups (n=5 per group), each group received a specific diet treatment. During the last two weeks, Wistar rats were given an injection every second day of 0.5 ml (20 µM/100 g of body weight) organic t-BHP or an injection of placebo made of 0.5 ml sterile PBS 1X (modified method of Aboua *et al.*, 2009).

Group A (CTRL): No OXs treatment, normal diet (25 g SRC daily) for six weeks. This group is a negative control group for oxidative stress. Rats received no induction of oxidative stress. They were injected with 0.5 ml placebo every second day during the last two weeks.

Group B (CTRL t-BHP): OXs treatment, normal diet (25 g SRC daily for six weeks). This second group is a positive control group for oxidative stress. Rats were injected with 0.5 ml t-BHP every second day during the last two weeks.

Group C (4WK RPO): No OXs treatment, specific diet (normal diet + 0.175g of 7g RPO/kg chow). Rats were fed with RPO-supplementation daily for the first four weeks. This group is a non-oxidative stress group. Rats were injected with 0.5ml placebo every second day during the last two weeks.

Group D (4WK RPO+ t-BHP): OXs treatment, specific diet (normal diet + 0.175g of 7g RPO/kg chow). Rats were fed with RPO-supplementation daily for the first four weeks. This group is an oxidative stress group. Rats were injected with 0.5ml t-BHP every second day during the last two weeks.

Group E (6WK RPO): No OXs treatment, specific diet (normal diet + 0.175g of 7g RPO/kg chow). Rats were daily fed with RPO-supplementation for six weeks. This group is a non-oxidative stress group. Rats were injected with placebo every second day during the last two weeks.

Group F (6WK RPO + t-BHP): OXs treatment, specific diet (normal diet + 0.175g of 7g RPO/kg chow). Rats were daily fed with RPO-supplementation for six weeks. This group is an oxidative stress group. Rats were injected with 0.5 ml t-BHP every second day during the last two weeks.

3.3 Chemicals

2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 6-hydroxydopamine (6-HD), diethylenetriaminepentaacetic acid (DETAPAC), 5,5'-Dithio-bis-(2-nitrobenzoic acid) reagent (DTNB), ethylenediaminetetraacetic acid (EDTA), 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 (KH_2PO_4), reduced β-nicotinamide adenine dinucleotide phosphate (NAD(P)H), sodium azide, sodium hydroxide (NaOH), sulphuric acid, superoxide dismutase standard, tertiary-butyl hydroperoxide (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. Greiner 96-well flat bottom, Costar 96-well UV flat bottom and Nunclon black 96-well flat bottom microplates were obtained from Sigma–Aldrich (Johannesburg, SA). Atlas Animal Foods (Cape Town, SA) supplied the standard rat chow (SRC). Red palm oil (*Elaeis guineensis*) was kindly donated by Carotino SDN BDH (Malaysia).

3.4 Blood collection and sample preparation

Blood sample collection was performed prior to any injections (t-BHP or PBS) at 4 weeks and at the end of the experimental period. At the end of each experiment, rats were anaesthetized using 1ml (± 60mg/kg) of sodium pentabarbitone. Once anaesthetized, blood samples were collected from the animal using sterile 10ml disposable syringes and 21G sterile hypodermic needles. Blood samples obtained were collected in EDTA containing tubes and in serum separator clot activator tubes (BD Vacutainers, Plymouth, UK) and placed on ice.

Plasma and serum were obtained after centrifugation at 4000rpm at 4°C for 10min within 6 hours of collection and stored at -80°C until further analysis. Plasma samples were prepared for the oxygen radical absorbance capacity (ORAC) assay with a ten-fold dilution in phosphate buffer (75mM, pH 7.4). Diluted samples were treated with 0.5% perchloric acid (PCA 70%) at a two dilution-fold (50:50, v/v) to precipitate protein compounds, vortexed for 10sec and centrifuged at 14000 rpm for 5min at 4°C. The supernatants were used for the assays. Plasma samples were used without any preparation for the FRAP assay. Serum samples were used for the determination of malondialdehyde, conjugated dienes and

homocysteine levels. Erythrocyte lysates were used for the analysis of SOD, GPx and GSHt. The buffy coat was removed and the packed erythrocytes were washed three times with an equal volume of potassium buffer saline with ten-fold dilution (PBS1X). Each centrifugation run was set at 4000rpm at 4°C for 5min. Erythrocytes were transferred into new tubes and stored at -80°C. On the day of analysis, erythrocyte lysate was obtained after adding 4 volumes of ice-cold distilled water, centrifuging at 10 000g for 10 min at 4°C and transferring supernatant to new tubes.

3.5 Evaluation of total antioxidant capacity

3.5.1 ORAC spectrofluorometric determination

Oxygen radical absorbance capacity (ORAC) is a method used to measure the antioxidant scavenging activity of a substance (lipophilic and hydrophilic). The ORAC assay is based upon the measurement of the inhibition of free radical damages to a fluorescent probe by antioxidants. The loss of the fluorescence intensity reflects the intensity of free radical damages and of their concentrations. A delay in the degradation of the fluorescent probe indicates the ability of the pre-existing antioxidant to scavenge free radicals activity. In this study, fluorescein was used as the probe and the assay is based upon the inhibition of the peroxyl-radical-induced oxidation of fluorescein (FL). The reaction was initiated by the thermal decomposition at 37°C of the azo-compound 2, 2'-azobis (2-amidino-propane) dihydrochloride (AAPH) which served as a source of peroxyl radicals. The protective effect of antioxidants in a sample was measured by comparing the areas under the fluorescence decay curve (AUC) obtained from samples to the areas obtained from control mixtures which were prepared using Trolox solution. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid) is a synthetic water-soluble antioxidant derivative of vitamin E. The ORAC assay combines both inhibition time and degree of inhibition into one quantity and the results were expressed as micromoles of Trolox® equivalents (TE) per millilitre of sample (µmol of TE/ml).

The procedure used was a modified method of Rautenbach and co-workers (2010). All reagents and standards (AAPH, FL and Trolox) were prepared in phosphate buffer (75mM, pH 7.4, ORAC buffer OB). One hundred and thirty eight microliters of fluorescein (final concentration 14µM per well) and 12µL of the sample were mixed in a black Nunclon 96-well plate. Stock solution of AAPH (500µM) was prepared and 50µl was added to the plate before readings. Control mixtures were prepared within a range of 0-417µM Trolox. Fluorescence readings were carried out on a fluoroskan ascent plate reader (Thermo Fisher Scientific, Waltham, Mass., U.S.A.) with 485 and 538nm as excitation and emission wavelengths. Each reading was taken after shaking at the end of every cycle (1min) for two hours. Antioxidant

activity was expressed in Trolox equivalents. One ORAC unit was assigned to the net protection area provided by a 1µM solution of Trolox. The final ORAC values were calculated using a regression equation ($y=a+bx+cx^2$) describing the relationship between the Trolox concentration (µM) and the net area under the fluorescence decay curve. Data are expressed as micromoles of Trolox equivalents (TE) per millilitres of plasma µMTE/ml. The area under the curve (AUC) is calculated as AUC = [(0.5 + f2/f1 + f3/f1 + f4/f1 +...+ fi/f1) x CT] where f1 is the initial fluorescence reading at cycle 1, +...+ is the interval/ratio from f4/f1 to infinity), fi is the fluorescence reading at cycle i and CT the cycle time in minutes. The net AUC was obtained by subtracting the AUC of the blank from that of the sample. The side wells (columns 1, 2, and 12) of the 96-well plate were not used and the cycle time was reduced to a minute to improve the accuracy of the results. Each sample was repeated in triplicate.

3.5.2 FRAP spectrophotometric determination

The ferric reducing ability of plasma or ferric ion reducing antioxidant power (FRAP) is a colorimetric spectrophotometric assay used to assess the antioxidant power of biological fluids. The principle of this assay is based upon the reduction ability of antioxidants to convert the ferric ion (Fe^{3+}) into its oxidised counterpart (Fe^{2+}) in acidic media. The electron transfer redox (oxidation/reduction) reactions occurring in this assay is signalled by the development of a characteristic blue coloration. At low pH, a ferric salt, ferric chloride hexahydrate $Fe^3(TPTZ)_2Cl_3$ (TPTZ= 2,4,6, Tripyridyl-s-triazine) used as an oxidant is reduced by biological antioxidant in a sample to give the blue-coloured ferrous tripyridyltriazine complex. The colour development occurs only in presence of electron donating antioxidant in the sample and is monitored by a spectrophotometer that measures the change in absorption maximum at 593nm (Benzie and Strain, 1996; Phipps *et al.,* 2007; Gupta *et al.,* 2009).

Ferric ion reducing antioxidant power reaction mix was prepared by mixing 30ml acetate buffer pH 3.6 (300mM), 3ml TPTZ (10mM), 3ml $FeCl_3$ (20mM), and 6.6ml distilled water (dH_2O). TPTZ was prepared in 40mM hydrochloric acid (HCl). All other reagents were prepared in water. L-(+) ascorbic acid was used to prepare aqueous antioxidant standard solutions in a range of 0-1000µM. In the 96-well plate, 10µl sample/standard and 300µl FRAP reaction mixture were mixed and incubated 30min at 37°C before readings. Each sample was run in triplicate and final results were obtained by comparison to the calibration curve standard using a regression equation (y = a + bx).

3.6 Evaluation of antioxidant status

3.6.1 Spectrophotometric determination of superoxide dismutase (SOD) activity

Superoxide dismutase activity was determined by a modified method from Ellerby and Bredesen (2000). In a 96-well plate, 170µl DETAPAC solution (0.1mM in SOD assay buffer (0.4mg in 10ml) was added to 6µl lysate and 24µl of superoxide dismutase buffer was added to each well. Each sample was run in duplicate. Fifteen microliters of stock 6-HD was finally added last to the previous mixture and read immediately at 490nm for 4min at 1min intervals. The activity of SOD was calculated from a linear calibration curve, in the range of 2-20U/mg. Samples were run in duplicate. SOD concentration was expressed as U/ml.

3.6.2 Spectrophotometric determination of total glutathione (GSHt) levels

Glutathione concentration was determined according to the method of Boyne and Ellman (1972). Erythrocyte lysates were diluted to 20 fold and was treated with metaphosphoric acid precipitating solution (0.167g of glacial metaphosphoric acid, 0.02g EDTA and 3g NaCl dissolved in 100ml milliQ water) and vortexed for a minute. After centrifugation, 100µl of lysate was mixed with 400µl 0.4M sodium phosphate buffer centrifuged at 12000g for 10min and the supernatant was transferred to new tubes and added to 96-well plates. Freshly prepared DTNB (5,5'-Dithio-bis-(2-nitrobenzoic acid)) reagent (40mg DTNB in 100ml of aqueous 1% trisodium citrate) was added last. The total content of glutathione was quantified using a spectrophotometer which monitored the reduction of DTNB at 412nm within 2min. Each sample was run in triplicate and final results were obtained by comparison to the calibration curve standard using a regression equation ($y = a + bx$). GSHt concentration was expressed as mmol/ml.

3.6.3 Ultraviolet (UV) spectrophotometric determination of glutathione peroxidase (GPx) activity

The activity of glutathione peroxidase is derived from the oxidation of reduced β-Nicotinamide adenine dinucleotide phosphate (NAD(P)H) in a conjugated glutathione reductase (GR) system using t-BHP (12mM) as a substrate. In a 96-well UV Costar plate, 210µl assay buffer (AB: 50mM potassium phosphate, 1mM EDTA, pH 7.0), 2.5µl GSH (30.7mg/ml in water), 2.5µl GR (0.1U/ml in AB), 2.5µl sample were read before adding 2.5µl NAD(P)H. Two readings were recorded (modified method of Ellerby and Bredensen, 2000). The first reading recorded the t-BHP non-dependent NAD(P)H oxidation at 340nm for 3min in 30sec intervals for samples (A_1) and blank (A_{1b}). The second reading was performed after adding 25µl of t-BHP. This reading monitored the decrease of t-BHP due to NAD(P)H

oxidation at 340nm for 2min in 30sec intervals for the same samples (A_2) and blank (A_{2b}). Samples were run in triplicate. GPx concentration was expressed as nmol/min/µl calculated according to Equation 3.1:

$$\frac{1000 \times [(A_2-A_1) - (A_{2b}- A_{1b}))]}{\xi} \quad X \quad \frac{\text{Reaction volume}}{\text{Sample volume}} \qquad \textbf{(Equation 3.1)}$$

Where A_{1b}: absorbance of first reading of blank at 340nm

A_1 : absorbance of first reading of sample at 340nm

A_{2b}: absorbance of second reading of blank at 340nm

A_2 : absorbance of second reading of sample at 340nm

ξ : coefficient of extinction= 6.22 $mM^{-1}.cm^{-1}$

Quoted ξ is based on a 500µl reaction; since 247.5µl is our final reaction volume, appropriate factoring was done in the calculations.

3.7 Evaluation of oxidative damages (lipid peroxidation evaluation)

One of the main standards of assessing the level of oxidative damage *in vivo* is to evaluate the level of lipid peroxidation. Lipid peroxidation activities in blood plasma were assayed by ultraviolet (UV) spectrophotometric measurements of conjugated dienes (initial-products of fatty acids peroxidation) and high performance lipid chromatography (HPLC) quantification of malondialdehyde (MDA) (end-product of fatty acids peroxidation).

3.7.1 HPLC determination of plasma malondialdehyde (MDA) levels

Plasma MDA was determined through a modern HPLC based TBA assay method. This method is highly specific because it quantifies the genuine MDA-$(TBA)_2$ adduct formed (Lykkesfeldt, 2001). The quantitative analysis of the plasma content of MDA was performed using a modified method of Cuny *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: flow rate 1mL/min, 15min run time, sample injection volume of 20µL and the mobile phases A and B were respectively made up of 50mM KH_2PO_4 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. Mixed stock solution of standards with concentration of MDA about 4.0M (in 0.1% sulphuric acid) was dissolved in

0.1% sulphuric acid to obtain 10, 5, 2, 1, 0.5µM solutions for construction of the calibration curve. HPLC mixture was made of 100µl of standards or plasma, 250µl TBA (40mM in 0.1N NaOH) and 750µl O-PA (0.2M), 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.

3.7.2 UV Spectrophotometric determination of plasma conjugated diene (CD) levels

Plasma CD levels were estimated using a UV spectrophotometric-modified method of Recknagel and Glende (1984). Four hundred and five microliters of chloroform-methanol (2:1, v/v) was added to 100µl plasma. This mixture was vortexed for 1min and centrifuged at 8000g for 15min at 4°C. After centrifugation, the mixture separated in three layers, namely a top aqueous layer, a protein layer and a lipid layer sitting at the bottom. The aqueous supernatant was discarded. The lipid layer was collected by inserting a pipet tip very gently along the wall of the tube. The lipid phase was then transferred to a new eppendorf tube and dried under nitrogen gas. One millilitre of cyclohexane was added unto dried tube and vortexed for 30sec. 300µl of this solution was transferred into UV Costar plate and read at 234nm. The concentrations of CD were calculated from Equation (3.2) using Microsoft Excel 2007 program.

$$\frac{A_{234}s - A_{234}b}{\xi} \quad X\ 10\mu mol/l\ plasma \qquad \textbf{(Equation 3.2)}$$

Where A_{234} s : absorbance of sample at 234nm

A_{234}b : absorbance of blank at 234nm

ξ : coefficient of extinction= 2.95×10^4

Quoted ξ is based on a 1cm cuvette; since 300µl in a plate well has a length of 0.9cm, appropriate factoring was done in the calculations.

3.8 Determination of homocysteine levels

A KAT homocysteine kit (KAT Medical, Gauteng, South Africa) was used for quantitative determination of total L-Homocysteine levels using a clinical chemistry automated analyser Vitalab Selectra E (Vital Scientific, The Netherlands). Each sample was run in duplicate and final results were obtained by comparison to the kit calibration curve standard.

3.9 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 readings 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.

CHAPTER 4

RESULTS

4.1 Effects of dietary RPO-supplementation on total antioxidant capacity of plasma

4.1.1 Effects of dietary RPO-supplementation on total antioxidant capacity at baseline level

Baseline values before any injections of placebo or t-BHP was measured in all groups. The results of RPO-supplementation on plasma total antioxidant capacity at baseline level are presented in Table 4.1 and Figure 4.1. FRAP and ORAC values for RPO supplemented and non-supplemented groups did not differ significantly ($p>0.05$).

Table 4.1: Effects of dietary RPO-supplementation on antioxidant capacity at baseline level

Treatment	ORAC	FRAP
	(µmolTE/ml)	(µmol/l)
AB: SRC	2.02 ± 0.09	145.20 ± 11.58
CDEF: SRC + RPO	1.96 ± 0.08	165.20 ± 10.27

Values in columns are expressed as means ± standard error of the mean (SEM). n=5 per group.

Abbreviations: µmol/l: micromole per litre; µmol TE/ml: micromole per millilitre Trolox equivalent. FRAP: ferric ion antioxidant power; ORAC: oxygen radical absorbance capacity.

Experimental groups:
AB: SRC : Groups A and B fed for 4 weeks with standard rat chow (SRC);
CDEF: SRC + RPO : Groups C, D, E and F fed SRC and supplemented with red palm oil (RPO) for 4 weeks.

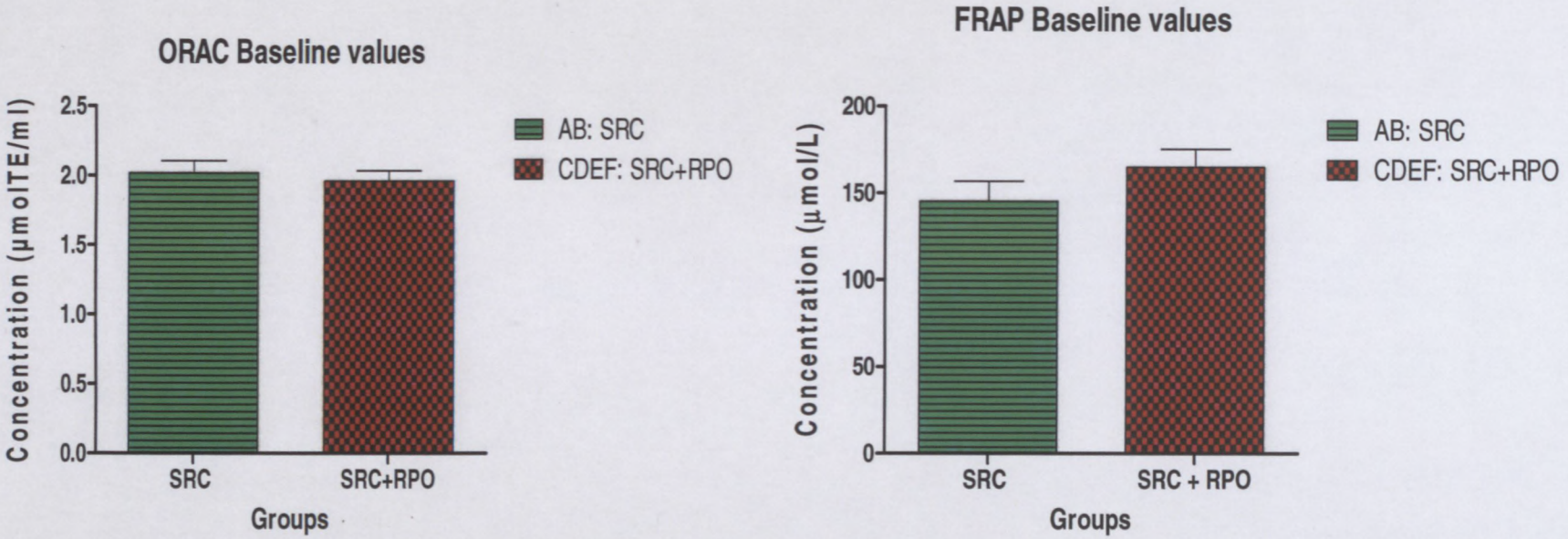

Figure 4.1: Effects of dietary RPO-supplementation on plasma antioxidant capacity at baseline level measuring ORAC and FRAP

Abbreviations: µmol/l: micromole per litre; µmol TE/ml: micromole per millilitre Trolox equivalent. FRAP: ferric ion antioxidant power; ORAC: oxygen radical absorbance capacity.

Experimental groups:
AB: SRC : Groups A and B fed for 4 weeks with standard rat chow (SRC);
CDEF: SRC + RPO : Groups C, D, E and F fed SRC and supplemented with red palm oil (RPO) for 4 weeks.

4.1.2 Effects of dietary RPO-supplementation on antioxidant capacity after t-BHP or placebo injections

The effects of RPO-supplementation on total antioxidant capacity after the two week injection period are presented in Table 4.2, Figures 4.2, 4.3, 4.4, and 4.5. FRAP and ORAC results showed no significant differences among groups ($p>0.05$).

Table 4.2: Effects of dietary RPO-supplementation on plasma antioxidant status after injection period of two weeks of either t-BHP or placebo, respectively

Treatment	ORAC	FRAP
	(µmol TE/ml)	(µmol/l)
A: CTRL Normal	1.70 ± 0.06	136.30 ± 8.64
B: CTRL t-BHP	1.60 ± 0.11	128.50 ± 7.72
C: 4WK RPO	1.88 ± 0.06	121.50 ± 9.51
D: 4WK RPO + t-BHP	1.57 ± 0.11	119.90 ± 14.70
E: 6WK RPO	1.59 ± 0.10	121.80 ± 10.20
F: 6WK RPO + t-BHP	1.40 ± 0.10	119.80 ± 13.60

Values in columns are expressed as mean ± SEM. n=5 per group.

Abbreviations: µmol/ml: micromole per millilitre; µmol TE/ml: micromole per millilitre Trolox equivalent. ORAC: oxygen radical absorbance capacity; FRAP: ferric ion antioxidant power; t-BHP: tertiary-butyl hydroxyperoxide; RPO: red palm oil.

Experimental groups

A: CTRL Normal : Control group A fed with standard rat chow (SRC);
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
C: 4WK RPO : Group C supplemented with RPO for 4 weeks;
D: 4WK RPO + t-BHP: Group D supplemented with RPO for 4 weeks and injected with t-BHP;
E: 6WK RPO : Group E supplemented with RPO for 6 weeks;
F: 6WK RPO + t-BHP: Group F supplemented with RPO for 6 weeks and injected with t-BHP.

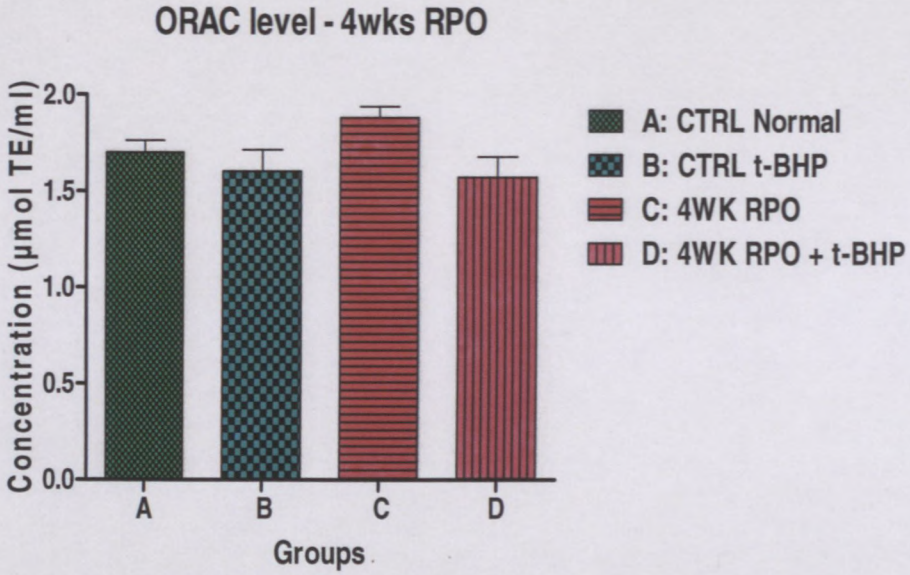

Figure 4.2: Effects of dietary RPO-supplementation on plasma ORAC values after 4 weeks consumption

Abbreviations: µmol TE/ml: micromole per millilitre Trolox equivalent. n=5 per group.

Experimental groups

A: CTRL Normal : Control group A fed with SRC;
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
C: 4WK RPO : Group C supplemented with RPO for 4 weeks;
D: 4WK RPO + t-BHP : Group D supplemented with RPO for 4 weeks and injected with t-BHP.

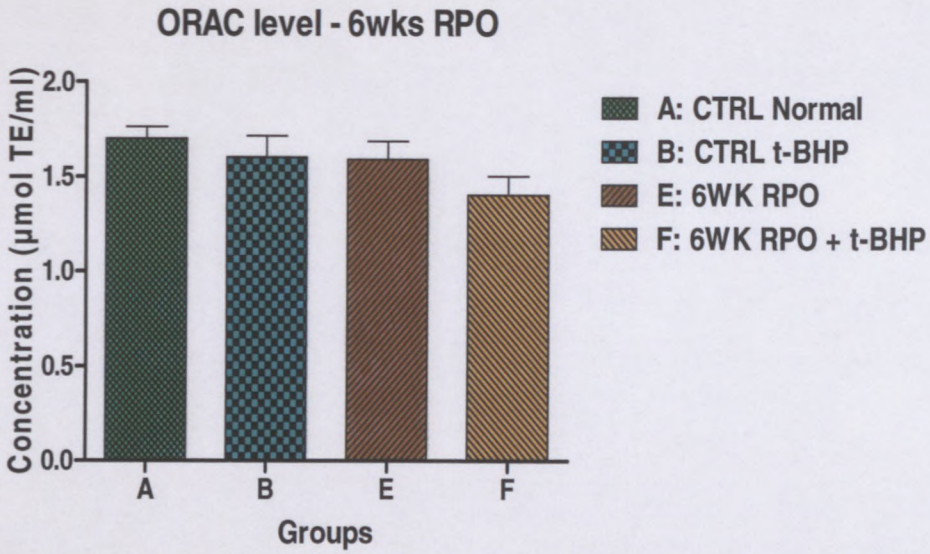

Figure 4.3: Effects of dietary RPO-supplementation on plasma ORAC values after 6 weeks consumption

Abbreviations: µmol TE/ml: micromole per millilitre Trolox equivalent. n=5 per group.

Experimental groups

A: CTRL Normal : Control group A fed with SRC;
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
E: 6WK RPO : Group E supplemented with RPO for 6 weeks;
F: 6WK RPO+ t-BHP: Group F supplemented with RPO for 6 weeks and injected with t-BHP.

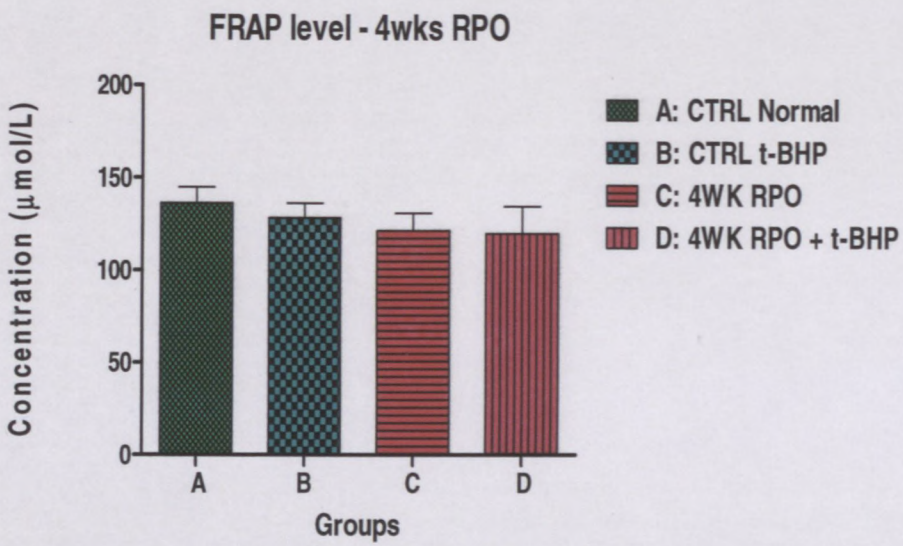

Figure 4.4: Effects of dietary RPO-supplementation on plasma FRAP values after 4 weeks consumption

Abbreviations: µmol/ml: micromole per millilitre. n=5 per group.

Experimental groups

A: CTRL Normal : Control group A fed with SRC;
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
C: 4WK RPO : Group C supplemented with RPO for 4 weeks;
D: 4WK RPO + t-BHP: Group D supplemented with RPO for 4 weeks and injected with t-BHP.

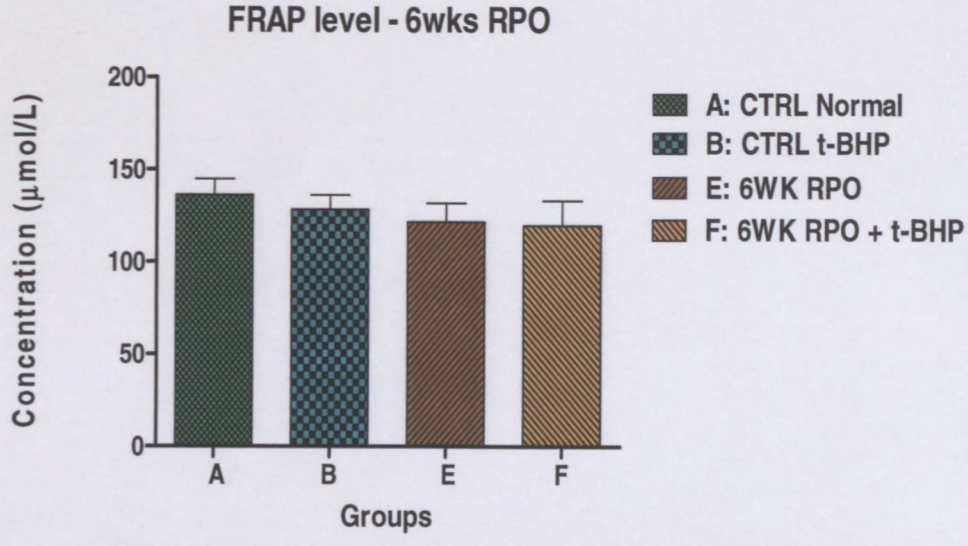

Figure 4.5: Effects of dietary RPO-supplementation on plasma FRAP values after 6 weeks consumption

Abbreviations: µmol/ml: micromole per millilitre. n=5 per group.

Experimental groups

A: CTRL Normal : Control group A fed with SRC;
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
E: 6WK RPO : Group E supplemented with RPO for 6 weeks;
F: 6WK RPO + t-BHP: Group F supplemented with RPO for 6 weeks and injected with t-BHP.

4.2 Effects of dietary RPO-supplementation on erythrocyte antioxidant parameters

4.2.1 Effects of dietary RPO-supplementation on antioxidant parameters at baseline level

At baseline level, results of dietary RPO intake on erythrocyte levels of SOD, GSHt and GPx are presented in Table 4.3 and Figure 4.6. SOD and GPx data for RPO supplemented and non-supplemented groups did not differ significantly ($p>0.05$). However, a significant increase ($p<0.05$) was observed in GSHt levels of RPO supplemented groups.

Table 4.3: Effects of dietary RPO-supplementation on erythrocytes' antioxidant status at baseline level

Treatment	SOD	GSHt	GPx
	(U/ml)	(mmol/ml)	(nmol/min/µl)
AB: SRC	898.10 ± 8.23	148.00 ± 5.92	9.40 ± 0.26
CDEF: SRC + RPO	900.20 ± 5.87	186.00 ± 13.00*	8.81 ± 0.22

Values in columns are expressed as means ± SEM. (*) Indicates significant difference at $p<0.05$.

Abbreviations: mmol/ml: millimole per millilitre; nmol/min/µl nanomole per minute per microliter; U/ml: unit/ millilitre. GSHt: total glutathione, GPx: glutathione peroxidase, SOD: superoxide dismutase.

Experimental groups:
AB: SRC : Groups A and B fed for 4 weeks with standard rat chow (SRC);
CDEF: SRC + RPO: Groups C, D, E and F fed SRC and supplemented with red palm oil (RPO) for 4 weeks

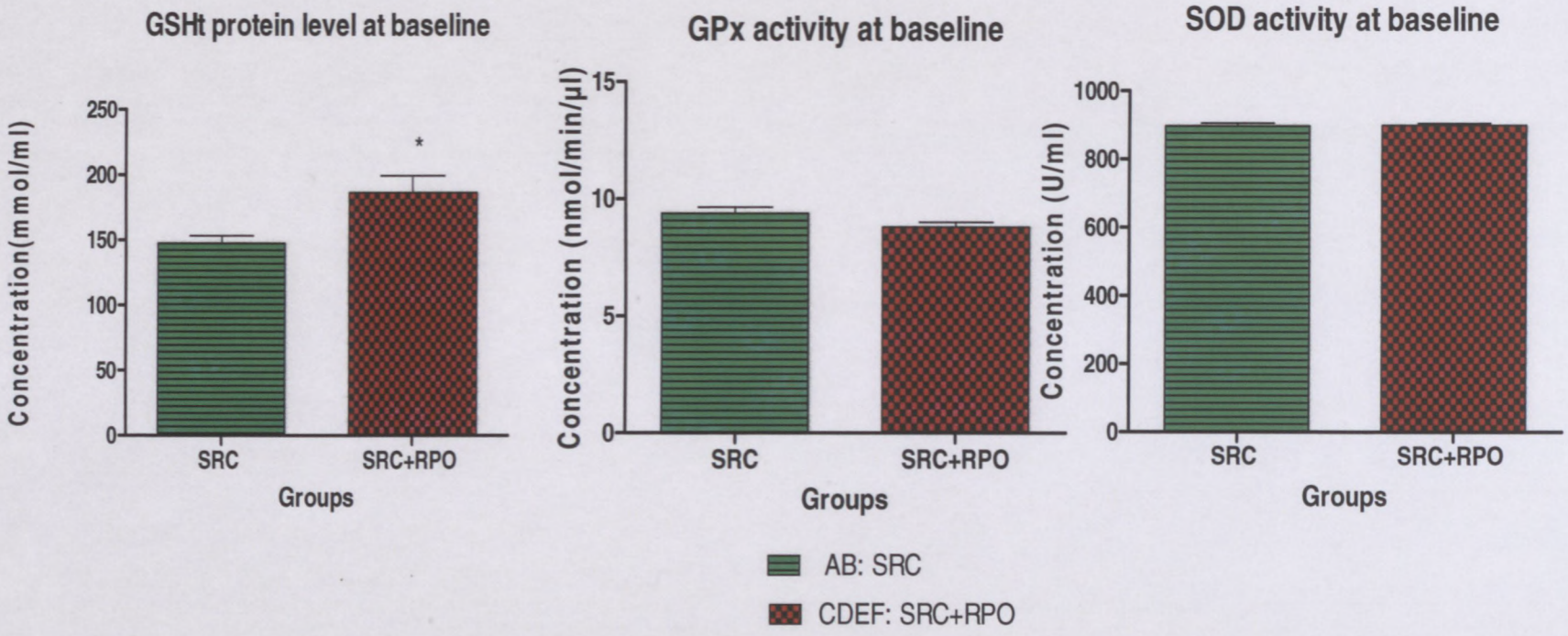

Figure 4.6: Effects of dietary RPO-supplementation on erythrocytes' antioxidant status at baseline level measuring GSHt levels, GPx and SOD activities

(*) Indicates significant difference at $p<0.05$

Abbreviations: mmol/ml: millimole per millilitre; nmol/min/µl nanomole per minute per microliter. GSHt: total glutathione, GPx: glutathione peroxidase.

Experimental groups:
AB: SRC : Groups A and B fed for 4 weeks with standard rat chow (SRC);
CDEF: SRC + RPO: Groups C, D, E and F fed SRC and supplemented with red palm oil (RPO) for 4 weeks.

4.2.2 Effects of dietary RPO-supplementation on superoxide dismutase and glutathione peroxidase after t-BHP or placebo injections

The effects of RPO-supplementation on SOD and GPx after the two week injection period are presented in Table 4.4 and Figures 4.7, 4.8, 4.9, 4.10, 4.11, 4.12.

After 4 weeks of RPO consumption, SOD activity in group C (4WK RPO) did not differ significantly ($p>0.05$) from the normal control group (A). Also, SOD activity in group D (4WK RPO + t-BHP) was not significantly different ($p>0.05$) when compared with the oxidative stress control group B (Figure 4.7). However, after 6 weeks of RPO consumption, a significant increase ($p<0.05$) in SOD activity was observed in group E (6WK RPO) when compared to its control group A (Figure 4.8).

No significant differences ($p>0.05$) was observed for GPx after 4 and 6 weeks of dietary supplementation with RPO between groups (Figures 4.9 and 4.10).

Table 4.4: Effects of dietary RPO-supplementation on erythrocytes' antioxidant status after injection period of two weeks of either t-BHP or placebo, respectively

Treatment	SOD	GSHt	GPx
	(U/ml)	(mmol/ml)	(nmol/min/µl)
A: CTRL Normal	536.20 ± 61.25	166.00 ± 9.57	12.87 ± 0.40
B: CTRL t-BHP	381.60 ± 68.47	184.70 ± 15.8	12.16 ± 0.33
C: 4WK RPO	335.90 ± 50.12	247.10 ± 11.10[a]	13.4 ± 0.53
D: 4WK RPO + t-BHP	448.20 ± 94.69	176.70 ± 9.10	11.26 ± 0.40
E: 6WK RPO	751.90 ± 37.47[ac]	180.60 ± 8.66	12.79 ± 1.00
F: 6WK RPO + t-BHP	492.90 ± 50.62	153.60 ± 6.94	11.97 ± 2.07

Values in columns are means ± SEM. (a) Indicates significant difference when compared to control group A at $p<0.05$. (c) Indicates significant difference when compared to group C at $p<0.05$. n=5 per group.

Abbreviations: mmol/ml: millimole per millilitre; nmol/min/µl: nanomole per minute per microliter; U/ml: unit/ millilitre. GSHt: total glutathione, GPx: glutathione peroxidase, SOD: superoxide dismutase. t-BHP: tertiary-butyl hydroxyperoxide; RPO: red palm oil.

Experimental groups

A: CTRL Normal : Control group A fed with standard rat chow (SRC);
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
C: 4WK RPO : Group C supplemented with RPO for 4 weeks;
D: 4WK RPO + t-BHP: Group D supplemented with RPO for 4 weeks and injected with t-BHP;
E: 6WK RPO : Group E supplemented with RPO for 6 weeks;
F: 6WK RPO + t-BHP: Group F supplemented with RPO for 6 weeks and injected with t-BHP.

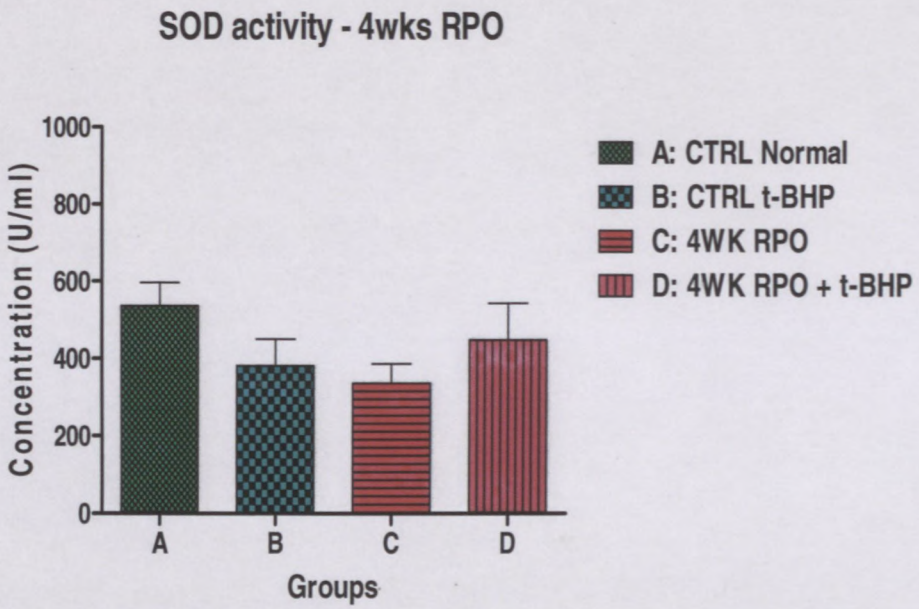

Figure 4.7: Effects of dietary RPO-supplementation on erythrocytes SOD activity after 4 weeks consumption

Abbreviations: U/ml: unit/millilitre. SOD: superoxide dismutase. n=5 per group.

Experimental groups

A: CTRL Normal : Control group A fed with SRC;
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
C: 4WK RPO : Group C supplemented with RPO for 4 weeks;
D: 4WK RPO + t-BHP: Group D supplemented with RPO for 4 weeks and injected with t-BHP.

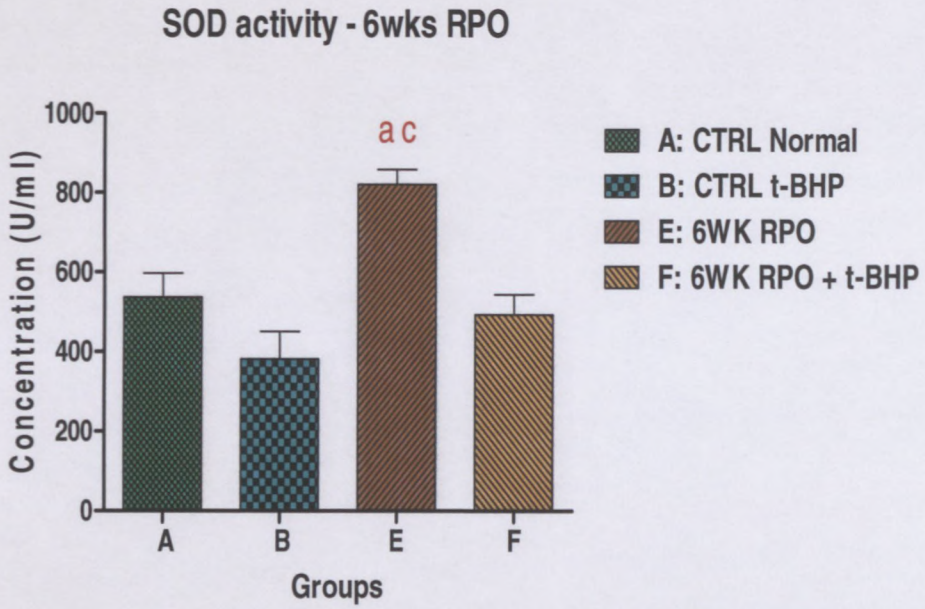

Figure 4.8: Effects of dietary RPO-supplementation on erythrocytes SOD activity after 6 weeks consumption

(a) Indicates significant difference when compared with control group A at $p<0.05$.
(c) Indicates significant difference when compared with group C at $p<0.05$.
n=5 per group.

Abbreviations: U/ml: unit/millilitre. SOD: superoxide dismutase.

Experimental groups

A: CTRL Normal : Control group A fed with SRC;
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
E: 6WK RPO : Group E supplemented with RPO for 6 weeks;
F: 6WK RPO + t-BHP: Group F supplemented with RPO for 6 weeks and injected with t-BHP.

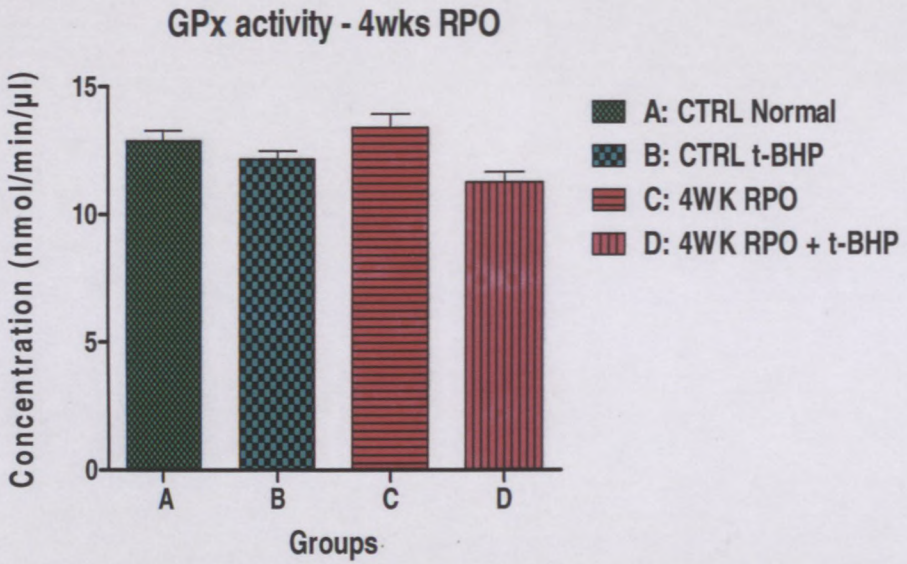

Figure 4.9: Effects of dietary RPO-supplementation on erythrocytes GPx activity after 4 weeks consumption

Abbreviations: nmol/min/µl: nanomole per minute per microliter. GPx: glutathione peroxidase, n=5 per group.

Experimental groups
A: CTRL Normal : Control group A fed with SRC;
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
C: 4WK RPO : Group C supplemented with RPO for 4 weeks;
D: 4WK RPO + t-BHP: Group D supplemented with RPO for 4 weeks and injected with t-BHP.

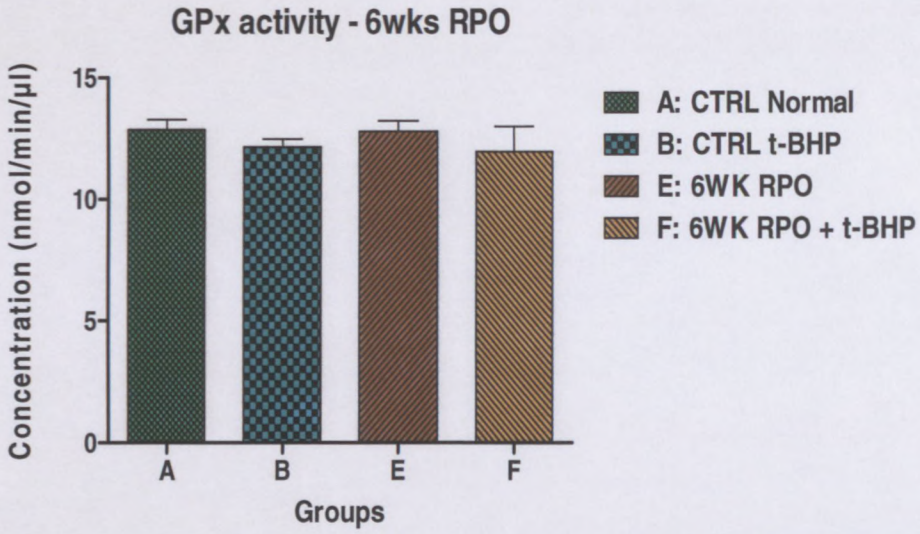

Figure 4.10: Effects of dietary RPO-supplementation on erythrocytes GPx activity after 6 weeks consumption

Abbreviations: nmol/min/µl: nanomole per minute per microliter. GPx: glutathione peroxidase, n=5 per group.

Experimental groups
A: CTRL Normal : Control group A fed with SRC;
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
E: 6WK RPO : Group E supplemented with RPO for 6 weeks;
F: 6WK RPO + t-BHP: Group F supplemented with RPO for 6 weeks and injected with t-BHP.

4.2.3 Effects of dietary RPO-supplementation on total glutathione levels after t-BHP or placebo injections

The effects of RPO-supplementation on GSHt levels after after the two week injection period are presented in Table 4.4 and Figures 4.11, 4.12.

Following 4 weeks of RPO consumption, GSHt levels in group C (4WK RPO) increased significantly ($p<0.05$) compared to the normal control group (A). GSHt levels in group D (4WK RPO + t-BHP) did not differ ($p>0.05$) from the oxidative stress control group B (Figure 4.11). After 6 weeks of RPO consumption, no significant differences ($p>0.05$) were observed in group E (6WK RPO) and group F (6WK RPO + t-BHP) when compared to the respective controls of group A and B (Figure 4.12).

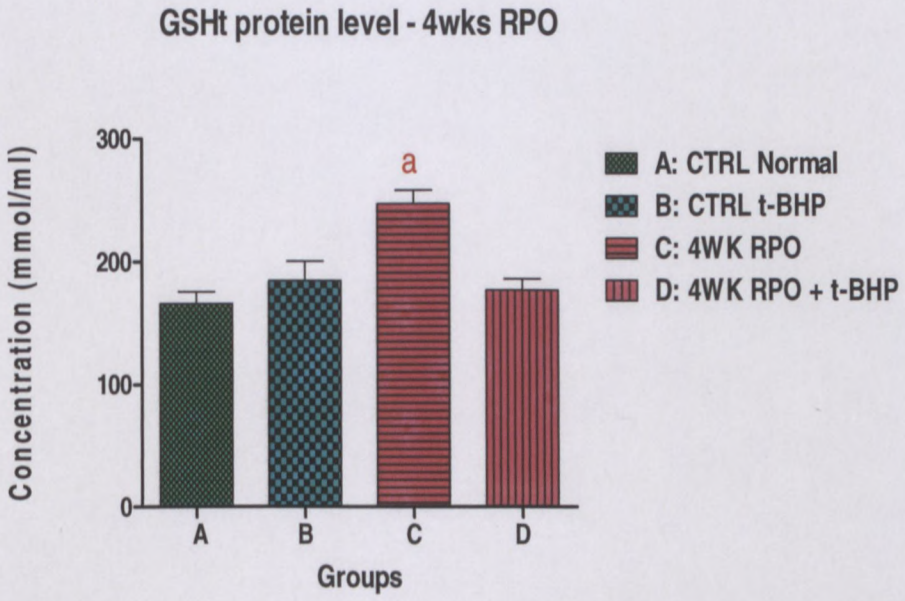

Figure 4.11: Effects of dietary RPO-supplementation on erythrocytes GSHt levels after 4 weeks consumption

(a) Indicates significant difference when compared with control group A at $p<0.05$. n=5 per group.

Abbreviations: mmol/ml: millimole per millilitre. GSHt: total glutathione.

Experimental groups
A: CTRL Normal : Control group A fed with SRC;
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
C: 4WK RPO : Group C supplemented with RPO for 4 weeks;
D: 4WK RPO + t-BHP: Group D supplemented with RPO for 4 weeks and injected with t-BHP.

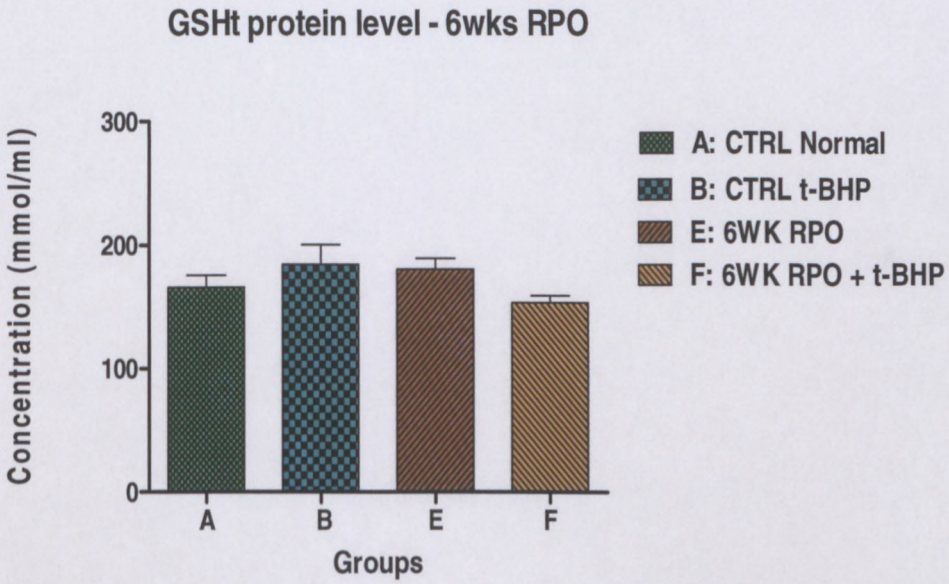

Figure 4.12: Effects of dietary RPO-supplementation on erythrocytes GSHt levels after 6 weeks consumption

Abbreviations: mmol/ml: millimole per millilitre. GSHt: total glutathione. n=5 per group.

Experimental groups
A: CTRL Normal : Control group A fed with SRC;
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
E: 6WK RPO : Group E supplemented with RPO for 6 weeks;
F: 6WK RPO + t-BHP: Group F supplemented with RPO for 6 weeks and injected with t-BHP.

4.3 Effects of dietary RPO-supplementation on oxidative status of plasma

4.3.1 Effects of dietary RPO-supplementation on oxidative status at baseline level

At baseline level, results of dietary RPO intake on rat plasma levels of CD and MDA are presented in Table 4.5 and Figure 4.13. After 4 weeks, no significant differences ($p>0.05$) were observed between groups for these two parameters.

Table 4.5: Effects of dietary RPO-supplementation on plasma oxidative status at baseline level

Groups/ Treatment	CD	MDA
	(µmol/l)	(µmol/l)
AB: SRC	2.02 ± 0.29	67.98 ± 11.13
CDEF:SRC + RPO	1.96 ± 0.30	75.78 ± 14.23

Values in columns are expressed as means ± SEM.

Abbreviations: µmol/l: micromole per litre. CD: conjugated diene, MDA: malondialdehyde.

Experimental groups:
AB: SRC : Groups A and B fed for 4 weeks with standard rat chow (SRC);
CDEF: SRC + RPO: Groups C, D, E and F fed SRC and supplemented with red palm oil (RPO) for 4 weeks.

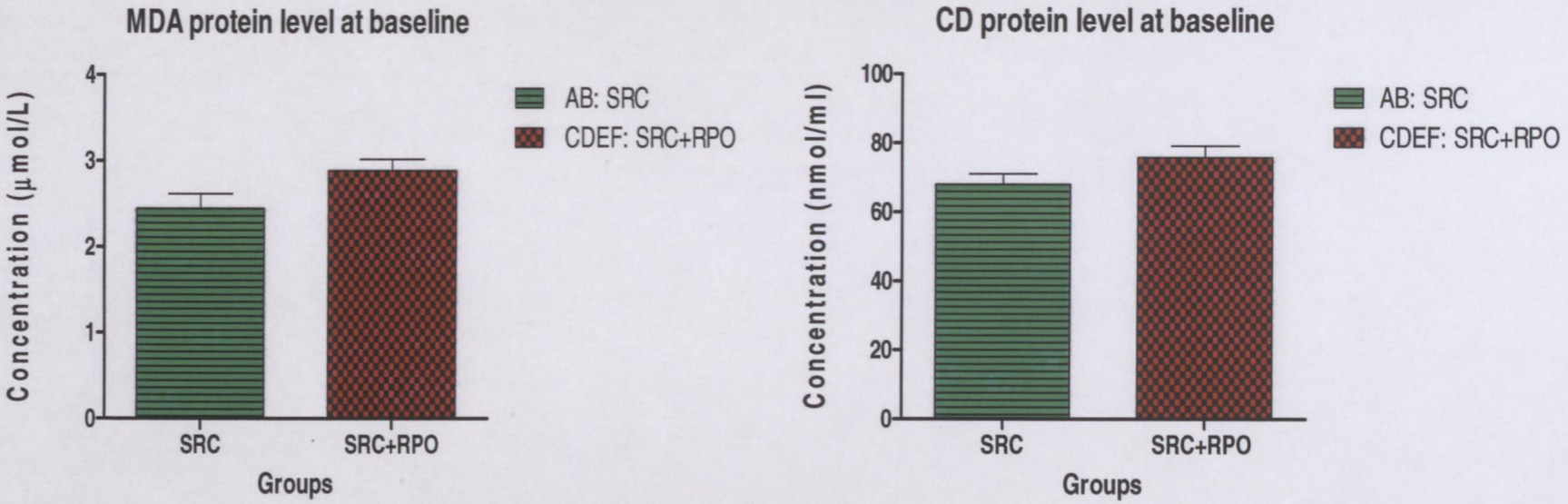

Figure 4.13: Effects of dietary RPO-supplementation on plasma lipid peroxidation at baseline level measuring MDA and CD levels

Abbreviations: µmol/l: micromole per litre. CDs: conjugated dienes, MDA: malondialdehyde. n=5 per group.

Experimental groups:
AB: SRC : Groups A and B fed for 4 weeks with standard rat chow (SRC);
CDEF: SRC + RPO: Groups C, D, E and F fed SRC and supplemented with red palm oil (RPO) for 4 weeks.

4.3.2 Effects of dietary RPO-supplementation on oxidative status after t-BHP or placebo injections

The effect of dietary RPO-supplementation on lipid peroxidation biomarkers MDA and CDs after the two week injection period are presented in Table 4.6 and Figures 4.14, 4.15, 4.16, 4.17.

After 4 weeks of RPO consumption, CD levels in oxidative-stress control (group B) was significantly higher ($p<0.05$) than in normal control (group A). Conjugated diene levels in group C (4WK RPO) was higher than normal control group A (Figure 4.14). CD levels in group D (4WK RPO + t-BHP) was not significantly different ($p>0.05$) from oxidative stress control group B (Figure 4.14). However, after 6 weeks of RPO consumption, a significant increase ($p<0.05$) was only observed in group E (6WK RPO) only when compared to control group A (Figure 4.15).

Following 4 weeks of dietary RPO-supplementation, MDA levels in oxidative stress control (group B) was significantly higher ($p<0.05$) than normal control (group A). Similar results were obtained after 6 weeks of dietary RPO-supplementation (Figure 4.16 and 4.17). This increase in oxidative status proved that the model used to induce oxidative stress was effective. There was a significant decrease ($p<0.05$) between MDA levels of group D (4WK RPO + t-BHP) subjected to oxidative stress and its corresponding control group B (t-BHP). Likewise, group F (6WK RPO + t-BHP) result was significantly lower ($p<0.05$) than control group B. No differences ($p>0.05$) were observed between the groups A (CTRL Normal) and C (4wk RPO), as well as groups A(CTRL Normal) and F (6wk RPO) for MDA levels (Figure 4.16 and 4.17).

Table 4.6: Effects of dietary RPO-supplementation on plasma oxidative status after injection period of two weeks of either t-BHP or placebo, respectively

Groups/ Treatment	CD	MDA
	µmol/L	µmol/L
A: CTRL Normal	39.96 ± 2.49	2.09 ± 0.16
B: CTRL t-BHP	59.04 ± 3.85[a]	3.53 ± 0.50[a]
C: 4WK RPO	66.64 ± 3.52	2.60 ± 0.11
D: 4WK RPO + t-BHP	66.23 ± 2.13	2.13 ± 0.11[b]
E: 6WK RPO	71.50 ± 8.23[a]	1.57 ± 0.08
F: 6WK RPO + t-BHP	51.49 ± 4.52	1.74 ± 0.13[b]

Values in columns are means ± SEM. (a) Indicates significant difference when compared to control group A at $p<0.05$. (b) Indicates significant difference when compared with control group B at $p<0.05$. n=5 per group.

Abbreviations: µmol/L: micromole per litre. CD: conjugated dienes, MDA: malondialdehyde.t-BHP: tertiary-butyl hydroxyperoxide; RPO: red palm oil.

Experimental groups

A: CTRL Normal : Control group A fed with standard rat chow (SRC);
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
C: 4WK RPO : Group C supplemented with RPO for 4 weeks;
D: 4WK RPO + t-BHP: Group D supplemented with RPO for 4 weeks and injected with t-BHP;
E: 6WK RPO : Group E supplemented with RPO for 6 weeks;
F: 6WK RPO + t-BHP: Group F supplemented with RPO for 6 weeks and injected with t-BHP.

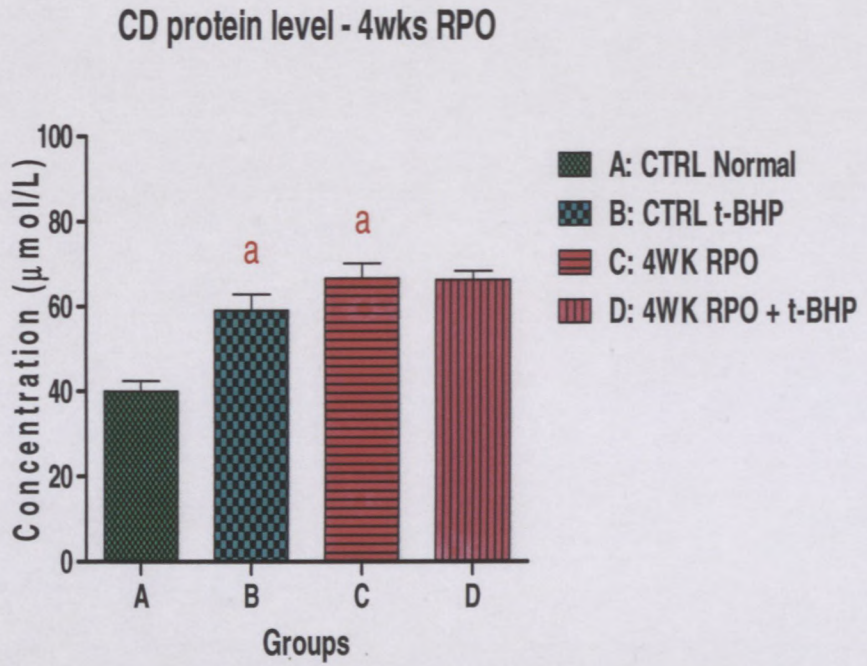

Figure 4.14: Effects of dietary RPO-supplementation on plasma CD levels after 4 weeks consumption

(a) Indicates significant difference when compared with control group A at $p<0.05$. n=5 per group.

Abbreviations: µmol/ml: micromole per millilitre; µmol TE/ml: micromole per millilitre Trolox equivalent .

Experimental groups

A: CTRL Normal : Control group A fed with SRC;
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
C: 4WK RPO : Group C supplemented with RPO for 4 weeks;
D: 4WK RPO + t-BHP: Group D supplemented with RPO for 4 weeks and injected with t-BHP.

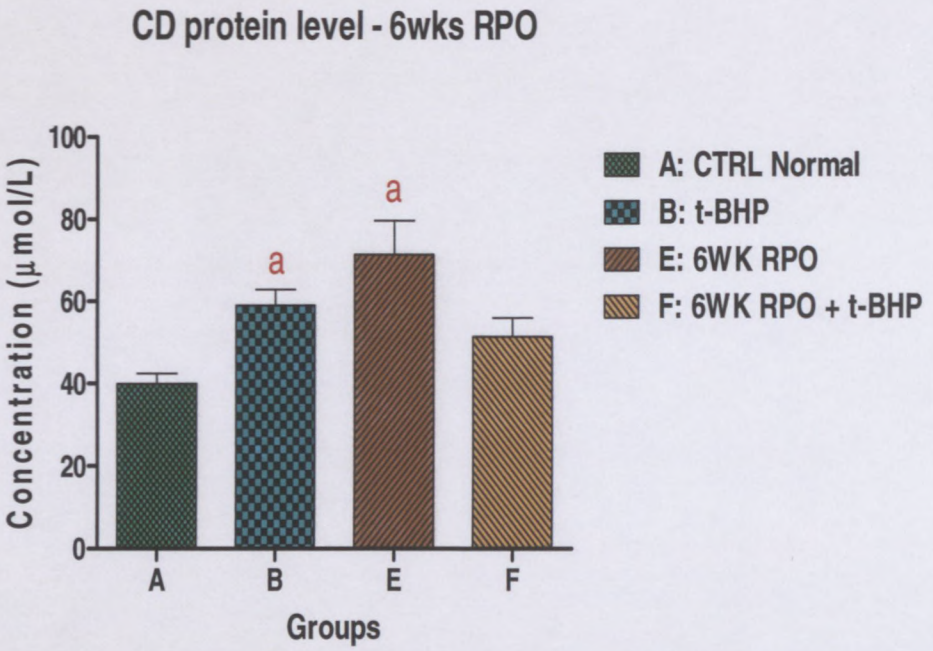

Figure 4.15: Effects of dietary RPO-supplementation on plasma CD levels after 6 weeks consumption

(a) Indicates significant difference when compared with control group A at $p<0.05$. n=5 per group.

Abbreviations: µmol TE/ml: micromole per millilitre Trolox equivalent.

Experimental groups

A: CTRL Normal : Control group A fed with SRC;
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
E: 6WK RPO : Group E supplemented with RPO for 6 weeks;
F: 6WK RPO + t-BHP: Group F supplemented with RPO for 6 weeks and injected with t-BHP.

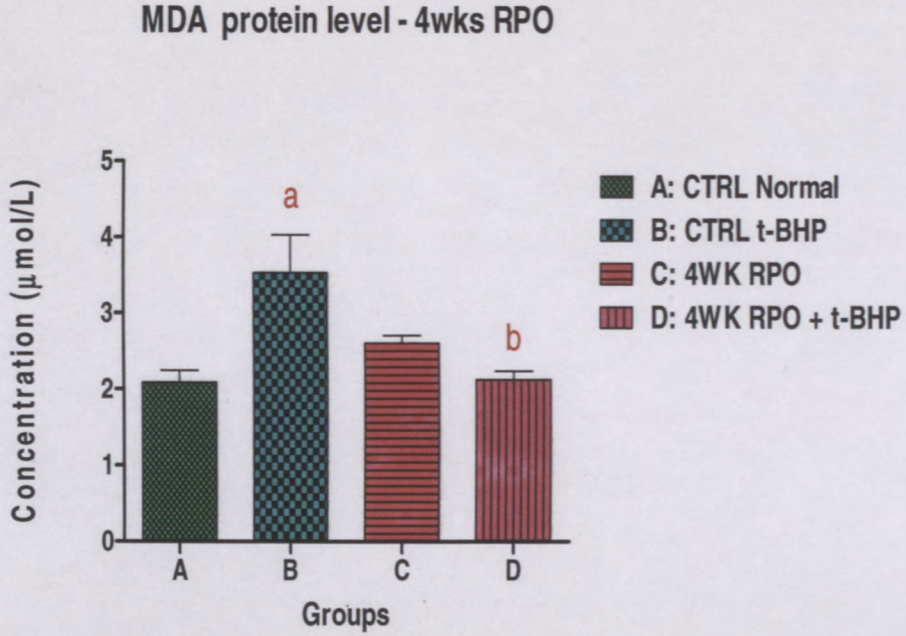

Figure 4.16: Effects of dietary RPO-supplementation on plasma MDA levels after 4 weeks consumption

(a) Indicates significant difference when compared with control group A at $p<0.05$.
(b) Indicates significant difference when compared with control group B at $p<0.05$.
n=5 per group.

Abbreviations: µmol/L: micromole per litre.

Experimental groups
A: CTRL Normal : Control group A fed with SRC;
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
C: 4WK RPO : Group C supplemented with RPO for 4 weeks;
D: 4WK RPO + t-BHP: Group D supplemented with RPO for 4 weeks and injected with t-BHP.

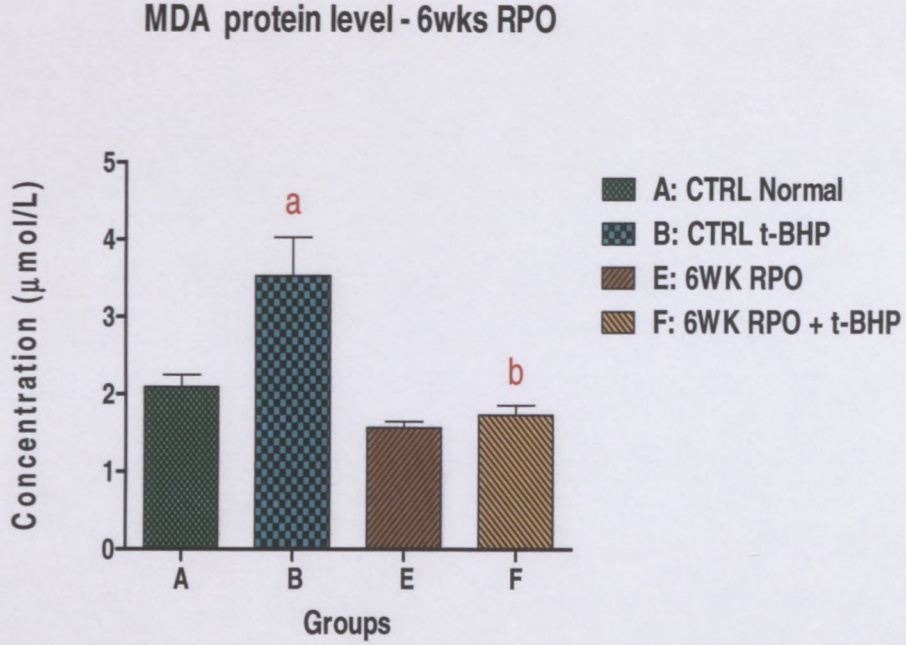

Figure 4.17: Effects of dietary RPO-supplementation on plasma MDA levels after 6 weeks consumption

(a) Indicates significant difference when compared with control group A at $p<0.05$.
(b) Indicates significant difference when compared with control group B at $p<0.05$.
n=5 per group.

Abbreviations: µmol/L: micromole per litre.

Experimental groups
A: CTRL Normal : Control group A fed with SRC;
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
E: 6WK RPO : Group E supplemented with RPO for 6 weeks;
F: 6WK RPO + t-BHP: Group F supplemented with RPO for 6 weeks and injected with t-BHP.

4.4 Effects of dietary RPO-supplementation on CVD risk factor in serum

4.4.1 Effects of dietary RPO-supplementation on CVD risk factor at baseline level

At baseline level, results of dietary RPO-supplementation in serum homocysteine levels are presented in Table 4.5. No significant differences ($p>0.05$) were observed between RPO supplemented and non-supplemented groups.

Table 4.7: Effects of dietary RPO-supplementation on serum homocysteine at baseline level

Groups/Treatment	Homocysteine (μmol/l)
AB: SRC	9.70 ± 1.31
CDEF: SRC + RPO	9.71 ± 1.41

Values in columns are expressed as means ± SEM. n=5 per group.

Abbreviations: μmol/l: micromole per litre.

Experimental groups:
AB: SRC : Groups A and B fed for 4 weeks with standard rat chow (SRC);
CDEF: SRC + RPO: Groups C, D, E and F fed SRC and supplemented with red palm oil (RPO) for 4 weeks.

4.4.2 Effects of RPO-supplementation on CVD risk factor after t-BHP or placebo injections

The effects of RPO-supplementation on homocysteine levels in rat plasma after the two week injection period are presented in Table 4.8 and Figure 4.18 and 4.19.

After 4 weeks of RPO consumption, homocysteine levels of group C (4WK RPO + t-BHP) was significantly increased ($p<0.05$) compared to normal control group A (Figure 4.18). After 6 weeks of RPO-supplementation, a significant increase ($p<0.05$) was observed in group E (6WK RPO) compared to control group A (Figure 4.19).

Table 4.8: Effects of dietary RPO-supplementation on serum homocysteine after injection period of two weeks of either t-BHP or placebo, respectively

Groups/Treatment	Homocysteine µmol/L
A: CTRL Normal	7.84 ± 0.48
B: CTRL t-BHP	9.42 ± 0.37
C: 4WK RPO	10.07 ± 0.65[a]
D: 4WK RPO + t-BHP	11.10 ± 0.55
E: 6WK RPO	10.00 ± 0.50[a]
F: 6WK RPO + t-BHP	9.18 ± 0.55

Values in columns are means ± SEM. (a) Indicates significant difference when compared to control group A at $p<0.05$. n=5 per group.

Abbreviations: µmol/L: micromole per litre. t-BHP: tertiary-butyl hydroxyperoxide; RPO: red palm oil.

Experimental groups

A: CTRL Normal : Control group A fed with standard rat chow (SRC) and injected with placebo;
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
C: 4WK RPO : Group C supplemented with RPO for 4 weeks;
D: 4WK RPO + t-BHP: Group D supplemented with RPO for 4 weeks and injected with t-BHP;
E: 6WK RPO : Group E supplemented with RPO for 6 weeks;
F: 6WK RPO + t-BHP: Group F supplemented with RPO for 6 weeks and injected with t-BHP.

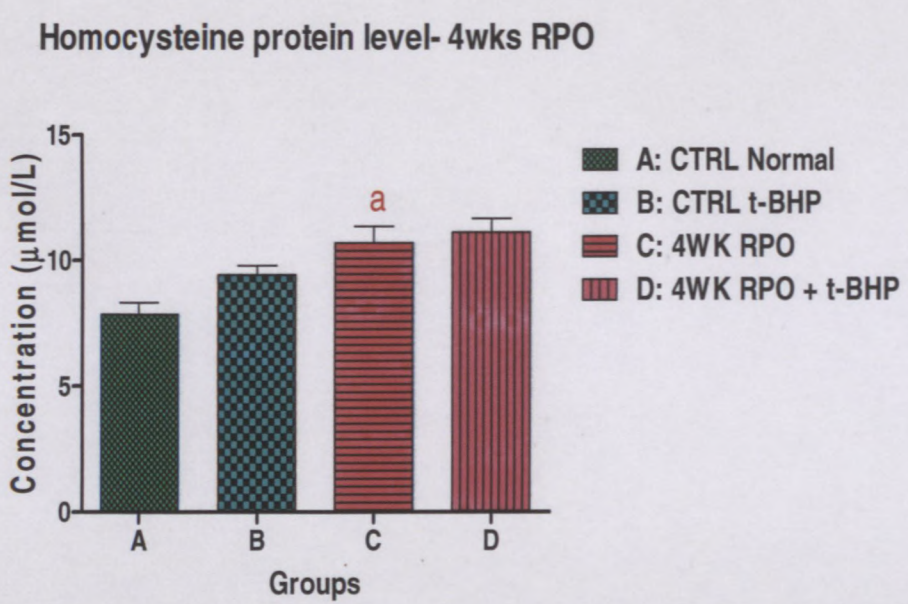

Figure 4.18: Effects of dietary RPO-supplementation on plasma homocysteine after 4 weeks consumption

(a) Indicates significant difference when compared with control group A at $p<0.05$. n=5 per group

Abbreviations: µmol/l: micromole per litre.

Experimental groups
A: CTRL Normal : Control group A fed with SRC and injected with placebo;
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
C: 4WK RPO : Group C supplemented with RPO for 4 weeks;
D: 4WK RPO + t-BHP: Group D supplemented with RPO for 4 weeks and injected with t-BHP.

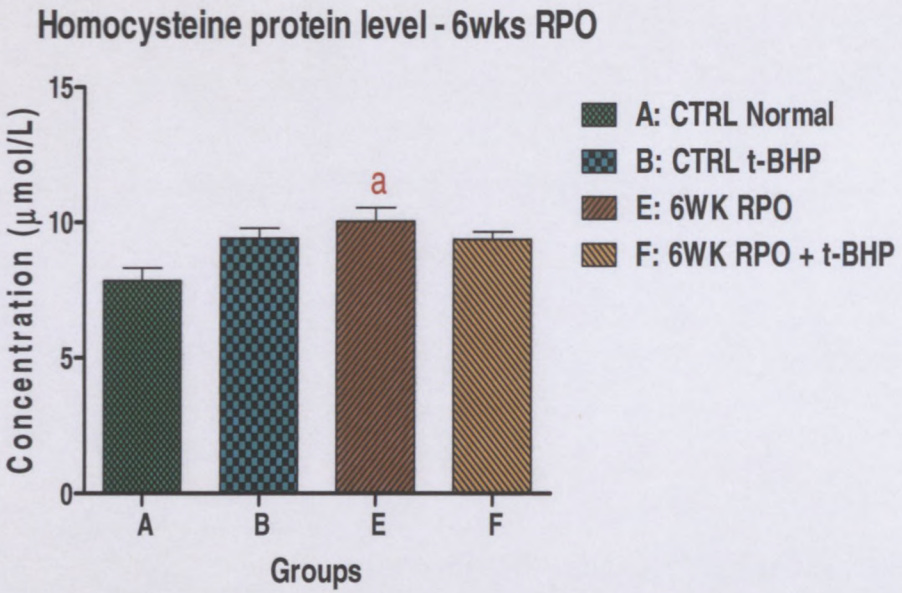

Figure 4.19: Effects of dietary RPO-supplementation on plasma homocysteine after 6 weeks consumption

(a) Indicates significant difference when compared with control group A at $p<0.05$. n=5 per group.

Abbreviations: µmol/l: micromole per litre

Experimental groups
A: CTRL Normal : Control group A fed with SRC and injected with placebo;
B: CTRL t-BHP : Control group B fed with SRC and injected with t-BHP;
E: 6WK RPO : Group E supplemented with RPO for 6 weeks;
F: 6WK RPO + t-BHP: Group F supplemented with RPO for 6 weeks and injected with t-BHP.

CHAPTER 5

DISCUSSION

Many studies have been done on RPO-supplementation and its role in oxidative stress-related diseases (Esterhuyse *et al.,* 2005, 2006; Oguntibeju *et al.,* 2010; Aboua *et al.,* 2012). However, little is known about RPO-supplementation and oxidative stress parameters in blood of oxidative stress induced-Wistar rats. Hence, the current investigation moved a step further to investigate the effects of RPO-supplementation on oxidative stress biomarkers and cardiovascular risk factors *in vivo*. We measured parameters such as superoxide dismutase, glutathione peroxidase, total glutathione, malondialdehyde, conjugated dienes and homocysteine. Our major finding was that RPO-supplementation influenced malondialdehyde levels, conjugated diene levels, superoxide dismutase activity and glutathione levels.

5.1 Effects of dietary RPO-supplementation on total antioxidant capacity (TAC)

Various methods have been used to determine and characterize the TAC, such as trolox equivalent antioxidant capacity (TEAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu, ferric ion reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC). Despite the diversity of assays, two general chemistry principles governed the measurement of antioxidant capacity and both are based on free radicals deactivation. The first principle is known as hydrogen atom transfer (HAT) and the second principle is named as electron transfer (ET) (Prior *et al.,* 2005; Neo *et al.,* 2010). The current study evaluated the TAC using ORAC and FRAP; two typical examples of HAT and ET free radicals deactivation respectively. Previous work performed on RPO did not focus on measuring plasma ORAC of rats supplemented with RPO, and very few reports have been documented on measuring plasma FRAP of rats supplemented with RPO. It is therefore important to point out that this study is the first of its kind as no results are available concerning the effect of RPO-supplementation on TAC in an oxidative stress-induced rat model. In this study, ORAC and FRAP results, as shown in Chapter 4, indicated that RPO did not significantly affect the TAC, irrespective of oxidative stress induction.

The results presented here did not fully support our hypothesis that RPO could increase total antioxidant capacity. This may suggest that antioxidant capacity alone may not be responsible for the level of protection offered by RPO. It could also be speculated that the

majority of RPO antioxidants, being lipophilic, are probably located in the lipophilic compartment of rat plasma, which can not be detected by our method of analysis.

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, for this *in vivo* model, separating hydrophilic from lipophilic measurements may show limitations. Actually, an investigation 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 relevant to exhaustively investigate a biological system. In a true biological system both hydrophilic and lipophilic antioxidants are communicating continuously. Therefore, by partitioning lipophilic from hydrophilic extracts, one could easily divert from our initial goal, which was to evaluate the effects of RPO on blood antioxidant capacity *in vivo*. These findings create opportunities for further investigations to elucidate the effects of RPO-supplementation in modulating the total antioxidant capacity of rat plasma.

5.2 Effects of dietary RPO-supplementation on antioxidant parameters (SOD, GPx and GSHt)

Tertiary-butyl hydroperoxide (t-BHP) is a prototypic initiator of oxidative stress (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 disintegrate 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 damages. 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, no significant reduction in SOD, GPx and/or GSHt were observed in t-BHP-induced control groups (Fig 4.7; 4.8; 4.9; 4.10; 4.11; 4.12). This is in contradiction to the results of Kumar (2007) and Aboua and colleagues (2012) who found decreased values of antioxidant enzymes activities associated witht-BHP-induced toxicity. Possible explanations for our results could be based on the following arguments.

We hypothesize that the dose and rate of t-BHP administration in the current study did not induce a severe enough state of oxidative stress. Although, the model of administration of t-

BHP used in this current study was modified from those used by Kumar (2007) and Aboua and colleagues (2012), the dose of t-BHP injected in this current study was exactly the same as the dose used in the study of Aboua and co-workers (2012). However, the main difference of administration of t-BHP was in the frequency of administration. In the present study, t-BHP injections were not administered daily but were administered every second day for two weeks. Hence, the oxidative stress induced may not have been enough to cause depletion of glutathione reserve and antioxidant enzymes activities. It may also indicate that method of t-BHP administration is important and may have a significant effect on the expected outcome. Secondly, we speculated that rats may be genetically predisposed to offer resistance to t-BHP induced oxidative stress. Hence, t-BHP-challenged rats might have inherited a defence mechanism to resist the state of oxidative stress (Loepfe *et al.,* 2010).

Moreover, it is possible that no depletion in GSHt levels was observed because of a cellular self-defence mechanism. In this case, following the injection of t-BHP, cellular defence mechanisms might have arisen in an attempt to detoxify t-BHP. We argue that GPx might have been used in order to breakdown t-BHP into less reactive compounds with subsequent formation of oxidised glutathione (Dringen *et al.,* 1998). Thereafter, a conversion of oxidised glutathione in reduced glutathione might have occurred. And this might have indirectly replenished the depleted glutathione. Similar results were found in GPx activity in all treatment groups at 4 and 6 weeks. This supports our hypothesis that more GPx will be produced in an attempt to defend against t-BHP-induced oxidative stress, the more the turn-over rate will also increase. Thus, this could easily maintain the activity of GPx compared to normal control.

Previous studies on dietary antioxidant supplementation have shown a positive correlation between natural dietary supplementation and increase in SOD activity and GSHt levels. Although there is a difference between enzyme levels and activities, similar increases in SOD levels and activity was observed after dietary antioxidant supplementation. For example, Ananthan and co-workers (2004) investigated supplementation with *Gymmema montanum* leaf extract which is an Indian medicinal plant. They demonstrated recovery from diabetic state (oxidative stress-related condition) associated with significant increase in SOD activity and GSHt levels. In another study, Awoniyi and co-workers (2012) demonstrated that rooibos tea significantly increased SOD levels after t-BHP induced-oxidative stress. Superoxide dismutase is a major scavenging enzyme which removes superoxide radicals, a toxic radical, by converting it into more stable compounds such as hydrogen peroxide and water. An increase in SOD activity or in GSHt levels could therefore be seen as a possible mechanism to reduce the risk of oxidative stress-induced damage.

The current study shows that RPO consumption for a period of 6 weeks significantly enhanced SOD activity when compared to normal control group A (Figure 4.8). Similarly, RPO consumption for a period of 4 weeks increased GSHt levels (Figure 4.11). These results indicate that RPO-supplementation has the ability to increase endogenous antioxidant activities and levels and enhance the antioxidant defence mechanism of the cell. It can be suggested that the potential mechanisms by which RPO enhanced endogenous detoxification could involve one or more antioxidant defence mechanism. Previous studies found that RPO antioxidant properties were adequate to potentially protect against oxidative stress induced-damages (Upritchard *et al.,* 2003; Aboua *et al.,* 2012).

5.3 Effects of dietary RPO-supplementation on oxidative stress damage parameters (MDA and CD)

One of the main indicators of oxidative stress induced damage is lipid peroxidation. Lipid peroxidation is a free radical mediated propagation of oxidative damage to polyunsaturated fatty acids (PUFAs), initiated through several types of free radicals. MDA is a by-product of lipid peroxidation. An increase in oxidative stress is related to a significant increase in MDA levels. This could easily be related to pathological conditions both in animals and humans. Additionally, it could also be related to exposure to high altitude (hypoxia) or environmental toxicants. For instance, under hypoxia-induced oxidative stress, an increase of the levels of plasma MDA was observed in Sprague-Dawley rats (Luo *et al.,* 2012). Wang and co-workers (2012) recently indicated a significant increase in MDA levels in haemolysed red blood cells of workers exposed to carcinogenic chromate compounds.

In this investigation, where oxidative stress was induced with t-BHP, plasma concentration of malondialdehyde (MDA) significantly increased in oxidative stress-control rats when compared to normal control group (Group A). However, when RPO was supplemented to the oxidative stress groups for 4 and 6 weeks, it was observed that MDA levels were significantly reduced to the value of normal control rats (Group A). The improvements observed in the lipid peroxidation status may be attributed to antioxidant properties of RPO by means of scavenging the effects of hydroperoxide resulting from induced-oxidative stress. Such findings agree with a recent study of Aboua and co-workers (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. Carotenes and vitamin E tocotrienols and tocopherols are considered among the most effective antioxidants (Van Stuijvenberg and Benade, 2000; Schroeder *et al.,* 2006; Varoglu *et al.,* 2010). It could therefore be argued that RPO carotenoids and vitamin E tocopherols and tocotrienols could have played an important role in protecting cellular membranes *in*

vivo. Previously, it has been 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-tocotrienol and β-carotene (Schroeder *et al.,* 2006). Red palm oil in contrast to other oils seems to possess a unique blend of vitamin E components. For example, Sundram and co-workers (2003) reported that RPO was not only rich in tocopherols, but also unique because of its contents in tocotrienols. Furthermore, although few studies have investigated the effect of RPO-supplementation on MDA measurement *in vivo*, administration of RPO fractions also showed a potential benefit. Budin and co-workers (2009) reported that tocotrienol rich fractions in the diabetes-induced rat model significantly decreased plasma MDA and other oxidative stress markers.

Previous studies have also used antioxidant treatment and supplementation to oppose oxidative stress. Recently, Khakpour and colleagues (2012) reported a significant decrease in MDA levels in mice following *Citrus aurantium* treatment. These findings support the finding of the current study despite using a different model. In a study with ischemic stroke patients, *Ginkgo biloba* extract coupled to conventional therapy 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 mostly of fat-soluble antioxidants. This argues 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 (Marnewick *et al.,* 2000; Wilson *et al.,* 2005; Shen *et al.,* 2012) reduced the accumulation of MDA. Therefore, as expected, our findings showed that RPO has the ability to protect against toxic damage of lipid peroxidation by decreasing the plasma concentration of MDA.

Furthermore, this study shows that CD levels in rats which consumed RPO for four and six weeks were significantly higher than normal control group (Group A) whereas CD levels of rats which were induced with oxidative stress and fed with red palm oil had a tendency to decrease to those of normal control group A (Figures 4.14; 4.15). To our knowledge, this is the first study to investigate the effects of RPO-supplementation on CD levels after oxidative stress induction with t-BHP in Wistar rats. Two mechanisms may be considered:

- The first mechanism concerns elevated CD levels found in groups of rats fed with RPO and not induced with oxidative stress. Red palm oil may have an ability to increase fatty acid levels in serum independent of its antioxidant content. We speculate that these higher lipid levels may be associated with an increased ability of fatty acid chains to evolve into conjugated dienic structures.

- The second mechanism concerns the fact that RPO only show effects when the experimental model used is challenged. In earlier studies by Esterhuyse and co-workers (2006) and Bester and co-workers (2006), it was found that RPO-supplementation had no effect on biological parameters when the experimental model was not challenged with oxidative stress-related condition. However, the moment when ischemia-reperfusion was induced, the protective effect of RPO was activated.

5.4 Effects of dietary RPO-supplementation on homocysteine levels

In this study, it was found that RPO-supplementation increased homocysteine levels when the experimental model was not challenged with oxidative stress. However, no significant changes were observed in homocysteine levels following the induction of oxidative stress and RPO-supplementation. Similar findings were reported by Jaarin *et al.,* (2006) in Sprague Dawley rats supplemented with oxidised vegetable oils. Nevertheless, the result on homocysteine level is inconclusive and further investigations are recommended.

5.5 Effects of dietary RPO-supplementation on feeding time period

It is inconclusive whether 4 or 6 weeks of RPO-supplementation is the best duration of supplementation. In this study, total antioxidant capacity, GPx activity, lipid peroxidation and homocysteine levels showed approximately similar results between 4 and 6 weeks of RPO-supplementation. However, we observed that effects of RPO-supplementation were enhanced for SOD activity after 6 weeks of supplementation compared to 4 weeks. These results confirm earlier results by Tamahane-Katengua (2010) which found that 6 weeks of RPO-supplementation achieved best results in protection against ischemia-reperfusion injury compared to 4 weeks and 8 weeks of supplementation (Tamahane-Katengua, 2010).

Additionally, in a study by Wergeland and co-workers (2011), RPO protection against cytotoxic effects of anthracycline in the heart was investigated using a 4 week supplementation period. Their results suggested that more clarification on the dosage and duration of RPO-supplementation in perfusion studies is needed. From our results, we propose that a supplementation period of 6 weeks may achieve better results than 4 weeks and argue that 6 weeks could be an optimal supplementation time for RPO to achieve beneficial results.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

In this study, we demonstrated how RPO could modulate oxidative stress biomarkers in an *in vivo* experimental animal model using Wistar rats.

The findings of this study demonstrated that RPO-supplementation could ameliorate antioxidant status and could provide defence against oxidative stress. Indeed, the current study demonstrated that dietary RPO-supplementation could have a protective effect against lipid peroxidation damages by restoring MDA levels of oxidative stress groups to those of control rats, which were not supplemented with RPO nor induced with oxidative stress. Moreover, it was also demonstrated that RPO-supplementation had a potential beneficial effect in improving some antioxidant status parameters such as superoxide dismutase and glutathione under normal conditions.

After induction of oxidative stress, RPO-supplementation did not show deleterious effects on homocysteine levels. It was observed that on the onset of oxidative stress, RPO-supplementation indicated potential ability to offer protection. In addition, this study acknowledged that RPO protection against oxidative stress conditions could manifest within 4-6 weeks of supplementation in Wistar rats, with 6 weeks of RPO-supplementation achieving the best results.

To our knowledge, this is the first time an investigation focused on measuring serum homocysteine, plasma malondialdehyde and blood antioxidant status in rats supplemented with RPO (4-6 weeks) under oxidative stress conditions *in vivo*. This explorative study has created opportunities for further investigations.

Small sample size and other factors such as the selection of ultimate tissue type (blood) contributed in not achieving clear results in all aspects of the study. Therefore, studies with larger sample size and further biological investigations are recommended.

CHAPTER 7

SCIENTIFIC RESEARCH OUTPUT

7.1 First scientific research output

❖ INTERNATIONAL CONFERENCE HOSTED IN SOUTH AFRICA

Authors: **OBL Alinde**, OO Oguntibeju, J van Rooyen, AJ Esterhuyse

Title: Effects of red palm oil on plasma antioxidant capacity in rat model of tertiary-butyl hydroxyperoxide-induced oxidative stress.

Conference: 2011 Joint Research Conference: 4th Walter Sisulu International Research Conference, 8th Society for Free Radical Research – Africa (SFRR), 31st African Health Sciences Congress (31st AHSC), 4th International Conference of the Promotion of Traditional Medicines (PROMETRA)

Location: East London, Eastern Cape, South Africa

Date: 17-19 August 2011

Presentation: Oral

7.2 Second scientific research output

- **LOCAL CONFERENCE**

Authors:	**OBL Alinde**, OO Oguntibeju, J van Rooyen, AJ Esterhuyse
Title:	Effects of palm oil on plasma antioxidant capacity in rat model of tertiary-butyl hydropeoxide-induced oxidative stress
Conference:	Laboratory Medicine Congress
Location:	Johannesburg, South Africa.
Date:	31 August - 4 September 2011
Presentation:	Oral

7.3 Third scientific research output

- **INTERNATIONAL CONFERENCE**

Authors:	OO Oguntibeju, **OBL Alinde**, J van Rooyen, AJ Esterhuyse
Title:	Effects of red palm oil on plasma antioxidant capacity in rat model of tertiary-butyl hydroxyperoxide-induced oxidative stress.
Conference:	International Conference and exhibition on nutritional science and therapy.
Location:	Philadelphia, USA
Date:	27-29 August 2011
Presentation:	Oral

7.4 Fourth scientific research output

❖ PUBLICATION OF A CHAPTER IN A BOOK

- **Chapter details**

Authors:	OO Oguntibeju, **OBL Alinde**, AJ Esterhuyse
Title:	Induced-oxidative stress and red palm oil (RPO) supplementation for 4 and 6 weeks in animal model: Any benefit?
Pages:	349-368

- **Book details**

Editors:	V.K. Gupta, S. Singh and A. Kaul
Publisher:	Daya Publishing House, New Delhi.
Pages:	433
Year:	2012
ISBN:	978-81-7035-768-1

7.5 Fifth scientific research output

❖ PUBLICATION OF AN ABSTRACT IN A JOURNAL

Authors: OO Oguntibeju, **OBL Alinde**, AJ Esterhuyse

Title: Induced-oxidative stress and red palm oil (RPO) supplementation for 4 and 6 weeks in animal model: Any benefit?

- **Journal details**

Journal name: Journal of Nutrition and Food Sciences

Page: 50

Year: 2012

Volume: 2 (7)

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