THE EFFECT OF RED PALM OIL SUPPLEMENTATION OF AN OXIDATIVE RISK INDUCED DIET AND A HIGH SATURATED FAT DIET ON ISCHAEMIA/REPERFUSION INJURY IN THE ISOLATED PERFUSED RAT HEART

by

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Dissertation presented for the Degree of

Magister Technologiae

(Biomedical Technology)

in the

Department of Biomedical Sciences

Faculty of Health Sciences

at

Cape Peninsula University of Technology

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January 2006

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α	Alpha
AA	Arachidonic acid
ALA	Alpha linolenic acid
AO	Aortic output
β	Beta
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CF	Coronary flow
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
Chol	Cholesterol
CoQ_{10}	Coenzyme Q_{10}
COX	Cyclooxygenase
CTEP	Cholesterylester transfer protein
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
EFA	Essential fatty acids
eNOS	Endothelial nitric oxide synthase
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated protein kinase
γ	Gamma

H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
HFD	High saturated fat diet
HFD+RPO	High saturated fat diet supplemented with RPO
HMG-CoA	3-Hydroxy-3methylglutaryl-coenzyme A
HR	Heart rate
JNK	c-Jun N-terminal kinase
K^+	Potassium ion
LA	Linoleic acid
LDL	Low density lipoprotein
LOX	Lipoxygenase
LPO	Lipid hydroperoxide
LTs	Leukotrienes
LVDevP	Left ventricular developed pressure
LVDP	Left ventricular diastolic pressure
LVSP	Left ventricular systolic pressure
MA	Myristic acid
MAPKs	Mitogen-activated protein kinases
MPOB	Malaysian palm oil board
MUFAs	Monounsaturated fatty acids
Na^+	Sodium ion
NO	Nitric oxide
NOS	Nitric oxide synthase

O_2^-	Superoxide
OA	Oleic acid
$\cdot$OH	Hydroxyl radical
ONOO$^-$	Peroxynitrite
ORD	Oxidative risk induced diet
ORD+RPO	Oxidative risk induced diet supplemented with RPO
oxLDL	Oxidized low density lipoprotein
PA	Palmitic acid
P/S	Polyunsaturated/saturated fatty acid ratio
P38	p38 Mitogen-activated protein kinase
PARP	Poly(ADP-ribose)polymerase
PGIs	Prostacyclines
PGs	Prostaglandins
PKB/Akt	Serine/threonine protein kinase, Protein kinase B or AKT
PKA	cAMP-dependent protein kinase
PPM	Parts per million
PUFAs	Polyunsaturated fatty acids
RBPO	Bleached and deodorized palm oil
RC	Rat chow
ROS	Reactive oxygen species
RPO	Red palm oil
RPP	Rate pressure product
SA	Stearic acid

SF	Saturated fat
SFAs	Saturated fatty acids
SOD	Superoxide dismutase
SRC	Standard rat chow
TC	Total cholesterol
TLC	Thin layer chromatography
TXA	Thromboxanes
TXA_2	Thromboxane A_2
U/S	Unsaturated/saturated ratio
WHO	World Health Organization

DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: ....   Date:....30/.04./.2006..

ACKNOWLEDGEMENTS

I would like to express the vital role which my family and friends have played in supporting me and inspiring me to do my best.

I thank Jesus Christ for placing me on this road and helping me to walk it; surely without Him none of this would have been possible.

ABSTRACT

Research has shown that the activation of the NO-cGMP pathway leads to myocardial protection from oxidative stress conditions, such as ischaemia and reperfusion. Few of these studies have however combined diet induced oxidative stress with ischaemia/reperfusion injury. Although little is known about the effects of supplements such as red palm oil (RPO) on the NO-cGMP pathway, research has shown that dietary RPO-supplementation improved reperfusion aortic output recovery through mechanisms that may include activation of the NO-cGMP- and inhibition of the cAMP pathway. RPO is an antioxidant-rich oil containing β-carotene and Vitamin E (tocopherols and tocotrienols). The aims of this study were to determine: 1) whether RPO-supplementation of an oxidative risk induced diet (ORD) and a high saturated fat diet (HFD) offers protection against ischaemia/reperfusion injury in the isolated perfused rat heart and 2) the possible mechanisms for this protection.

Male Wistar rats were randomly divided into four groups for a period of 14 weeks according to the dietary supplementation they received. The control groups received either an oxidative risk induced diet (ORD) or a high saturated fat diet (HFD), while the experimental groups received an ORD supplemented with RPO (ORD+RPO) or a HFD supplemented with RPO (HFD+RPO). After the diet feeding period the rats were sacrificed and hearts were perfused on a working heart perfusion apparatus. Myocardial functional recovery was measured after a 25-minute global normothermic ischaemic period and hearts were then freeze-

clamped for determination of myocardial phosholipid fatty acid composition, cAMP/cGMP concentrations, total myocardial nitric oxide concentrations, and superoxide dismutase- and nitric oxide synthase activity.

Our data show that dietary RPO-supplementation offers protection of hearts from rats against ischaemia/reperfusion injury in both the ORD and the HFD, as reflected by improved aortic output recovery (83.6 ± 1.8 % *versus* 61.4 ± 3.8 % for ORD groups and 66.2 ± 2.8 % *versus* 50.0 ± 1.6 % for HFD groups; $P<0.05$). This was associated with an increase in cGMP early in ischaemia in both RPO-supplemented groups (195.4 ± 18.3 % *versus* 90.3 ± 12.8 % for the ORD groups and 133.0 ± 12.4 % *versus* 50.1 ± 10.4 % for the HFD groups, $P<0.05$) indicating that the NO-cGMP pathway is possibly involved in the cardioprotection of both groups. Phospholipid fatty acid changes may also play a role in cardioprotection. This was indicated by the fact that RPO-supplemented groups had increased PUFA concentrations associated with better protection against ischaemia/reperfusion injury. Furthermore, antioxidants provided in dietary RPO-supplementation may also protect PUFAs from oxidation and allow for preservation of their beneficial effects.

Based on our results we propose that the myocardial protection offered by RPO-supplementation of rats on an ORD or a HFD may involve 1) the activation of the NO-cGMP pathway and/or, 2) changes in myocardial phospholipid fatty acid composition during ischaemia/reperfusion.

CHAPTER 1

Introduction

A potential risk factor that has been identified with respect to cardiovascular disease includes a high fat intake, especially saturated fats, and cholesterol (Elson and Quereshi, 1995). Diniz and co-workers (2004) showed that changes in dietary fatty acid composition affect cardiac oxidative stress. These authors showed that despite their beneficial effects on serum lipid concentrations, diets rich in polyunsaturated fatty acids (PUFAs) are deleterious to the heart by increasing cardiac susceptibility to lipid peroxidation. It can therefore be concluded that individuals following a diet rich in PUFAs or high in fat may be at risk of developing cardiovascular disease. Refined sugar is also a potential hazard as it may lead to a loss of glucose homeostasis followed by diabetes and ultimately cardiovascular dysfunction (Ely, 1996). An atherogenic Western diet causes an increased rate of atherosclerotic plaque growth and hypertension which leads to cardiovascular disease (reviewed by Simopoulos, 2002; Woods *et al.*, 2004). A Western diet is associated with an increased incidence of insulin resistance, type two diabetes and obesity and will further increase the risk of cardiovascular disease (Ely, 1996). According to a study done by Woods and co-workers (2004) this may be due to the high fat content of the typical Western diet. They concluded that a high fat diet leads to increased weight, increased body fat, hyperinsulinaemia, hyperleptinaemia and insulin resistance, even if the animals did not become obese. They also found that the hypothalamic apolipoprotein A-IV system, which plays a role in controlling the feeling of satiety, is down-regulated by a high fat diet. This may lead to the hyperphagia that is normally associated with a high fat diet. A review article by Greenwood and Winocur (2005) stated that a high fat diet may lead to development of type two diabetes which is

associated with various abnormalities, some of which may be detrimental to cardiovascular function.

The World Health Organization (WHO) recommends that humans obtain their energy from a diet with the following composition: protein; 10 - 15 %, carbohydrates; 55 - 75 %, total fat; 15 - 30 %, saturated fat (SFAs); less than 10 %, polyunsaturated fat (PUFAs); 3 - 7 %, mono-unsaturated fat (MUFAs); more than 10 %, sugar; 0 - 10 % and cholesterol; 0 - 300 mg (Sizer and Whitney, 2000).

It is believed that atherosclerotic plaque formation is linked to increased levels of total serum cholesterol (TC) and low-density lipoprotein (LDL). Specifically oxidized LDL (oxLDL) has been associated with increased atherosclerotic plaque formation (reviewed by Pryor, 2000; Siebert and Kruk, 2004). This damage may be limited if sufficient concentrations of antioxidants (such as vitamin E) are added to the diet. It is therefore clear that antioxidants can play a prominent role in both inhibiting atherosclerotic plaque formation and prevention of subsequent or ensuing cardiovascular disorders (Chen *et al.*, 1999; reviewed by Pryor, 2000).

It is known that necrosis reduces the amount of salvageable myocardium during reperfusion. Calcium overload, mitochondrial dysfunction and defective myocardial lipid metabolism contribute to this injury. Formation of reactive oxygen species (ROS) is one of the primary causes of myocardial injury (Illarion *et al.*, 2002; reviewed by Fishmeister *et al.*, 2005). It has been shown that oxidative stress associated with increased production of superoxide in the presence of cholesterol leads to increased peroxynitrite (ONOO⁻) production and decreased nitric oxide (NO) concentrations which may cause

tissue damage (Onody *et al.*, 2003). An increase in ROS is also associated with tissue damage caused by ischaemia and reperfusion and this is thought to be the major cause of the morbidity that accompanies these conditions (reviewed by Galinanes *et al.*, 2004). Oxygen free radicals such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (^-OH) may react with intracellular nucleic acids, proteins and lipids, resulting in damage to the intracellular organelles and cell membranes (Gilham *et al.*, 1997).

Red Palm Oil (RPO) is an edible oil that is often perceived to increase serum total cholesterol concentrations due to its total fatty acid composition which consists of 51% SFAs, 38% MUFAs and 11% PUFAs (Nagendran *et al.,* 2000; Sundram *et al.,* 2003). However, research has shown that dietary RPO-supplementation does not raise TC or LDL- cholesterol concentrations to the extent expected from its fatty acid composition when a moderate fat, moderate cholesterol diet is consumed and this may be explained by the composition of its SFAs (Elson and Quereshi, 1995; Quereshi *et al.*, 2002). The major saturated fatty acid found in RPO is palmitic acid (PA) (44 % out of approximately 51 %) with only small amounts of stearic acid (SA) (5 %) and myristic acid (MA) (1 %). PA is considered to have much lower cholesterol raising effects than MA (Hayes *et al.*, 1991). It should also be taken into consideration that the glyceride of palm oil contains predominantly oleic acid (OA) at the sn-2 position, with PA and SA at the sn-1 and sn-3 positions. This allows for the ready absorption of the sn-2 monoglycerols, while the saturated free fatty acids remain poorly absorbed. This configuration of the palm oil triacylglycerols allows it to behave like a monounsaturated oil, instead of a saturated oil,

and therefore the perception that RPO is hypercholesterolaemic is debatable (reviewed by Ong and Goh, 2002).

Apart from the fatty acid effects of RPO, the micronutrient components, such as β-carotene, monoterpines and tocotrienols may also have a hypocholesterolaemic effect. Tocotrienols have been shown to suppress 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is a rate limiting enzyme for cholesterol biosynthesis (Khor et al., 1995; Theriault, 1999; Sundram and Basiron, 2004; O'Byrne et al., 2000; Quereshi et al., 2002). However, when used in hypercholesterolaemic subjects with high fat formula diets, palm oil supplementation has been shown to raise TC and LDL cholesterol (Sundram and Basiron, 2004).

RPO contains a potent mixture of antioxidants including tocopherols, tocotrienols and carotenoids (mostly β-carotenoids) (Farombi and Britton, 1999). Serbinova and co-workers (1991) focused their research on the protective effects of the vitamin E anti-oxidants contained in RPO against ischaemia/reperfusion injury, and found that a palm oil vitamin E mixture containing both α-tocopherol and α-tocotrienol improved reperfusion functional recovery in a Langendorff perfused rat heart. They hypothesized that the free radical scavenging action of α-tocopherols and α-tocotrienols during ischaemia and reperfusion induced the protective effect, but that α-tocotrienols offered more efficient protection. Esterhuyse and co-workers (2005 a and b) found that dietary RPO-supplementation in rats fed a standard rat chow (control) and hypercholesterolaemic diet protected against ischaemia/reperfusion injury as reflected by improved aortic output recovery. This was associated with an increase in cGMP early

in ischaemia and a decrease in cAMP during ischaemia in the RPO-supplemented group versus the control group. Their results also demonstrated that the mitogen activated protein kinase- (MAPK), protein kinase B or Akt (PKB/Akt) and caspase activation pathways have been affected by RPO-supplementation and may have influenced myocardial protection during ischaemia/reperfusion (Fujio *et al.*, 2000; Engelbrecht *et al.*, 2006).

NO is a vital signalling molecule that is present in small amounts in the cardiovascular system (reviewed by Bolli, 2001). The functions of NO include a vasodilator-, anti-platelet- anti-neutrophil, antithrombotic and antioxidant effect (Hare and Comerford, 1995; Xie and Wolin, 1996). One of the important signalling pathways associated with NO is the NO-cGMP pathway. Cyclic guanosine monophosphate (cGMP) is a molecule that is known to offer myocardial protection (Rubbo *et al.*, 1994). NO can combine with O_2^-, to form $ONOO^-$. By doing this, NO quenches the potentially deleterious O_2^-, but in the process $ONOO^-$ is formed which will rapidly decompose to other potentially hazardous radicals such as H_2O_2 and peroxynitrous acid (ONOOH) which can undergo homolysis to a molecule with ˙OH like reactivity (Deliconstantinos *et al.*, 1995). Therefore, myocardial NO concentrations need to be regulated with extreme precision and can lead to exacerbated cardiac damage if it is not well regulated.

NO offers cardioprotection against ischaemia/reperfusion injury (Maulik *et al.*, 1995; Williams *et al.*, 1995; Araki *et al.*, 2000; reviewed by Bolli, 2001). It is well known that NO increases the concentrations of myocardial cGMP. It has been suggested that a mechanism secondary to the stimulation of guanylyl cyclase within the vascular wall or

in ventricular myocytes may be responsible for the protective effects of NO from ischaemia/reperfusion injury (Beresewics et al., 1995; Maulik et al., 1995; Deprè et al., 1996). The protective effects of NO may be attributed to the suppression of cyclic adenosine monophosphate (cAMP) induced by both NO and cGMP (reviewed by Fishmeister et al., 2005). cAMP increases heart rate and contractile force by opening calcium ion (Ca^{2+}) channels, which could ultimately lead to ventricular fibrillation. cGMP has the opposite effect, by opening potassium- and sodium channels and closing Ca^{2+} channels. This is achieved by stimulation of soluble guanylate cyclase which brings about reduction of Ca^{2+}, partly through activation of cGMP-dependent protein kinase and termination of chain propagating lipid radical reactions caused by oxidative stress (Rubbo et al., 1994). It has been suggested by Du Toit and co-workers (2001) that the cAMP-to cGMP ratio may play an important role in NO induced cardioprotection.

It is well known that a high fat and high cholesterol diet may induce hyperlipidaemia and increased oxidative stress which develops into pathological disorders such as cancer, cardiovascular disease and neuronal degradation (reviewed by Pryor, 2000). Research has shown that most unsaturated fatty acids may have hypocholesterolaemic effects on the serum lipid profile (Diniz et al., 2004; reviewed by Nettleton and Katz, 2005). However, these same authors have shown that high concentrations of PUFAs in the diet will lead to oxidative stress, associated with various pathological conditions, including cardiovascular disease.

We speculate that dietary induced oxidative stress, associated with increased production of ROS may interfere with NO-cGMP pathway function. However, Esterhuyse and co-workers (2005 a and b) suggested that RPO offers cardioprotection from

ischaemia/reperfusion injury by providing antioxidants that quenches the ROS which is produced during ischaemia and reperfusion. Engelbrecht and co-workers (2006) showed that dietary RPO-supplementation of a standard rat chow diet affects the MAPK by causing increased phosphorylation of p38 and PKB and reduced phosphorylation of JNK which is associated with myocardial protection against apoptosis. Their results also showed a decrease in Poly(ADP-ribose)polymerase (PARP) cleavage with RPO-supplementation which could lead to the inhibition of apoptosis. RPO-supplementation may thus induce protection from ischaemia/reperfusion injury by the inhibition of myocyte apoptosis. These effects on the MAPK may be due to the antioxidants present in RPO, as antioxidants have been shown to interfere with JNK activation of cultured myocytes, subjected to hypoxia/reoxygenation (Landeroute and Webster, 1997).

Research done by Esterhuyse and co-workers (2005 a and b) used a standard rat chow- and a hypercholesterolaemic diet. These studies do not provide us with information regarding the effect of RPO-supplementation of diets rich in saturated- or polyunsaturated fatty acids. The diets were also not isocaloric, and dietary RPO intake could only be viewed as a daily supplement to the diet.

However, in this study isocaloric diets including an oxidative risk induced diet (rich in PUFAs) and a high saturated fat diet (rich in SFAs) were developed by our research group as control groups and supplemented with RPO to serve as experimental groups.

1.1 Aims of the study

To our knowledge little is known about the effects of RPO-supplementation in an oxidative risk induced diet or a high saturated fat diet. The aims of this study were to determine whether: 1) RPO-supplementation of an oxidative risk induced diet and a high saturated fat diet offers protection against ischaemia/reperfusion injury in the isolated perfused rat heart; 2) the NO-cGMP pathway can be considered as a possible mechanism of protection; and 3) changes in the myocardial total phospholipid fatty acid composition during ischaemia contribute to this protection.

CHAPTER 2

Literature review

2.1 Fats and oils in modern diets

Dietary oils have been used as a source of fat intake for many years, and are used in many countries as a major source of energy and essential fatty acid in lower socio-economic communities (reviewed by Cottrell, 1991). The need to consume a large enough dietary fat or oil portion is recognized by the WHO who recommends a human consumption of 20 - 25 kg of oils and fats per capita per year (reviewed by Ong and Goh, 2002).

The level of saturation of a fat or oil is often used as a measure to determine whether it will be detrimental to cardiovascular health or not. Saturated fats and oils (consisting of 50% or more saturated fatty acids) are considered more detrimental to cardiovascular health than unsaturated fats and oils (Charnock *et al.*, 1991). However, many oils rich in SFAs that are regularly consumed also consist of micronutrient components such as vitamins and other trace elements which are beneficial to health. Table 2.1 summarizes the level of saturation of commonly used fats and oils.

There are many edible oils available for human consumption, but 60 - 70 % of the world's oil production is made up of soybean, palm oil, sunflower seed oil and rapeseed oil (reviewed by Ong and Goh, 2002).

Research has confirmed that red palm oil (RPO) is cholesterol-free, *trans*-free and not genetically modified. It contains beneficial micronutrient compounds, such as α-

carotene, β-carotene, vitamin E (tocopherol and tocotrienol), lycopene and other carotonoids (Goh *et al.*, 1985, Sundram *et al.*, 2003). Although RPO is classified nutritionally as saturated oils, it can be considered highly structured, usually having the sn-2 positional fatty acids unsaturated and the 1,3-fatty acids saturated on the triacylglycerol. This distribution is unique to plant oils and confers a decreased impact on serum cholesterol (Elson and Qureshi, 1995).

Many unsaturated oils are subjected to hydrogenation in order to solidify them without the addition of SFAs. This process however results in high concentrations of *trans* fatty acids which have been shown to increase low density lipoprotein (LDL), whilst decreasing the high density lipoprotein (HDL) or at least increasing the LDL/HDL ratio (Van Tol *et al.*, 1995; Judd *et al.*, 1996; reviewed by Katan, 2000). By doing so, it is not only increasing the oxidative risk, but also removing possible protection offered by HDL. Many epidemiologic studies have shown that *trans* fatty acids associated with oxidative stress lead to an increased risk of developing cardiovascular disease (Van Tol *et al.*, 1995; Judd *et al.*, 1996; reviewed by Katan, 2000).

A review article by Simopoulos (2002) stated that *trans* fatty acids are detrimental for cardiovascular health, as it interferes with desaturation and elongation. These metabolic processes take place within the body in order to form fatty acids that are needed. Furthermore, research has also confirmed that total serum cholesterol is raised by *trans* fatty acids (Van Tol *et al.*, 1995; Simopoulos, 2002). These authors also found that despite the fact that *trans* fatty acids are formed naturally in the gut of some animals (eg. sheep and cows), an increase in *trans* fatty acids leads to increased cholesterylester transfer protein (CTEP) activity associated with increased LDL and decreased HDL

concentrations. It is however important to note that high concentrations of *trans* fatty acids are required in order for them to have a detrimental effect (Van de Vijver *et al.*, 1996).

Table 2.1. Fatty acid content of various edable fats and oils.

Fat or Oil	SFA	MUFA	PUFA	P/S ratio	U/S ratio	P2/S2 ratio	U2/S2 ratio
Rapeseed	5,0	71,0	24,0	4,8	19,0	95,0	166,0
Canola	7,0	61,0	32,0	4,67	13,3	155,0	330,0
Sunflower	11,7	18,0	68,6	5,9	7,4	-	-
Olive	13,0	79,1	7,9	0,6	6,7	10,6	73,0
Corn	13,3	28,4	58,3	4,4	6,5	28,0	39,0
Soybean	16,0	23,5	60,5	3,8	5,3	64,0	85,0
Groundnut	20,0	38,7	41,3	2,1	4,0	14,8	38,0
Cottonseed	27,7	19,8	52,5	1,9	2,6	6,32	8,8
Lard	43,0	47,0	10,0	0,2	1,3	0,04	0,2
Palm olein	46,8	41,5	12,0	0,3	1,1	6,9	22,0
Palm oil	49,5	40,3	9,6	0,2	1,0	1,7	6,7
Red palm oil	50.8	38.3	10.9	0.2	1.0	-	-
Cocoa butter	60,0	36,5	3,4	0,2	0,7	2,3	24,0
Butter	63,4	32,5	4,5	0,1	0,6	0,03	0,2
Hydrogenated soybean [a]	64,0+ *trans*	26,0	4,0	0,1	0,5	-	-
Palm kernel	84,0	14,0	2,0	0,02	0,2	-	-
Coconut	92,2	6,2	1,6	0,02	0,1	0,02	0,05

P/S and U/S: polyunsaturated /saturated and monounsaturated+ polyunsaturated/ saturated fatty acid ratios, respectively. P2, S2, U2: polyunsaturated, saturated and total unsaturated fatty acids, respectively, at position sn-2 of the triacylglycerol molecule

[a] Typical sample, saturated 22%, trans fatty acids 42%.

(reproduced from Ong and Goh, 2002.)

2.2 Serum lipids

Fatty acids are transported in the circulation as macromolecules (referred to as serum lipids) and can be divided into different classes according to their size, density, content

and functions. The most important lipids are HDL, LDL, and triacylglycerol. The parameters measured with regard to serum lipids include total cholesterol, triacylglycerols and HDL, while serum LDL content can be calculated.

Serum lipids play an important role in the etiology of cardiovascular disease and increased total cholesterol and LDL concentrations with decreased HDL concentrations are considered to be major risk factors for development of cardiovascular disease (Van Tol et al., 1995; Hu et al., 2000; Quereshi et al., 2002). The presence of oxLDL is a major risk factor for cardiovascular disease. Antioxidant enzymes contained in HDL have been proposed to play a protective role by decreasing the occurrence of LDL oxidation (Kontush et al., 2005). These authors showed that decreased HDL is associated with an increased risk of coronary heart disease and premature atherosclerosis. Intracellular oxLDL may be associated with direct myocardial damage when deposited in the myocardium which leads to expression of the oxLDL receptor, followed by apoptosis and cardiac dysfunction (Onody et al., 2003). Therefore, oxidized LDL may be seen as the major risk factor for atherosclerosis development (reviewed by Pryor, 2000; Siebert and Kruk, 2004).

High serum cholesterol concentrations are considered a risk factor for cardiovascular disease. Research has shown that most unsaturated fatty acids will have cholesterol lowering effects, while SFAs will have cholesterol raising effects (Diniz et al., 2004). However, high dietary concentrations of PUFAs will lead to oxidative stress associated with cardiovascular disease (reviewed by Nettleton and Katz, 2005).

Decreased HDL-cholesterol associated with increased serum triacylglycerol concentrations is another important form of dislipidaemia. Abeywardena (2003) found among Sri Lankans that a diet containing low concentrations of fat (although rich in SFAs) and high concentrations of carbohydrates, increased oxidative stress. These researchers also found that high concentrations of SFAs led to development of insulin resistance and hypertension (due to increased angiotensin production) and a decrease in nitric oxide (NO) production. Another study found that decreased HDL, even in the presence of normal serum triacylglycerol concentrations led to increased oxidative stress (Kontush et al., 2005) These authors attributed the protective effects of HDL to both the cholesterol lowering effect of HDL and the fact that HDL transports antioxidative enzymes which protects LDL from oxidation. Increased concentrations of triacylglycerols in non-adipocyte somatic cells are associated with apoptosis (Aronis et al., 2005).

Atherosclerosis is directly related to a loss of regulation of serum lipid concentrations (O'Byrne et al., 2000) and is closely linked to oxidation of LDL (Kontush et al., 2005; reviewed by Pryor, 2000; Siebert and Kruk, 2004). Atherosclerosis is considered to be normal if the rate of plaque formation is not abnormally high. However, if the rate of atherosclerotic plaque formation is markedly increased, it may lead to further cardiovascular pathology such as ischaemic heart disease or acute myocardial infarction. It has been shown that atherosclerosis has a negative effect on cardiac function (Tokuno et al., 2001).

2.3 Fatty acids

Fatty acids are long chain carbon based biomolecules that can be metabolized to yield large amounts of energy. Various fatty acids exist and they are classified into different

groups according to their level of saturation and the position of the double bonds within the molecule. Fatty acids provide cell structure by forming an integral part of cell membranes and serve as an efficient source of energy in the body. They are used to produce hormones and other signalling molecules such as eicosanoids. Membrane-associated functions e.g. receptors, ion channels and membrane bound enzymes are regulated by membrane lipid components such as cholesterol and fatty acids within the cell membrane (Clandinin et al., 1991).

There are three major types of fatty acids, namely saturated fatty acids, mono-unsaturated fatty acids and polyunsaturated fatty acids. Polyunsaturated fatty acids (PUFAs) found within mammalian tissues are further subdivided into three families (n-3, n-6 and n-9 PUFAs), depending on where the double bond lies within the molecule. These different classes of fatty acids can be converted to other forms within the body through the action of converting enzymes such as desaturases and elongases as illustrated by Figure 2.1 (Pereira et al., 2003). The n-6 fatty acids cannot be converted to n-3 fatty acids in mammals due to lack of n-3-desaturases (reviewed by Simopoulos, 2002). The differences in length and levels of saturation of fatty acids affect cardiovascular health (Nair et al., 1997).

It has been shown that the phospholipid fatty acid content of the cardiomyocyte cell membrane is dependent on dietary fatty acid supplementation (reviewed by Simopoulos, 2002) and phospholipid fatty acids play an active role in signal transduction and other biochemical reactions of the cell.
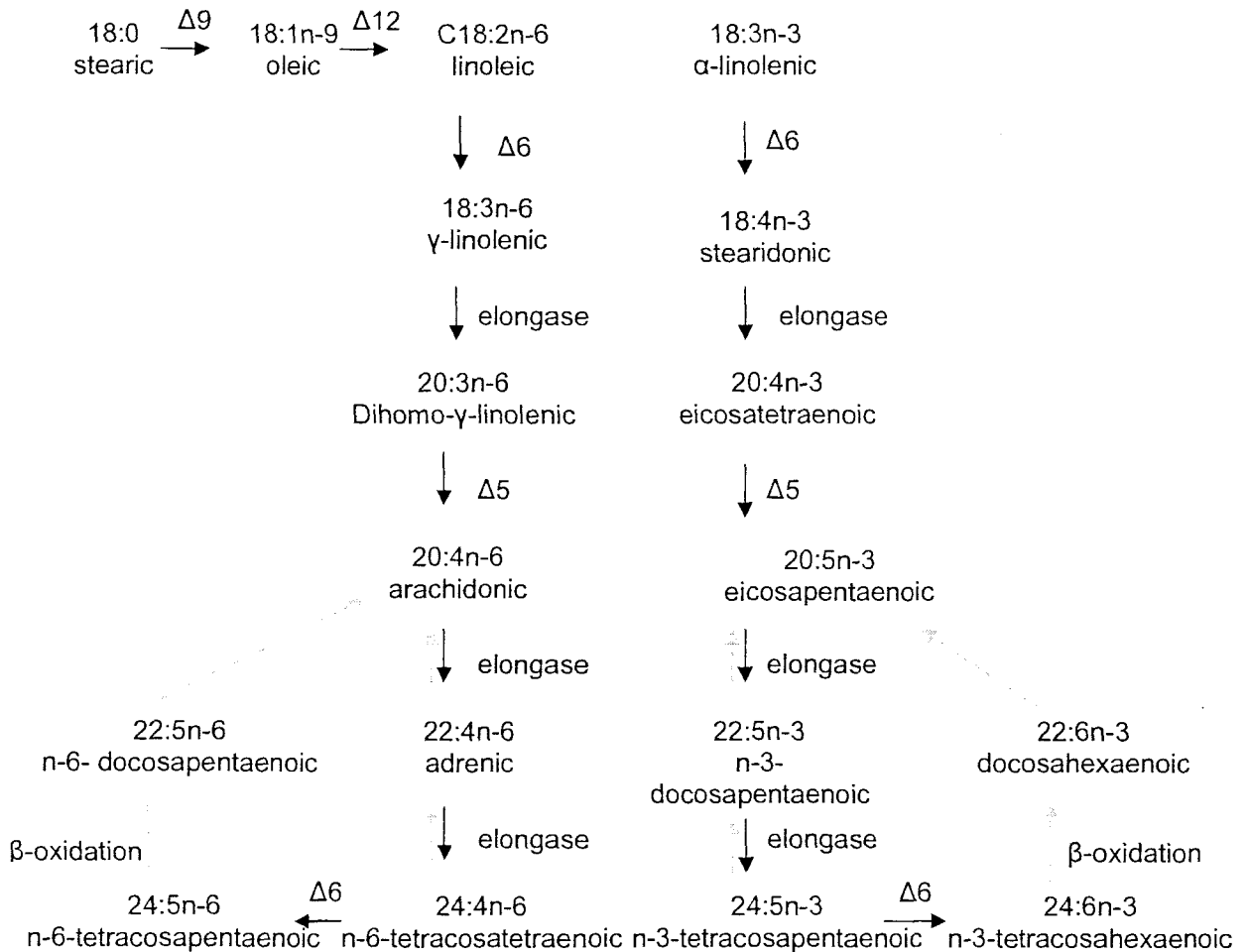
Figure 2.1. Fatty acid biosynthesis by the action of elongases and desaturases (Reproduced and simplified from Pereira et al., 2003.)

2.3.1 Saturated- and polyunsaturated fatty acids and cardiovascular health

Many studies have investigated the effects of dietary supplementation with different fatty acid components. Results have shown that specific fatty acids could make a significant difference to the health state of the animal or individual when incorporated in their diet. We therefore need a balanced dietary fatty acid intake in order to achieve optimum health (reviewed by Simopoulos, 2002).

Different types of fatty acids seem to have functions within the body that are complimentary, interdependent or even antagonistic. It is unclear how these fatty acids interact, but many studies have shown that imbalances in certain fatty acid ratios lead to disease states (reviewed by Simopoulos, 2002).

The SFA:PUFA ratio of the blood or tissue plays an important role in cardiovascular health. In the past it was thought that PUFAs were more beneficial to health than SFAs as reflected by effects on the serum lipid profile (Diniz et al., 2004). Recent studies have shown that increased PUFAs in the membranes of cardiomyocytes lead to increased lipid peroxidation and therefore increased susceptibility to cardiac injury (Diniz et al., 2004; O'Farrel and Jackson, 1997). A protective role for cholesterol molecules has been shown in the membrane, as it protects PUFAs from being oxidized (Tirosh et al., 1997). Therefore, changes in dietary fatty acids affect cardiac oxidative stress and research has shown that diets rich in PUFAs, despite beneficial effects on serum lipids, were deleterious when compared with SFAs in the heart by measuring cardiac susceptibility to lipid peroxidation. Furthermore, it is important to note that decreased SFAs, along with increased exercise, are associated with a decreased risk of developing type two diabetes, which would in turn lead to an increased risk of developing cardiovascular disease (reviewed by Nettleton and Katz, 2005). Abeywardena and Charnock (1995) showed that SFAs and MUFAs are pro-arrhythmic, while PUFAs are anti-arrhythmic.

Research has shown that a balanced n-6- and n-3 PUFA diet leads to improvement of certain pathological conditions and often to total restoration of normal function in previously dysfunctional systems (reviewed by Simopoulos, 2002). Some disorders that were investigated include cancer, cardiovascular disease and auto-immune

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inflammatory diseases. These authors suggested that a n-6 to n-3 PUFA ratio of 1:1 would be ideal for normal functioning in humans. Effects of n-6 and n-3 PUFAs are linked to the regulation and production of prostaglandins and prostacyclins. O'Farrel and Jackson (1997) linked improved health associated with a decreased n-6- to n-3 PUFA ratio, to increased prostacyclin production and lower thromboxane (TXA) concentrations. Therefore, eicosanoid products of the two groups of PUFAs have opposing effects. It is thought that the products of n-3 PUFAs are more beneficial than those of n-6 PUFAs. This may be due to the fact that n-6 PUFAs are consumed in high concentrations in Western diets, which leads to increased blood viscosity, vasospasm, vasoconstriction, decreased bleeding time, a pro-thrombotic and pro-agregatory effect (reviewed by Simopoulos, 2002). However, overproduction of any of these products will lead to a loss of homeostasis and disease. Research has shown that n-3 PUFAs obtained from marine diets will lead to a decreased risk of cardiovascular disease (McLennan *et al.*, 1996; reviewed by Abeywardena and Head, 2001). It should however be kept in mind that many studies of this kind were done using individuals that were following a Western diet associated with a n-6- to n-3 PUFA ratio of 15:1 to 16.7:1 (reviewed by Simopoulos, 2002). Interestingly, n-6 PUFAs increase Ca^{2+} uptake into the cell while n-3 PUFAs decrease the Ca^{2+} uptake. In this way n-3 PUFAs may protect the cell directly by preventing Ca^{2+} overload, which is associated with ventricular fibrillation in the heart (reviewed by Pepe, 2005). Das (2000) proposed that protection offered by n-3 fatty acids may be associated with regulation of sodium ion (Na^+) channels, inhibition of tumour necrosis factor-α, interleukin 1 and interleukin 2 production, while stimulating the release of acetylcholine.

Eicosanoid production from PUFAs plays a major role in the maintenance of cardiovascular health. The n-6 and n-3 PUFAs are the major sources of eicosanoids, with LA and AA being the major n-6 fatty acids used for eicosanoid production and EPA and DHA for the n-3 PUFAs (Figure 2.2). The eicosanoids produced from the n-3 sources are thought to be more favourable than those of the n-6 PUFAs, although research has shown that arachidonic acid (AA) is the preferred substrate for eicosanoid production by cyclooxygenases (COX) and lipoxygenases (LOX). Dietary supplementation with n-3 PUFAs leads to the inhibition of platelet TXA and thus induces an anti-thrombotic action. EPA and DHA are associated with an increased production of PGI_2 and PGI_3 (reviewed by Abeywardena and Head, 2001). High concentrations of dietary n-3 PUFA supplementation lead to decreased incorporation of n-6 PUFAs into cell membranes. This replacement of n-6 PUFAs is associated with a decline in AA with respect to ALA, and is thought to be due to a suppressive effect of n-3 PUFAs on Δ6-desaturase (O'Farrel and Jackson, 1997). Abeywardena and Head (2001) showed that NO decreased COX activity due to a release of metabolites. Urquhart and co-workers (2001) showed improved health by the replacement of n-6 PUFAs, and more specifically AA, with n-3 PUFAs. They concluded that eicosanoid production from AA leads to inflammatory disease states.
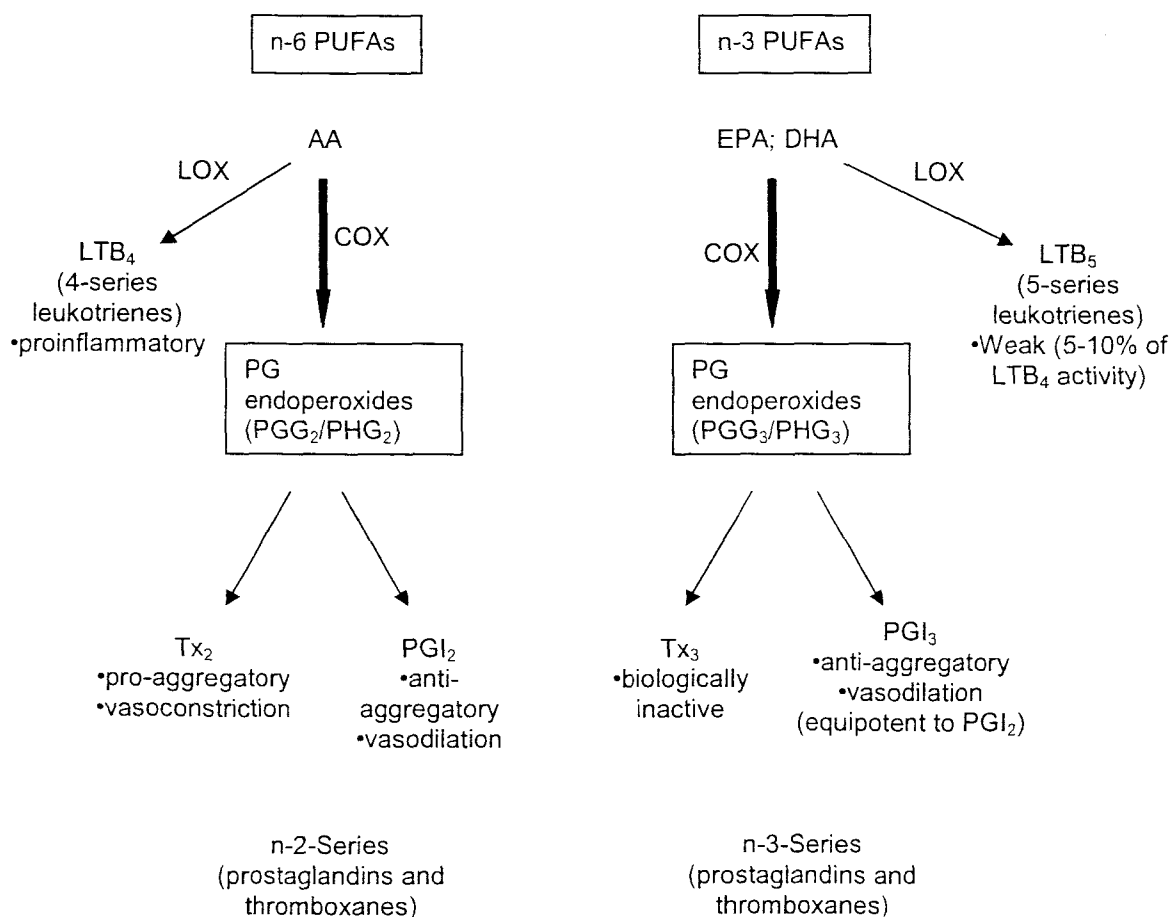
Figure 2.2. Major eicosanoid metabolites produced from n-3 and n-6 PUFAs, and their major functions (Reproduced from Abeywardena and Head, 2001.)

COX=cyclooxygenase, LOX=lipoxygenase, LT=leukotrienes, PG=prostaglandin, TX=thromboxane

Studies performed on the protective effects of dietary fish oil supplementation have shown that both docosahexaenoic acid (DHA) and EPA offer cardiovascular protection. The mechanisms of protection induced by DHA include inhibition of calcium influx into the cell, retardation of hypertension and inhibition of TXA production (which leads to improved regulation of contraction). EPA is also associated with retardation in hypertension, but to a lesser extent than that of DHA. It has also been shown that DHA is better incorporated into the membrane than EPA. It is therefore concluded that both EPA and DHA present in fish oil offers protection, but DHA proved to be the more

effective product (McLennan *et al.*, 1996). Wilkinson and co-workers (2005) showed that fish oil supplementation offered more effective protection than α-linoleic acid (ALA). This may be due to increased DHA incorporation into cell membranes associated with fish oil supplementation. O'Farrel and Jackson (1997) speculated that due to the high rate of oxidation of n-3 PUFAs, they may act as oxidative buffers for other biomolecules by reacting with the bulk of the radicals in the cell.

2.3.2 Effects of a high fat diet

A study done by Woods and co-workers (2004) concluded that a high fat diet leads to increased weight and body fat, hyperinsulinaemia, hyperleptinaemia and insulin resistance, even if the animals did not become obese. They also found that the hypothalamic apolipoprotein A-IV system, which plays a role in controlling the feeling of satiety, is down-regulated by a high fat diet. This may lead to the hyperphagia that is normally associated with a high fat diet (Woods *et al.*, 2004). High fat diets lead to lower levels of satiety when compared with high protein or high carbohydrate diets and therefore, increase meal frequency (reviewed by Westerterp-Plantenga, 2004). Potential risk factors that have been identified with respect to cardiovascular disease include a high fat intake, especially saturated fats and cholesterol. High concentrations of lard in the diet lead to increased LDL concentrations, and therefore increase the risk of cardiovascular disease (Elson and Qureshi, 1995).

It is well known that a high fat diet may induce hyperlipidaemia and increased oxidative stress which leads to increased DNA damage. The oxidation of lipids in the body does not take place at random, but appears to target certain areas and to bring about specific disorders, namely cancer, cardiovascular disease and neuronal degradation (reviewed

by Pryor, 2000). This damage may be limited if sufficient concentrations of antioxidants (such as vitamin E) are supplemented along with the high fat diet (Chen *et al.*, 1999). High fat diets, accompanied by a high sugar intake, are also associated with increased rates of atherosclerotic plaque formation. This is due to the antagonising effect of fat on platelet aggregation and the sugar's impairment of phagocytic removal of thrombi. Other pathological conditions (e.g. organ damage) that may result from high concentrations of sugar in the diet include accelerated aging, birth defects, cancer, diabetes, infectious diseases, vascular disease, neurological and psychiatric disorders (Ely, 1996).

It is important to note that a diet low in fat but high in carbohydrates will lead to increased insulin resistance and increased risk of developing cardiovascular disease (reviewed by Simopoulos, 2002).

2.3.3 Essential fatty acids and their physiological role

Essential fatty acids (EFA) are defined as fatty acids that perform a necessary function within the body but cannot be produced in sufficient quantities to fulfill these needs. These fatty acids are needed for various physiological processes such as growth and maintenance of health (Horribon, 1990). It is therefore of utmost importance that these fatty acids are included in the diet in sufficient amounts.

The two main essential fatty acids are Linoleic Acid (C18:2n-6; LA) and α-Linolenic acid (C18:3n-3). Desaturation and elongation of these fatty acids results in the production of other fatty acids such as AA, EPA and DHA (Nair *et al.*, 1997). The fatty acids formed from these EFAs play a vital role in the maintenance of homeostasis in various body systems, especially in the cardiovascular system. The protaglandins and prostacyclins

formed from AA, EPA and DHA regulate coagulation, inflammation, chemotaxis and vasodilation (O'Farrel and Jackson, 1997). The effects of the products play a unique role and therefore a balanced composition of these fatty acids is needed within the body. To achieve this, a balanced amount of LA and ALA needs to be included in the diet, as they compete for binding sites on desaturases and elongases.

2.4 The oxidative risk induced- and high saturated fat diets

Diets with a high fat content are known to lead to cardiovascular disorders (Chen et al., 1999). High dietary intake of either SFAs or PUFAs lead to increased oxidative stress in cardiac tissue due to increased production of ROS, which is stimulated much more by dietary PUFA-supplementation when compared with SFA-supplementation (Diniz et al., 2004). These authors have shown that even though PUFAs will have beneficial effects on serum lipids when compared to SFAs, they were far more deleterious in the heart by providing cardiac susceptibility to lipid peroxidation.

Nettleton and Katz (2005), defined oxidative stress as the abundance of free radicals or highly reactive oxygen species that can result from lipid peroxidation. These authors linked oxidized lipids to increased risk of developing cardiovascular disease, and expressed concern that increased consumption of PUFAs may increase oxidative stress due to their high level of unsaturation.

Pryor (2000) stated in a review article that oxidation within the body does not occur at random, but rather that the oxidation of specific bio-molecules will lead to chronic, life shortening disorders, including heart disease. These authors also stated that vitamin E

was able to retard the oxidation of lipids, and therefore reduce oxidative stress (reviewed by Pryor, 2000).

Chen and co-workers (1999) endeavored to show that dietary PUFA- and vitamin E supplementation was effective in altering oxidative stress. Although these authors were unable to show that oxidative stress has been attenuated by altering the Vitamin E:PUFA ratio, they demonstrated that DNA oxidative damage was modified. A shortcoming of this study was that it was conducted over a short period of 20 days only.

In our study the efficacy of an antioxidant rich RPO-supplement was evaluated when used as a supplement to an oxidative risk induced diet (ORD). This name was derived from the fatty acid content of the diet, which was rich in PUFAs, and poor in MUFAs and SFAs. In contrast with this diet, we also designed a high saturated fat diet (HFD) which was rich in SFAs, and poor in MUFAs and PUFAs. Both of these diets, as well as RPO-supplemented groups were designed to be isocaloric. The effect of RPO-supplementation on these diets was determined to evaluate whether RPO is effective in offering myocardial protection from ischaemia/reperfusion injury, irrespective of the fat content of the diet.

2.5 Cholesterol supplemented diets

A high-cholesterol diet is regarded as an important factor in the development of cardiovascular disease since it leads to development of hyperlipidaemia, atherosclerosis and ischaemic heart disease (Puskas *et al.*, 2004; Onody *et al.*, 2003).

2.5.1 Introduction to cholesterol supplemented diets

There are many factors to be considered when a cholesterol supplemented diet is being formulated. When designing such a diet it must be kept in mind that cholesterol metabolism varies greatly between species. Rats for example have low serum LDL concentrations and show little change in their serum LDL concentrations even with high levels of dietary cholesterol supplementation (Diniz et al., 2004). This is also due to the lack of cholesterylester transfer protein (CTEP) activity in the plasma of the rat. The lack of CTEP leads to decreased LDL concentrations and increased HDL concentrations and resistance to atherosclerosis (Van Tol et al., 1995). Esterhuyse and co-workers (2005 b) have shown that a cholesterol supplemented diet significantly affected aortic output recovery after ischaemia and reperfusion with no change in serum lipid concentrations. Therefore, we could assume that regardless of the serum lipid concentrations, high levels of dietary cholesterol supplementation will alter physiological function. Onody and co-workers (2003) also suggested that the amount of cholesterol that is incorporated in myocardial membranes may give rise to higher $ONOO^-$ concentrations which is associated with tissue damage.

2.5.2 Cardiovascular effects of cholesterol supplemented diets

The consumption of a diet supplemented with cholesterol shows detrimental effects on cardiovascular function after ischaemia/reperfusion injury. Hypercholesterolaemia has been reported to be associated with an increased rate of atherosclerotic plaque formation (O'Byrne et al., 2000).

A possible reason for the poor aortic output recovery of hearts of animals fed a cholesterol supplemented diet after global ischaemia has been proposed by Onody and

co-workers (2003). Their work showed increased superoxide (O_2^-) production with no increase in SOD activity in rats fed a cholesterol supplemented diet. This leads to the formation of peroxynitrite which is an unstable and damaging radical. In the process of peroxynitrite formation, NO is depleted, as it reacts with superoxide to form peroxynitrite. These authors suggest that decreased NO associated with hyperlipidaemia leads to atherosclerosis and showed that the increased ONOO⁻ leads to increased left ventricular end diastolic pressure. This leads to decreased cardiac output caused by a decrease in stroke volume. The depletion of NO also leads to loss of cardioprotection and induced cGMP production (Ferdinandy *et al.,* 1997; Szekeres *et al.,* 1997). Other proposed effects of a cholesterol supplemented diet include inhibition of the mevalonate pathway (Ferdinandy *et al.,* 1998; Puskas *et al.,* 2004), inhibition of heat shock response (Csont *et al.,* 2002), and expression of oxidized low-density lipoprotein receptors which induce apoptosis (Chen *et al.,* 2002). Recent studies also identified changes in gene activity in atherosclerotic plaques in human and animal blood vessels and rat hearts (Puskas *et al.,* 2004).

The decrease in NO release from the endothelium, which is associated with hyperlipidaemia, has a negative effect on the reactivity of the vasculature (reviewed by Galinanes *et al.,* 2004). These authors also suggest that the increase in SOD activity which can be found in hyperlipidaemic rabbits, is linked to an increase in oxidative stress. This increase in oxidative stress is suggested to be caused by increased free radical production by the enzyme xanthine oxidase which is speculated to be absent in many species, including the rat and human. This finding has been confirmed by Onody and co-workers (2003), who suggested that NADPH oxidase acts as a free radical producer.

Cholesterol induced hyperlipidaemia is associated with interference of myocardial contraction by increasing left ventricular end diastolic pressure and a change in the structure of myocytes. This is also linked to an increase in mitochondrial damage in myocytes and therefore a decrease in energy production which leads to functional deterioration (Puskas *et al.*, 2004). These authors also found that NO and cGMP metabolism were decreased in cholesterol induced hyperlipidaemia and can be associated with an increase in LDL oxidation and ONOO⁻ production, which both lead to increased risk of developing cardiovascular disease.

High concentrations of cholesterol in the diet can also be linked to diseases such as atherosclerosis, diabetes, ischaemic heart disease and decreased adaptability to oxidative stress (Onody *et al.*, 2003). This may largely be attributed to a high level of LDL-cholesterol and a low level of HDL-cholesterol, which is associated with an increase in oxidative stress and is known to be proinflammatory (Yücel *et al.*, 2005). It has been found that hypercholesterolaemia results in larger myocardial infarcts in rabbits subjected to ischaemia after they have been fed a short term cholesterol diet (reviewed by Galinanes *et al.*, 2004).

2.6 Red palm oil

2.6.1 Introduction

Red palm oil (RPO) is an edible oil that is produced from the fruit of the *Elaeis guineensis* tree (Hariharan *et al.*, 1996; Nagendran *et al.,* 2000; Sundram *et al.,* 2003). It is a solid at room temperature and is red/orange in colour. Crude palm oil is produced by

removing the core from the fruit and RPO is produced from crude palm oil by a process of raffination (Nagendran et al., 2000).

Most of the RPO production in the world occurs in Malaysia. The oil palm tree originated from West-Africa and was exported to Malaysia where it is now known as the most prolific oil bearing crop in the world. An oil palm tree bears 10-12 fruit bunches annually, each weighing between 20-30 kg and continues to bear fruit for 20-30 years.

Palm oil has a long history of food use dating back over 5 000 years and is now one of the 16 edible oils possessing an FAO/WHO Food standard under the Codex Alimentarius Commission Programme, which comprises 122 member countries (Codex Alimentarius, 1983).

2.6.2 Composition

RPO is a semi-solid oil that consists of 11% PUFAs, 38% MUFAs and 51% SFAs and a range of micronutrient constituents, such as carotonoids and vitamin E (tocotrienols and tocopherols) (Ooi et al., 1996; Nagendran et al., 2000; Sundram et al., 2003). The vitamin E content of RPO is unique because very few natural sources of vitamin E contain such a large percentage of tocotrienols in comparison with tocopherols (Nagendran et al., 2000; Sundram et al., 2003). The major SFAs contained in RPO are palmitic acid (44 %) and some stearic acid (5 %). The MUFAs are mostly made up of oleic acid (39 %), with linoleic acid (10 %) and α-linolenic acid contributing the most of the PUFA content (reviewed by Ong and Goh, 2002). RPO contains high concentrations of carotonoids, with 80 to 90 % of these carotonoids being α- or β-carotenes in a ratio of 2:1, respectively (Farombi and Britton, 1999).

The fatty acid composition of RPO could be considered hypocholesterolaemic, as both palmitic acid and stearic acid do not alter serum cholesterol significantly, while oleic and linoleic acid decrease serum cholesterol concentrations. Therefore, dietary palm oil in balanced diets (when a moderate-fat, moderate-cholesterol diet is consumed) generally reduces blood cholesterol and triacylglycerol, while raising the HDL-cholesterol (reviewed by Ong and Goh, 2002).

2.6.3 Cardiovascular protection offered by red palm oil

Little research has been done with RPO to investigate its cardioprotective role. Esterhuyse and co-workers (2005 a) were able to demonstrate that dietary RPO-supplementation protected the isolated perfused rat heart against ischaemia/reperfusion injury. In another study, these authors showed that dietary RPO-supplementation offered protection even when cholesterol was added to the diet (Esterhuyse et al., 2005 b). They concluded that this protection was offered via more than one pathway. Proposed mechanisms of protection include the NO-cGMP pathway, antioxidant action of RPO as well as pathways involving the MAPK's and caspase signalling. It is well known that the production of ROS during ischaemia and reperfusion is one of the major causes of myocardial injury. ROS are also formed during normal physiological functions and other pathological conditions, and are capable of altering virtually all classes of biomolecules (McCall and Frei, 1999). It is for this reason that the antioxidant properties of RPO are thought to offer protection during ischaemia and reperfusion.

Research has shown that vitamin E fractionated from RPO offers cardio protection from ischaemia/reperfusion injury (Serbinova and Packer, 1994; Mutalib et al., 2003).

Serbinova and co-workers (1991) showed that a palm oil vitamin E mixture containing both α-tocopherol and α-tocotrienol improved reperfusion functional recovery in the Langendorff perfused rat heart.

Engelbrecht and co-workers (2006) showed in a novel study that RPO-supplementation affected the MAPK and PKB signal transduction and increased phosphorylation of p38 and PKB and reduced phosphorylation of JNK with RPO-supplementation and also a decrease in Poly(ADP-ribose)polymerase (PARP) cleavage. These effects on the MAPK may be due to the antioxidants present in RPO which have been shown to interfere with JNK activation of cultured myocytes, subjected to hypoxia/reoxygenation (Landeroute and Webster, 1997).

Dietary RPO-supplementation does not increase serum cholesterol unless used with high concentrations of lard (Elson and Quereshi, 1995). High concentrations of tocotrienol in RPO inhibit the cholesterol producing enzyme HMG-CoA indirectly. This is achieved by converting farnesyl (an intermediate product in formation of cholesterol) to farnesol, which down-regulates the activity of HMG-CoA (Khor *et al.*, 1995; Theriault, 1999; Sundram and Basiron, 2004; O'Byrne *et al.*, 2000; Quereshi *et al.*, 2002).

2.7 Protection offered by red palm oil components

There are various micronutrients contained within RPO that offers protection not only against ischaemia/reperfusion injury, but also against other diseases such as breast cancer. Some of these micronutrients are carotonoids, vitamin E and ubiquinones (Sundram and Basiron, 2004; Bagchi and Puri, 1998).

2.7.1 Protection offered by carotonoids

Carotonoids are a group of pigments ranging from red to yellow in color which are found in red, yellow and orange vegetables. They are effective antioxidants, especially β-carotene which is one of the most effective quenchers of singlet oxygen (Farombi and Britton, 1999). The carotenes can thus prevent formation of ROS and prevent tissue damage that is associated with free radical formation (Bagchi and Puri, 1998). It has been shown that β-carotene protects LDL-cholesterol against oxidation (Hariharan et al., 1996; Siebert and Kruk, 2004). Although α-carotene's antioxidant properties have not yet been fully elucidated, it is thought that it may be more effective than β-carotene in quenching peroxyl radicals (Farombi and Britton, 1999).

RPO is one of the best sources of carotenes, containing 15 times more carotenes than carrots and up to 50 times more than tomatoes (Van Stuijvenberg et al., 2005) (Table 2.2).

Table 2.2 β-carotene content of RPO relative to other vegetable sources

Food	Portion size (g)	β-carotene (µg)	β-carotene content relative to (100g) carrots
Red Palm Oil	100	30000	2.1
Red Palm Oil	20	6000	0.4
Carrots	100	14380	1
Spinach	100	2040	0.1
Butternut	100	1615	0.1
Papaya	100	606	0.04
Tomatoes	100	225	0.02

(Reproduced from Van Stuijvenberg et al., 2005.)

2.7.2 Protection offered by vitamin E

Vitamin E is a highly lipophillic group of eight compounds that can be subdivided into four tocopherols and four tocotrienols. These compounds are thought to be the major lipid-soluble chain-breaking antioxidant found in blood plasma. Oxidation of PUFAs within the cell membrane leads to loss of membrane structure and function, but vitamin E intercalates with these PUFAs to prevent oxidation (O'Farrel and Jackson, 1997). Vitamin E is highly effective in protecting PUFAs from auto-oxidation and it has been suggested that this may be the major biological function of vitamin E (Burton and Ingold, 1981; Bagchi and Puri, 1998; Theriault *et al.*, 1999). Pryor (2000) argued that vitamin E is the best lipid soluble antioxidant in humans and contributes some of the efficacy of vitamin E (as antioxidant) to its ability to be recycled by vitamin C. α-Tocopherol is thought to be the more active form of vitamin E, however it has been suggested that tocotrienol is a better antioxidant than tocopherol (Serbinova *et al.*, 1991; Suzuki *et al.*, 1993; Mutalib *et al.*, 2003). Vitamin E compounds are effective singlet oxygen quenchers (Kamal-Eldin and Appelqvist, 1996). A study by McCall and Frei (1999) showed that a decrease in dietary vitamin E intake leads to higher concentrations of oxidation products *in vivo*. Vitamin E has also been shown to be anti-atherogenic and anti-inflammatory (Szeto *et al.*, 2004).

Recently it has been suggested that vitamin E may act as a gene regulator which affect mRNA and protein translation stability. These changes could be related to regulation of gene transcription, mRNA stability, protein translation, protein stability and post translational events (Ricciarelli *et al.*, 2001; Azzi *et al.*, 2002). Tocotrienols have been associated with several other functions, namely a hypocholesterolaemic effect and the ability to reduce the atherogenic apolipoprotein B and lipoprotein (a) concentrations in

blood serum (Hood, 1995). Tocotrienols have also been linked with anti-thrombotic and anti-tumour effects, making it a suitable agent for the treatment of cardiovascular disease and cancer (Guthrie *et al.*, 1995; Quereshi *et al.*, 1997).

Epidemiological studies indicate that vitamin E is associated with a decreased risk of developing coronary heart disease. Research has shown that administration of vitamin E reduced cardiovascular deaths as well as non-fatal myocardial infarctions significantly (Stephens *et al.*, 1996). Vitamin E is also known to provide protection against ischaemia and reperfusion injury (Bagchi and Puri, 1998). Vitamin E protects against atherosclerosis and oxidation of lipids, which would lead to pathological conditions including cardiovascular disease, cancer and neuronal degeneration (reviewed by Pryor, 2000). This author also stated that vitamin E is superior to carotonoids and vitamin C because it can give rise to a decrease in platelet aggregation ability. Tocopherols are also effective in protecting LDL from oxidation, and thereby preventing disorders such as atherosclerosis (Liu *et al.*, 2004).

Palm oil is unique in the way that it contains both tocopherols and tocotrienols, while other vegetable oils contain mostly tocopherols (Kamen, 2000). In rat ischaemia/reperfusion studies done by Serbinova and co-workers (1991), it was found that α-tocotrienol protected more effectively than α-tocopherol, as shown by improved reperfusion recovery in a Langendorff perfused rat heart. This superior antioxidant activity of tocotrienols, when compared with tocopherols, has been ascribed to the more uniform distribution of tocotrienols in the cell membrane and also the greater recycleability of tocotrienol (Serbinova *et al.*, 1991; Theriault *et al.*, 1999).

A study comparing the effects of tocotrienols and tocopherols as antioxidants showed the following: 1) tocopherols and tocotrienols exerted the same effects on free radical scavenging and lipid-peroxidation in solution and liposomal membranes; 2) tocopherols increased the rigidity of liposomal membranes more significantly than tocotrienols; 3) tocopherols and tocotrienols showed similar mobilities within the liposomal membranes, however, tocotrienols were more readily transferred between the membranes and more frequently incorporated into cultured cells than tocopherols. Therefore, tocotrienols appear to be more effective antioxidants than tocopherols due to higher uptake (Yoshida *et al.*, 2003). Mutalib and co-workers (2003) also showed that tocotrienols are more effective antioxidants than tocopherols, even though they are absorbed poorly from the diet. These authors attribute the efficacy of tocotrienols to better motility in the membrane and better ability to be recycled when compared with tocopherols. They also found that tocotrienols are especially effective in preventing the oxidation of LDL.

Although both tocopherols and tocotrienols are natural antioxidants, the "antioxidant activities" of tocopherols and tocotrienols may vary depending on the experimental conditions applied. The inconsistent results reported previously for the antioxidant activities of tocopherols and tocotrienols may be ascribed partly to the different experimental conditions and evaluation methods used (Yoshida *et al.*, 2003).

2.7.3 Protection offered by ubiquinones

Crude palm oil contains small quantities of ubiquinones of which coenzyme Q_{10} (CoQ_{10}) is the most common. Although it is present at relatively low concentrations in crude palm oil, CoQ_{10} has been reported to boost the immune system, relieve angina and offers

protection against heart disease and reduction of high blood pressure (Nagendran *et al.*, 2000).

CoQ$_{10}$ plays an important role in the mitochondrial electron transport system and as an antioxidant protects the ischaemic/reperfused myocardium in rats (Hano *et al.*, 1994). Yokoyama and co workers (1996) proposed that CoQ$_{10}$ is a free radical scavenger and preserves coronary vessel mechanical function following ischaemia/reperfusion injury via a direct antioxidant mechanism.

2.8 Role of nitric oxide in ischaemia and reperfusion

2.8.1 Introduction

Nitric oxide (NO) is an important regulator of both cardiac and vascular function as well as tissue reperfusion. The major functions of NO include vasodilation, anti-platelet and anti-neutrophil effects and acting as an antioxidant (Hare and Comerford, 1995; Xie and Wolin, 1996). NO performs many of the above mentioned functions as an entity however, some of these functions of NO are mediated by the formation of cGMP by guanylyl cyclase, which is induced by NO (Rubbo *et al.*, 1994; Maulik *et al.*, 1995; Williams *et al.,* 1995; Araki *et al.,* 2000; reviewed by Bolli, 2001). NO may also play a protective role by reversing effects of atherosclerosis (Tokuno *et al.*, 2001). High concentrations of NO may however be hazardous as the highly reactive radical ONOO⁻ will be formed in high concentrations (Tokuno *et al.*, 2001).

NO is formed by a group of enzymes called nitric oxide synthase (NOS) which can be further subdivided into different sub groups (Desrois *et al.*, 2004). NO is formed in most cells throughout the cardiovascular system, including the myocytes and is present in

high concentrations in endothelial cells (Curtis and Pabla, 1997). There are three isoforms of NOS, with the NOS-1 and NOS-3 isoforms leading to the production of NO for use in neurotransmission and cardiovascular signalling, and the NOS-2 isoform leading to increased production of NO which leads to ONOO⁻ formation (Yücel *et al.*, 2005). The same author linked the activity of the NOS-2 isoform to high concentrations of cholesterol in the diet.

2.8.2 Cardiovascular protection offered by nitric oxide

A review written by Ferdinandy and Schultz (2003) emphasized the importance of cardioprotective agents for clinical use. This intervention is needed for chronic conditions like atherosclerosis, hyperlipidaemia, hypertension and diabetes which is common in modern society, especially in the western world. These conditions may give rise to the development of ischaemic heart disease, which is characterized by a lack of sufficient blood supply to the myocardium and can lead to severe cardiac disfunction. Cardioprotective agents decrease the amount of damage caused by ischaemic heart disease and also prevent cardiac arrhythmias during ischaemia and reperfusion.

Over the past decade many studies have focused on the role of NO in myocardial ischaemia. The overwhelming majority of the studies published support a cyto-protective role for NO (either endogenous or exogenous) in myocardial ischaemia/reperfusion injury, both *in vitro* and *in vivo* (reviewed by Bolli, 2001).

NO is produced within endothelial cells of both the vasculature and the heart, cardiac nerves and cardiomyocytes (Curtis and Pabla, 1997) and offers cardioprotection against ischaemia/reperfusion injury (Maulik *et al.*, 1995; Williams *et al.*, 1995; Araki *et al.*, 2000;

Reviewed by Bolli, 2001). Several mechanisms of cardioprotection exist which include 1) stimulation of soluble guanylate cyclase thus bringing about reduction of intracellular Ca^{2+}, 2) activation of cGMP-dependent protein kinase and 3) termination of chain propagating lipid radical reactions caused by oxidative stress (Rubbo *et al.*, 1994). The protective effects of NO can also be attributed to the suppression of cAMP production induced by both NO and cGMP (reviewed by Fishmeister *et al.*, 2005). cAMP increases heart rate and contractile force by opening Ca^{2+} channels, which will ultimately lead to ventricular fibrillation. cGMP has the opposite effect, by opening potassium- and sodium channels and closing Ca^{2+} channels. These changes in ion channel regulation also lead to protection against cardiac hypertrophy, induced by cGMP and NO (reviewed by Fishmeister *et al.*, 2005; Csont and Ferdinandy, 2005). They used the term "Ying Yang principle", to describe the interaction of the opposite effects that are exerted by cAMP and cGMP molecules. NO interferes with caspase 3 activation, leading to a decrease in apoptosis (Smith *et al.*, 2005). The same authors showed a decrease in c-Jun N-terminal kinase (JNK) activation associated with NO and speculated that it may also affect extracellular signal-regulated protein kinase (ERK) and p38 mitogen-activated protein kinase (p38) activation.

NO may however play a detrimental role when it is combined with O_2^- to form peroxynitrite, which rapidly decomposes to highly reactive oxidant species leading to tissue injury (Figure 2.3). There is a critical balance between cellular concentrations of NO, O_2^- and SOD which under physiological conditions favors NO production, but in pathological conditions such as ischaemia and reperfusion, results in $ONOO^-$ formation (reviewed by Ferdinandy and Schultz, 2003). Illarion and co-workers (2002) reviewed the role of reactive oxygen species, focusing mainly on O_2^-, H_2O_2 and OH, which have

long been implicated in the pathogenesis of ischaemia/reperfusion injury. These oxygen free radicals can react with nucleic acids, proteins and lipids, resulting in damage to the cell membrane or intracellular organelles. Vitamin E acts as a free radical scavenger that can react with oxygen, superoxide anion radicals and hydroxyl radicals and prevent intracellular damage by these radicals (Wall, 2000; Abuda *et al.*, 2004) (Figure 2.3).
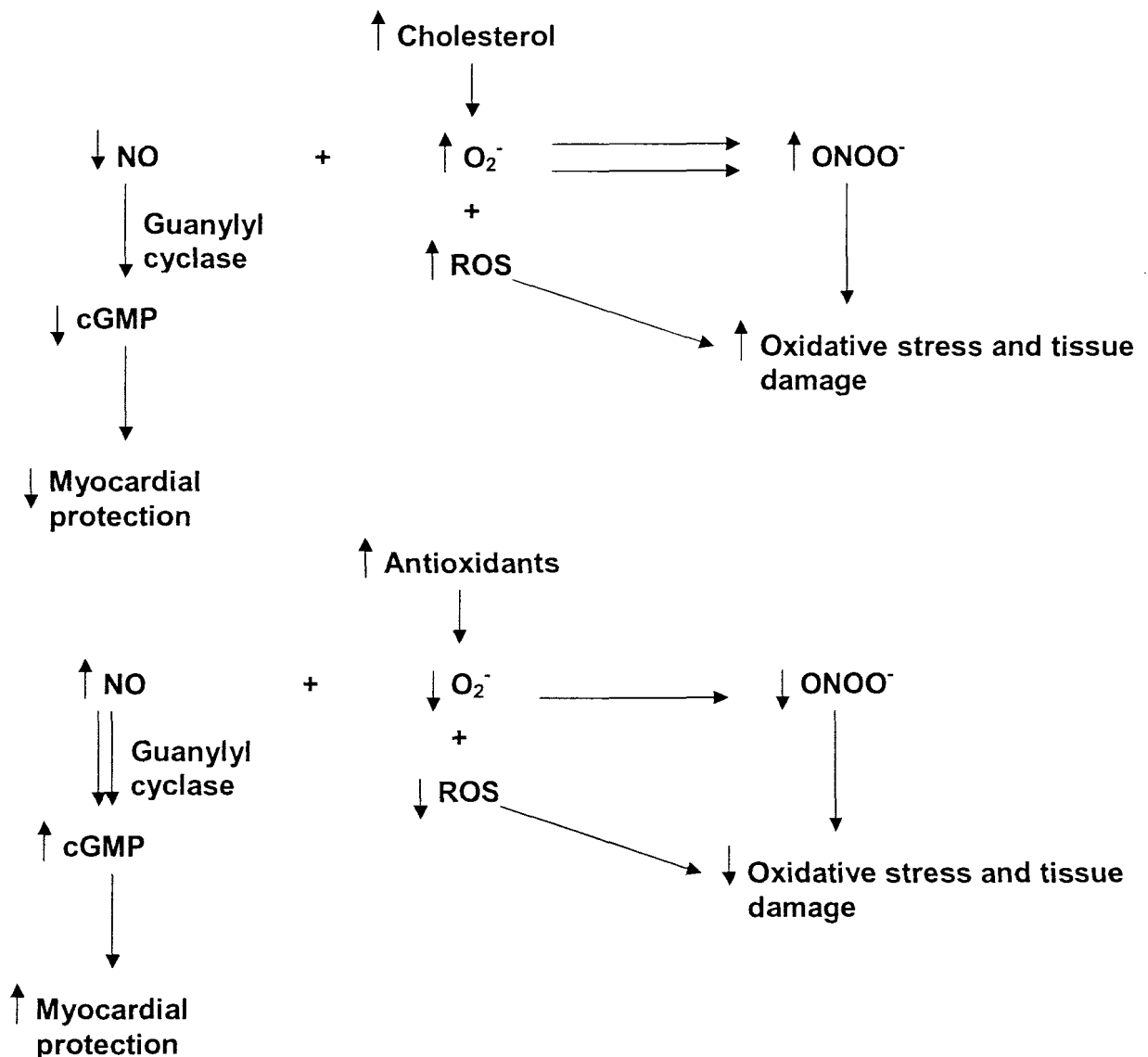
Figure 2.3. Mechanisms of nitric oxide (NO), superoxide (O_2^-) and peroxynitrite ($ONOO^-$) modulation by cholesterol and antioxidants

Smith and co-workers (2005) showed that endothelial NOS (eNOS) activity may offer myocardial protection. The protective effects induced by eNOS activity include a decrease in NADPH activity which leads to less O_2^- production, decreased risk of cardiac hypertrophy, decreased left ventricular developed pressure (LVDevP), increased cardiac function and decreased JNK and caspase 3 activation.

2.8.3 Nitric oxide-cGMP pathway signalling

NO and/or its second messenger, cGMP have been shown to exert a number of actions that are expected to be beneficial during myocardial ischaemia, including inhibition of Ca^{2+} influx into myocytes, antagonism of the effects of β-adrenergic stimulation, decreasing myocardial contractility and opening of sarcolemmal K_{ATP} channels. The reduced Ca^{2+} current may alleviate the Ca^{2+} overload associated with acute myocardial ischaemia, one of the major mechanisms of ischaemic injury (reviewed by Bolli, 2001; Csont and Ferdinandy, 2005). The NO-cGMP pathway also plays an important role in vasodilation by controlling the potassium (K^+) and Ca^{2+} transport channels (Schuldt *et al.*, 2005).

NO is known to increase myocardial cGMP and it can be speculated that the protective effect of NO is related to a mechanism secondary to the stimulation of guanylyl cyclase within the vascular wall or in ventricular myocytes (Beresewics *et al.*, 1995; Maulik *et al.*, 1995; Depré *et al.*, 1996) (Figure 2.3). NO donors administered during ischaemia possibly protect the myocardium by increasing tissue cGMP and decreasing cytosolic Ca^{2+} overload (Du Toit *et al.*, 2001). These authors (2001) found that nitric oxide donor treatment reduces ischaemia/reperfusion injury by increasing cGMP concentrations and

suggested that the cAMP-to-cGMP ratio might play an important role in cardio-protection. Maulik and co-workers (1995) showed that NO plays a significant role in transmembrane signalling in the ischaemic myocardium. They suggested that NO signalling is switched off due to inactivation of NO by reactive oxygen species and was the first to suggest that reactive oxygen species may alter NO-cGMP signalling.

Increases in cAMP concentrations associated with ischaemia would increase Ca^{2+} concentrations and exacerbate ischaemic/reperfusion injury (Du Toit *et al.*, 2001). In this regard it is possible that cGMP may attenuate this type of injury by inhibiting the cAMP induced increase in slow inward calcium current, thus leading to a decrease in cytosolic calcium levels (Sumii and Sperelakis, 1995). Therefore, cGMP appears to be an endogenous intracellular cardioprotectant (Pabla *et al.*, 1995; Pabla and Curtis, 1995).

2.8.4 The effect of dietary cholesterol supplementation on the nitric oxide-cGMP pathway

Research has shown that a cholesterol supplemented diet leads to a decrease in NO, even when the NOS activity is not decreased (Ferdinandy *et al.*, 1997; Onody *et al.*, 2003). The above-mentioned authors attributed this to the increased formation of $ONOO^-$ which is linked to the increased O_2^- associated with a cholesterol supplemented diet (Onody *et al.*, 2003).

It is well known that high cholesterol levels influence the NO-cGMP signalling pathway. Therefore, cardiac stress adaptation is possibly jeopardized in hyperlipidaemia due to altered NO-cGMP pathway function in vascular and myocardial tissue. Szilvassy and co-workers (2001) found that a cholesterol-enriched diet decreased both vascular NO and

cGMP levels and increased aortic O_2^- and $ONOO^-$ production. Several other studies have also shown that a high-cholesterol diet impairs NO-cGMP signalling in both endothelium (Deliconstantinos *et al.,* 1995) and non-endothelial cells with a significant decrease in cardiac NO levels (Ferdinandy *et al.,* 1997; Szekeres *et al.,* 1997; Esterhuyse *et al.,* 2005 b). Giricz and co-workers (2003) found that although the cardiac NO-concentrations in cholesterol-fed rats were decreased, nitric oxide synthase activity was unchanged which may suggest that NO synthesis was not impaired. The mechanism leading to decreased cardiac NO level in hyperlipidaemia remains unknown. However, it is well known that hyperlipidaemia leads to increased production of reactive oxygen species in the vasculature (Kojda and Harrison, 1999). For example, hyperlipidaemia stimulates $ONOO^-$ generation in the heart which leads to myocardial dysfunction. The formation of $ONOO^-$ is linked to oxidation of cellular constituents, but also increases left ventricular end diastolic pressure (Onody *et al.,* 2003). It could be speculated that increased O_2^- production is responsible for the decreased NO concentrations in the hyperlipidaemic myocardium. This is due to increased NADPH oxidase activity (which is a major source of increased O_2^- production) caused by hyperlipidaemia (Warnholtz *et al.,* 1999; Giricz *et al.,* 2003).

2.8.5 Antioxidant properties of nitric oxide

NO has been shown to quench free radicals, especially the highly reactive O_2^- radical. Superoxide dismutase plays an important physiologic role by reducing the concentrations of free O_2^-, but when O_2^- production is too high, NO will react with the excess O_2^- to form $ONOO^-$ (Onody *et al.,* 2003; Ferdinandy *et al.,* 1997). In addition to its protective effect against O_2^-, NO is able to terminate $ONOO^-$ mediated lipid radical chain propagation (Rubbo *et al.,* 1994).

2.8.6 Effects of nitric oxide on the prostanoids

A previously unrecognized mechanism by which NO protects the ischaemic myocardium has recently emerged, namely, stimulation of cyclooxygenases activity with consequent production of cytoprotective prostanoids such as prostaglandin (PGE_2 and PGI_2) (Shinmuri et al., 2000).

2.8.7 Therapeutical potential of nitric oxide

Research has shown that NO plays a fundamental biological role in protecting the heart against ischaemia/reperfusion injury. The recognition that production of NO represents nature's own protective mechanism against ischaemia/reperfusion injury offers fertile practical implications. Many opportunities loom on the horizon for enhancing NO availability in a manner that would be therapeutically desirable (reviewed by Bolli, 2001).

2.9 Effect of red palm oil on NO-cGMP pathway signalling

Natural antioxidants can act as scavengers of damaging oxygen free radicals (Cottrell, 1991, Chandrasekharan, 1999; Theriault et al., 1999; Kritchevsky, 2000; Wall J, 2000). Vitamin E acts as a free radical scavenger that can react with oxygen, superoxide and hydroxyl radicals (Wall, 2000; Abuda et al., 2004) (Figure 2.3). Due to its lipid solubility, it is predominantly a chain breaking antioxidant within the lipoprotein (Wall, 2000; Abuda et al., 2004). Chow and co-workers (2002) reported that dietary vitamin E is capable of reducing the production and/or availability of not only O_2^-, but also NO and $ONOO^-$. By reducing available O_2^- and NO, vitamin E may alleviate nitric oxide toxicity via reduced formation of reactive $ONOO^-$. However, it is not clear if the action of vitamin E to reduce the generation of O_2^- and other ROS species is independent of its antioxidant function

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(Chow and Hong, 2002). Newaz and co-workers (2003) showed an antioxidant protection mechanism by γ-tocotrienols in hypertensive rats when compared with control rats. These authors suggest that improved NOS activity in blood vessels and increased NO availability may be mediated through the antioxidant properties of γ-tocotrienol where it effectively scavenges the free radicals. Venditti and co-workers (1999) also reported that vitamin E treatment offers protection against ischaemia/reperfusion-induced oxidative stress. However, the precise mechanism of action is unknown.

2.10 Effects of eicosapentaenoic acid and docosahexaenoic acid on signal transduction

The n-3 fatty acids have an inhibitory effect on cAMP-dependent protein kinase, protein kinase C (PKC), Ca^{2+}/calmodulin dependent protein kinase 2 and the MAPKs (Mirnikjoo *et al.,* 2001). These changes have a significant effect on signal transduction and may influence cell survival, or the type of cell death that may occur. Mirnikjoo and co-workers (2001) showed that kinases are inhibited by DHA and EPA. Denys and co-workers (2002) found that human T-cell function is regulated by this modulation of MAPK through DHA and EPA. Cheng and co-workers (2003) also showed that EPA decreases hypoxia/reoxygenation injury by reducing phosphorylation of p38.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental groups

Seven-week old male Wistar rats weighing approximately 200 g, were randomly divided into four groups and fed a specific diet for a period of fourteen weeks according to the dietary supplementation they received (Figure 3.1).
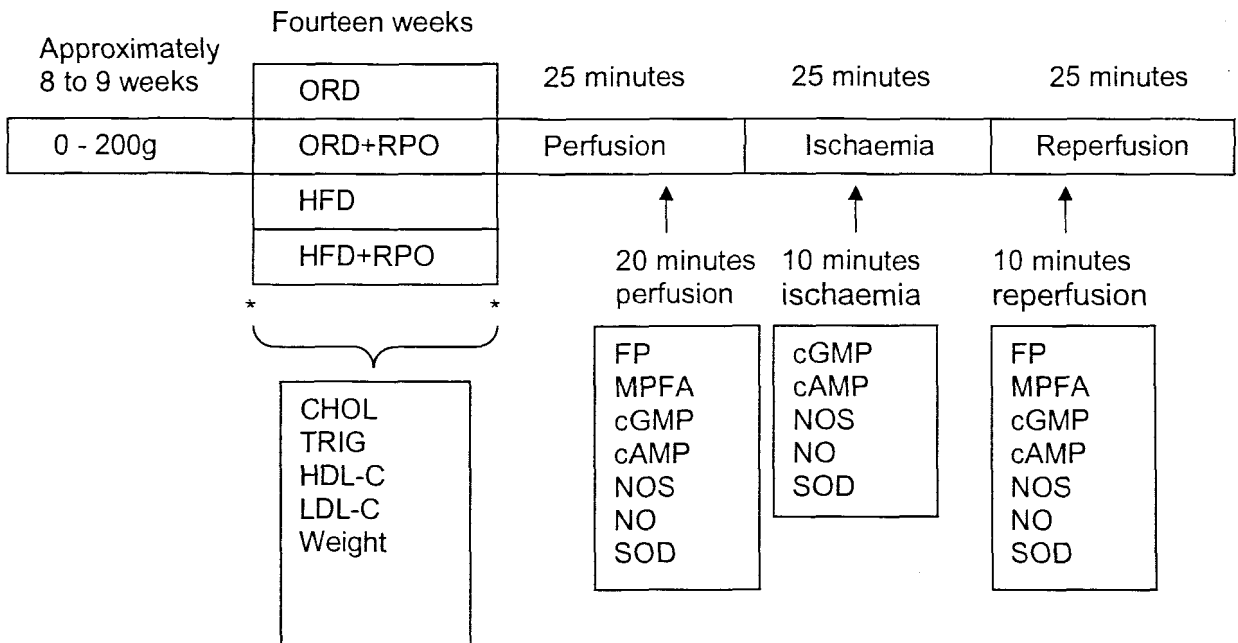
CHOL: Total cholesterol
TRIG: Triacylglycerols
HDL-C: High density lipoprotein cholesterol
LDL-C: Low density lipoprotein cholesterol
NO: Nitric oxide
ORD: Oxidative risk induced diet
ORD+RPO: ORD with RPO-supplementation
HFD+RPO: HFD with RPO-supplementation
*: Measurements was made before and after the fourteen week diet period

FP: Functional parameters
MPFA: Myocardial phospholipid
        fatty acids
NOS: Nitric oxide synthase
SOD: Superoxide dismutase
HFD: High saturated fat diet

Figure 3.1. Study design of experimental model

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Group 1: Received an Oxidative risk induced diet (ORD) which is high in PUFAs, low in MUFAs and low in SFAs.

Group 2: Received an ORD plus RPO (ORD+RPO).

Group 3: Received a high saturated fat diet (HFD) which is high in SFAs, PUFAs and cholesterol, but low in MUFAs.

Group 4: Received a HFD plus RPO (HFD+RPO).

All the diets designed for this study was isocaloric and were prepared on a daily basis to prevent it from spoiling. Diets contained a large portion of refined sugar that exceeded the energy recommendations of the WHO. Rats in all the experimental groups were given their supplements in the morning and after consumption they were allowed *ad libutim* access to standard rat chow. For composition of standard rat chow diet, see Table 3.1. Red Palm Oil baking fat used in this study was supplied by Carotino SDN BHD (company no. 69046-T), Johore Bahru, Malaysia. Table 3.2 shows the composition of the RPO used in this study. The total energy content (kJ) and macronutrient content (%) of rat diets as compared to recommendations made by the WHO are given in Table 3.3. with the assumption that rats consume an average of 25g of food per day.

Table 3.1 Composition of the standard rat chow used in this study

Substance		
Protein		180 g/kg diet (min)
Moisture		120 g/kg diet (min)
Fibre		60 g/kg diet (max)
Calcium		18 g/kg diet (max)
Phosphorus		7g/kg diet (min)
Total fat		25 g/kg diet (min)
%Fatty Acids		
Total SFA		21.9
Total MUFA		27.8
Total PUFA		48.8
Total n-6		40.6
Total n-3		8.2
14:0	Myristic acid	1.30
16:0	Palmitic acid	16.67
18:1	Oleic acid	25.47
18.2 n-6	Linoleic acid	40.32
18.3 n-3	α-Linoleic acid	2.14
20:4 n-6	Arachidonic acid	0.29
20.5 n-3	Eicosapentaenoic acid	2.81
22:6 n-3	Docosahexaenoic acid	2.96

Table 3.2 Composition of the RPO used in this study.

Substance		
Fatty Acid (%)		
14:0	Myristic acid	1.25
16:0	Palmitic acid	43.81
18:1	Oleic acid	38.00
18:2	Linoleic acid	9.73
18:3	Alpha linoleic acid	0.24
20:4	Arachidonic acid	0.20
20:5	Eicosapenteinoic acid	0.28
22:6	Docosahexaenoic acid	0.11
Carotenoids ppm		500
β-carotene (%)		60
α-carotene (%)		25
Vitamin E ppm		500
Tocotrienols (%)		75
Tocopherols (%)		25

Table 3.3 Summary of total energy content (kJ) and macronutrient content (%) of rat diets as compared to recommendations made by the World Health Organization

	ORD	ORD+RPO	HFD	HFD+RPO	WHO
Total energy	240.50 kJ	242.30 kJ	230.70 kJ	232.60 kJ	-
Total protein	15.50 %	15.40 %	13.60 %	13.50 %	10-15 %
Total carbohydrate	61.90 %	61.40 %	58.60 %	58.10 %	55-75 %
Total fat	22.60 %	23.20 %	27.80 %	28.40 %	15-30 %
SFA	4.10 %	3.30 %	10.40 %	9.60 %	<10 %
PUFA	12.60 %	14.10 %	7.70 %	9.30 %	3-7 %
MUFA	4.90 %	4.50 %	6.80 %	6.40 %	>10 %
Refined sugar	10.50 %	10.50 %	11.00 %	10.90 %	0-10 %
Cholesterol	-	-	2.20 mg/d*	2.20 mg/d*	0-300mg/d$^\#$

*These values are the actual amounts of cholesterol fed to the rats.
$^\#$ This value is recommended for human daily intake by the WHO.

ORD: Oxidative risk induced diet   HFD: High saturated fat diet
ORD+RPO: ORD with RPO-supplementation   HFD+RPO: HFD with RPO-supplementation

3.2 Animal Care

All animals received humane care in accordance with the Principles of Laboratory Animal Care of the National Society of Medical Research and the Guide for the Care and use of Laboratory Animals of the National Academy of Sciences. (National Institutes of Health publications no. 80 – 23, revised 1978).

3.3 Working heart perfusion

At the end of the feeding program, rats weighing 450 g - 600 g were anaesthetized by injecting 0.5 ml sodium thiopentone intraperitoneally and 400 units of heparin

intravenously. Hearts were rapidly excised and placed in ice-cold Krebs-Henseleit buffer and transferred to the standard working heart perfusion apparatus where they were perfused with a Krebs-Henseleit buffer equilibrated with 95% O_2 and 5% CO_2 at 37 °C (118,5 mM NaCl; 4,75 mM KCl; 1,2 mM MgCl . 6 H_2O; 1,36 mM $CaCl_2$; 25,0 mM $NaHCO_3$; 1,2 mM KH_2PO_4; 11,0 mM glucose) at a perfusion pressure of 100 cm H_2O (Figure 3.2). The aorta was cannulated and retrograde perfusion with Krebs-Henseleit buffer was initiated. During this initial perfusion in the Langendorff mode, excess tissue was removed from the hearts and the opening to the left atrium was cannulated *via* the pulmonary vein (Figure 3.3).
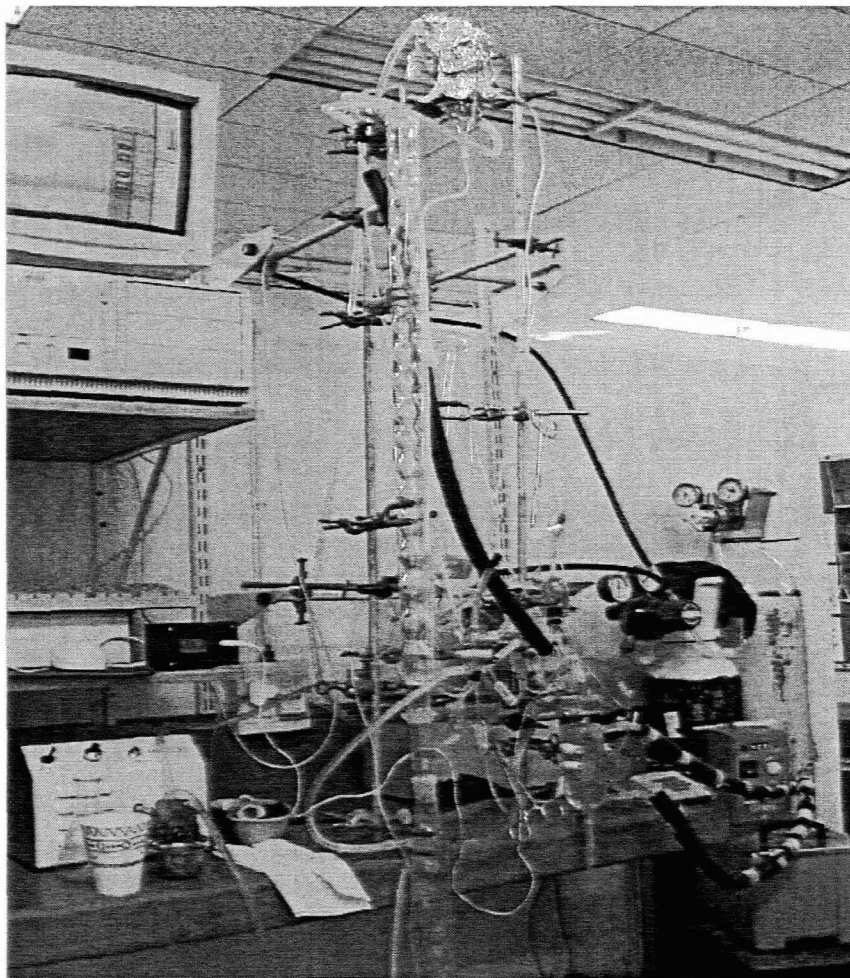
Figure 3.2. The working heart perfusion apparatus.
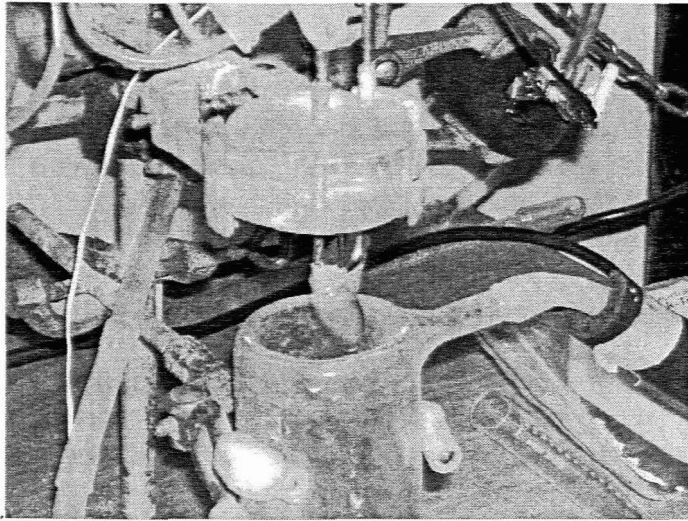
Figure 3.3. A heart mounted on the working heart perfusion apparatus.

Following a five-minute stabilization period in the Langendorff mode, hearts were switched to the working mode. The temperature of both the perfusate and the air surrounding the heart were thermostatically controlled and checked at regular intervals to ensure that the temperature was maintained at 37°C irrespective of coronary flow. A cannula, connected to a pressure transducer, was inserted through the apex of the heart into the left ventricle. Left ventricular systolic and diastolic pressure, coronary flow (CF), heart rate (HR) and aortic output (AO) were measured at five-minute intervals during the first 25 minutes of perfusion. Hearts were then subjected to 25 minutes of global ischaemia at a temperature of 34°C. At the end of ischaemia, hearts were reperfused in the Langendorff mode for 10 minutes. In order to reduce the incidence of reperfusion arrhythmias, 2% lignocaine solution was used for the initial three minutes of reperfusion of all hearts. This was followed by a 15-minute working heart perfusion period during which cardiac function was measured. To assess fatty acid composition and other biochemical markers, hearts were freeze-clamped using Wollenberger clamps pre-

cooled in liquid nitrogen, at various time points throughout the perfusion experiment (Figure 3.1).

3.4 Left ventricular developed pressure (LVDevP) (mmHg)

Left ventricular systolic-(LVSP) and diastolic (LVDP) pressure were monitored at 5-minute intervals during the 25 minute stabilization perfusion period and again during reperfusion after 25 minutes of global sustained ischaemia. LVDevP was calculated by subtracting LVDP from LVSP, and the values obtained were then used as a measure of mechanical function of the heart (n=6 per group per time point).

3.5 Rate pressure product (RPP)

Mechanical function was expressed as the percentage rate pressure product (RPP) recovery. RPP was calculated by multiplying the LVDevP with the heart rate (HR) at the following time points: 20 minutes perfusion, 15 minutes reperfusion, 20 minutes reperfusion and 25 minutes reperfusion. The results were converted to percentages of baseline values (n=6 per group per time point).

3.6 Aortic output recovery (%)

In order to compare the functional recovery of the hearts in the different groups, the aortic output (ml/min) was measured by collecting one-minute samples of the effluent. Aortic output (AO) recovery was then calculated by dividing the AO after the ischaemic period by the AO before the ischaemic period and expressing these values as a percentage recovery (n=6 per group per time point).

3.7 Biochemical analyses

3.7.1 cGMP assay

The assay is based on the competition between unlabelled cGMP present in the sample and a fixed quantity of ^{125}I-labeled cGMP for a limited number of binding sites on a cGMP-specific antibody. With fixed amounts of antibody and radioactive cGMP, the amount of radioactive cGMP bound to the antibody will be inversely proportional to the concentration of non-radioactive cGMP present in the sample. After centrifugation the bound fraction forms a stable pellet and the unbound fraction is discarded together with the supernatant. Measurement of the radioactivity in the pellet enables the amount of labeled cGMP in the bound fraction to be calculated. The concentration of unlabelled cGMP in the sample is then determined by interpolation from a standard curve. The acetylation buffer was used in order to obtain a standard curve in the range of 2 to 128 fmol/tube.

For cGMP assays, freeze-clamped hearts were used and 150 mg of wet tissue was homogenized, extracted in 5% trichloroacetic acid and centrifuged. The supernatant of the extracted sample was ether-washed three times for five-minute wash cycles. These samples were then diluted 1:10 (V/V) and acetylated for the ^{125}I-labeled cGMP assay. The centrifugation separation method was used, in order to separate the bound and unbound fractions of cGMP. The amount of radioactivity present in each sample was determined by counting each tube in a gamma scintillation counter for 60 seconds. The IC_{50} for the cGMP assay was 25 pmol/tube (Du Toit *et al.*, 1999).

The cGMP assay was performed using a test kit provided by Amersham (cGMP (^{125}I) assay system with Amerlex-M^{TM} magnetic separation, code RPA 525). The test kit

contained an acetylation standard, a non-acetylation standard, a tracer (radio-labeled cGMP), antiserum (rabbit anti-cGMP), assay buffer, a second antibody (donkey anti-rabbit Amerlex-M), acetic anhydride and triethylamine. The complete assay methodology is described in the instruction booklet provided within the above-mentioned test kit. (n=5 per group per time point).

3.7.2 cAMP assay

The assay is based on the competition between unlabelled cAMP in the sample and a fixed quantity of the tritium labelled compound for binding to a protein which has a high specificity and affinity for cAMP. The amount of labelled protein-cAMP complex formed is inversely related to the amount of unlabelled cAMP present in the assay sample. Measurement of the protein-bound radioactivity enables the amount of unlabelled cAMP in the sample to be calculated. Separation of the protein bound cAMP from the unbound nucleotide is achieved by adsorption of the free nucleotide onto coated charcoal, followed by centrifugation. An aliquot of the supernatant is then removed and added to scintillation fluid for liquid scintillation counting. The concentration of unlabelled cAMP in the sample is then determined from a linear standard curve.

For the cAMP assays, 150 mg tissue was homogenized, extracted with perchloric acid, neutralized using sodium hydroxide and assayed. The amount of radioactivity present in each sample was determined by counting each tube in a beta scintillation counter for 2 minutes. The IC_{50} for this assay was 1,92 mmol/tube (Du Toit et $al.,$ 1999).

The cAMP assay was performed using a test kit provided by Amersham (cAMP (^{3}H) assay system, code TRK 432). The test kit contained a standard, a tracer (radio-labeled

cAMP), assay buffer, binding protein (purified from bovine muscle) and charcoal. The complete assay methodology is described in the instruction booklet provided within the above-mentioned test kit (n=5 per group per time point).

3.7.3 Measurement of cardiac nitric oxide concentrations

Myocardial NO levels were determined using a Nitrate/Nitrite kit (Cayman Chemicals, Cayman Islands, catalog no. 780001), which provides an accurate and convenient method for measurement of total nitrate/nitrite concentration in a simple two-step process. The first step is the conversion of nitrate to nitrite utilizing nitrate reductase. The second step is the addition of the Griess Reagents (Sulfanilamide and N-(1-Naphtyl) ethylenediamine), which converts nitrite into a deep purple azo-compound. Photometric measurement of the absorbance at 540 nm due to this chromophore accurately determines nitrite concentration.

Sample preparation entailed homogenization of 200 mg of wet cardiac tissue in 0,5 ml PBS (pH 7,4), followed by centrifugation at 10 000 x g for 20 minutes. The supernatant was ultra-filtered using a 30 kDa molecular weight cut-off filter (Millipore) and 40 µl of the filtrate was assayed. The assay was performed using a 96 well plate and photometric measurement was performed using a plate reader with light filters set at 540nm.

The test kit contained a nitrate/nitrite assay buffer, nitrate reductase enzyme preparation, a nitrate reductase cofactor preparation, a nitrate standard, the two different Griess reagents and a 96 well plate. The complete assay methodology is described in the instruction booklet provided within the above-mentioned test kit (n=5 per group per time point).

3.7.4 Measurement of cardiac nitric oxide synthase activity

Total NOS activity was measured using a NOS assay-kit, which is based on biochemical conversion of L-arginine to L-citrulline by NOS (Cayman Chemicals, Cayman Islands, catalog no. 781001). This reaction, which represents an enzymatic process, involves a five-electron oxidation of guanidino nitrogen of L-arginine to NO, together with the stoichiometric production of L-citrulline Several co-factors are needed for this reaction, such as calmodulin, NADPH and calcium. Advantages of this NOS kit include the use of radioactive substrate [^{14}C] arginine which enables sensitivity in the picomole range, as well as the specificity of the assay for the NOS pathway due to the direct enzymatic conversion of arginine to citrulline in eukaryotic cells. Furthermore, easy separation of neutrally charged citrulline from positively charged arginine allows multiple assays to be performed with ease. The NOS activity is quantified by measuring the radioactivity in the eluate after performing the assay.

For this assay 100 mg of wet cardiac tissue was homogenized in 0,5 ml homogenization buffer (1:10 dilution of stock solution), supplied by Cayman Chemicals and centrifuged at 10 000 x g for 15 minutes at 4 °C. For the assay 250 μl of supernatant was used. A reaction mixture was prepared, containing the necessary co-factors, reaction buffer and ^{14}C labeled arginine. Samples were added to the reaction mixture and allowed to incubate for 60 minutes, after which the reaction was stopped by addition of the stop buffer. Separation of citrulline from arginine was achieved by addition of an equilibrated resin (which binds to the arginine), followed by filtration, making use of microfuge spin cups and holders. Quantification of radiation is then achieved by adding scintillation fluid

and counting each tube in a beta scintillation counter for 2 minutes. The amount of arginine that is converted to citrulline is proportional to the NOS activity.

The test kit contained rat cerebellum extract, calmodulin, reaction buffer, N^G-nitro-L-arginine methyl ester (L-NAME), a homogenization buffer, a stop buffer, equilibrated resin, calcium chloride, an elution buffer and spin cups with cup holders. The complete assay methodology is described in the instruction booklet provided within the above-mentioned test kit (n=5 per group per time point).

3.7.5 Measurement of cardiac superoxide dismutase activity

Total activity of SOD was measured using a SOD kit (Cayman Chemicals, Cayman Islands, catalog no. 706002) that utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthase. One unit of SOD is defined as the amount of enzyme required to exhibit 50% dismutation of the superoxide radical. The SOD assay measures all three types of SOD.

Approximately 200 mg of cardiac tissue was homogenized in 1.0 ml ice-cold HEPES buffer and centrifuged at 1 500 x g for 5 minutes at 4 °C. For the assay 10 µl of the supernatant was used. The sample is added to radical detector, after which the reaction is initiated by the addition of xanthine oxidase. The plate was then incubated for 20 minutes at room temperature before absorbance was measured using a plate reader with the light filters set at 450nm. SOD activity can then be calculated by interpolation from a standard curve.

The test kit contained an assay buffer, a sample buffer, a radical detector, SOD standard, xanthine oxidase and a 96 well plate. The complete assay methodology is described in the instruction booklet provided within the above-mentioned test kit (n=5 per group per time point).

3.8 Serum lipids

Rats were weighed weekly during the diet period and blood was collected before and after the fourteen-week period from their tail veins. The blood was centrifuged for 10 minutes at 3 000 x g, serum collected and analysed for lipid profiles i.e. total cholesterol, high-density lipoprotein-cholesterol and triacylglycerols. The serum lipid profile was determined using a Ciba Corning Express 550-instrument and Ciba Corning reagents employing enzymatic and colorimetric methods. The parameters measured were then used to calculate low-density lipoprotein-cholesterol (n=9 per group per time point).

3.9 Heart muscle total phospholipid fatty acids

Hearts of rats that received the various dietary supplements for 14 weeks (4 groups) were freeze-clamped before and after ischaemia and used to determine myocardial total phospholipid fatty acids. Tissue samples were extracted with chloroform: methanol (2:1; v/v) according to a modified method of Folch and co-workers (1957). Neutral lipids were separated from total phospholipids by thin layer chromatography (TLC) and the total phospholipid fraction analyzed for fatty acids by gas chromatography. A fatty acid mixture was prepared from individual fatty acids (Sigma. St. Louis, MO, USA) and used as a standard (Folch *et al.*, 1957; Smuts *et al.*, 1992; Van Jaarsveld *et al.*, 2000) (n=5 per group per time point).

3.10 Statistical methods

GraphPad Prism version 2.01 was used to do statistical analysis of data. Values are presented as mean ± standard error of the mean (SEM). One way ANOVA with Bonferroni's post-hoc correction was used to measure significance between the groups and for paired comparisons the Student's t test was used. $P< 0.05$ was considered significant. Some values were presented as a percentage change from the baseline values.

CHAPTER 4

Results

4.1 Animal weight (g)

Weight was significantly increased after the diet period in the HFD when compared to the ORD (543.6 ± 8.7 g *versus* 486.6 ± 6.8 g, *P*<0.05). There was also a significant weight increase between the HFD+RPO and the ORD+RPO after the diet period (523.9 ± 7.1 g *versus* 472.2 ± 4.3 g, *P*<0.05), which shows that the HFD is more likely to lead to increased body mass when compared to the ORD. Addition of RPO per *se* had no effect on the weights (n=25 per group per time point) (Figure 4.1).
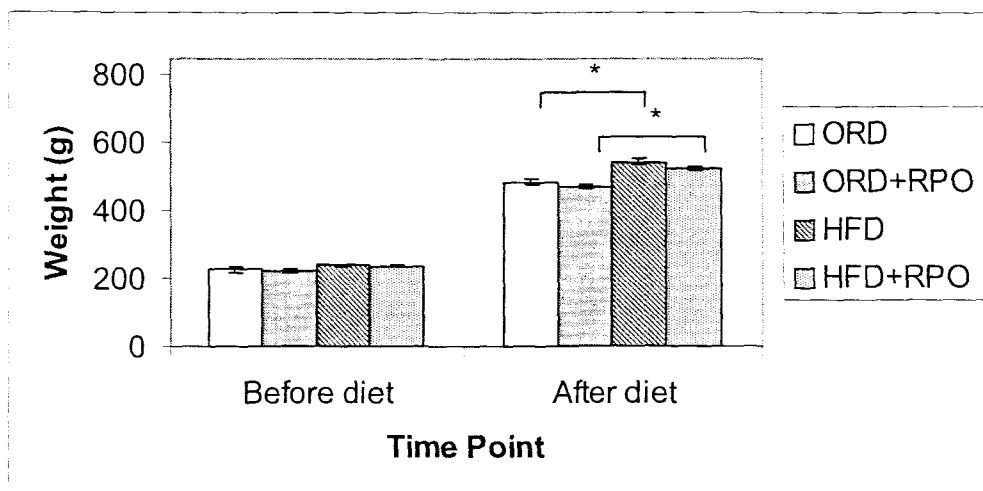
Figure 4.1. Weight of rats before and after fourteen week supplemented diet period. (n=25 for each group at each time point)(*P<0.05 for the group *versus* the indicated diet group)(mean ± SEM)
ORD: Oxidative risk induced diet   HFD: High saturated fat diet
ORD+RPO: ORD with RPO-supplementation   HFD+RPO: HFD with RPO-supplementation

4.2 Functional parameters

Functional parameters measured throughout the perfusion protocol included aortic output (AO), coronary flow (CF), heart rate (HR), left ventricular systolic pressure (LVSP) and left ventricular diastolic pressure (LVDP). These results are presented in Table 4.1

at 20 minutes perfusion and 25 minutes reperfusion. There was no significant difference in the coronary flow of any group at any time point, indicating that none of the changes in cardiac function was due to vascular changes. The heart rate of the HFD was significantly decreased when compared to the HFD+RPO at 25 minutes reperfusion (234.03 ± 6.71 bpm *versus* 258.97 ± 8.12 bpm, $P<0.05$). The LVDP of the ORD+RPO was significantly decreased when compared to the HFD+RPO at the 20 minutes perfusion time point (-14.06 ± 1.33 mmHg versus -8.25 ± 1.71 mmHg, $P<0.05$). The LVDP of the ORD was significantly decreased when compared to the HFD at the 25 minutes reperfusion time point (-8.08 ± 0.70 mmHg *versus* -0.83 ± 1.35 mmHg, $P<0.05$) The LVDP of the HFD+RPO was also decreased when compared to the HFD at the same time point (-6.68 ± 1.17 mmHg *versus* -0.83 ± 1.35 mmHg, $P<0.05$). The LVDP of the HFD was significantly increased at the 25 minutes reperfusion time point when compared to values of the 20 minutes perfusion time point (-0.83 ± 1.35 mmHg *versus* - 6.1 ± 0.83, $P<0.05$). The LVSP of the ORD+RPO was significantly decreased at the 25 minutes reperfusion time point when compared to the 20 minutes perfusion time point (145.91 ± 2.65 mmHg *versus* 155.50 ± 2.69 mmHg, $P<0.05$). The aortic output of the ORD+RPO was significantly increased when compared to the ORD at the 20 minutes perfusion time point (53.50 ± 1.45 ml/min *versus* 47.80 ± 1.50 ml/min, $P<0.05$). At the 25 minute reperfusion time point, the aortic output of both the RPO-supplemented groups were increased when compared with the groups that did not contain RPO (ORD+RPO: 43.00 ± 1.38 ml/min *versus* 28.30 ± 2.78 ml/min; HFD+RPO: 35.50 ± 2.62 ml/min *versus* 25.20 ± 1.53 ml/min, $P<0.05$) (n=6 per group per time point) (Table 4.1).

Table 4.1 Functional parameters measured in perfused rat hearts at 20 minute perfusion and 25 minutes reperfusion time points (n=6 per group per time point)

Time Point	Group	HR (bpm)	CF (ml/min)	AO (ml/min)	LVDP (mmHg)	LVSP (mmHg)
20 minutes perfusion	ORD	216.80±16.44	20.70 ± 1.05	47.80±1.50 [a,d]	-10.35 ± 1.18	155.17 ± 6.61
	ORD+RPO	229.35±7.28	19.63±0.73	53.50±1.45 [d]	-14.06 ± 1.33 [c]	155.50 ± 2.69 [d]
	HFD	223.98±11.57	21.00±1.34	50.40±1.75 [d]	-6.10 ± 0.83 [d]	134.50 ± 4.58
	HFD+RPO	250.75±14.65	20.50±1.20	53.17±3.05 [d]	-8.25 ± 1.71	141.07 ± 5.74
25 minutes reperfusion	ORD	230.48±10.25	20.70±1.28	28.30±2.78 [a]	-8.08 ± 0.70 [b]	137.82 ± 5.39
	ORD+RPO	228.11±10.52	19.13±0.93	43.00±1.38	-10.76 ± 1.17	145.91 ± 2.65
	HFD	234.03±6.71 [a]	21.00±1.34	25.20±1.53 [a]	-0.83 ± 1.35 [a]	124.95 ± 3.04
	HFD+RPO	258.97±8.12	21.50±2.11	35.50±2.62	-6.68 ± 1.17	133.53 ± 5.83

a: *P*<0.05 for the group *versus* its RPO supplemented group of the same diet
b: *P*<0.05 for the group *versus* the group of the other diet without RPO-supplementation
c: *P*<0.05 for the group *versus* the RPO supplemented group of the other diet
d: *P*<0.05 for the group before ischaemia *versus* the same group after ischaemia

ORD: Oxidative risk induced diet
ORD+RPO: ORD with RPO-supplementation
HR: Heart rate
LVDP: Left ventricular diastolic pressure
AO: Aortic output

HFD: High saturated fat diet
HFD+RPO: HFD with RPO-supplementation
CF: Coronary flow
LVSP: Left ventricular systolic pressure
bpm: Beats per minute

4.3 Left ventricular developed pressure (LVdevP)

The baseline LVDevP values showed no significant differences between any of the groups (ORD: 165.52 ± 7.44 mmHg; ORD+RPO: 169.56 ± 3.76 mmHg; HFD: 140.60 ± 5.39 mmHg; HFD+RPO: 149.32 ± 7.33 mmHg). The LVdevP of the hearts of the rats fed the ORD, ORD+RPO and HFD, was significantly decreased at the 15 minutes reperfusion time point when compared to the 20 minutes perfusion time point (ORD:

165.52 ± 7.44 mmHg *versus* 144.39 ± 5.42 mmHg; ORD+RPO: 169.56 ± 3.76 mmHg

versus 154.65 ± 3.55 mmHg; HFD: 140.60 ± 5.39 mmHg *versus* 122.15 ± 3.25 mmHg,

$P<0.05$) (Figure 4.2). The LVDevP of all groups at the 20- and 25 minutes reperfusion

time points were similar to that of the 15 minutes reperfusion time point which was

significantly decreased when compared with the 20 minute perfusion time point (n=6 per

group per time point).
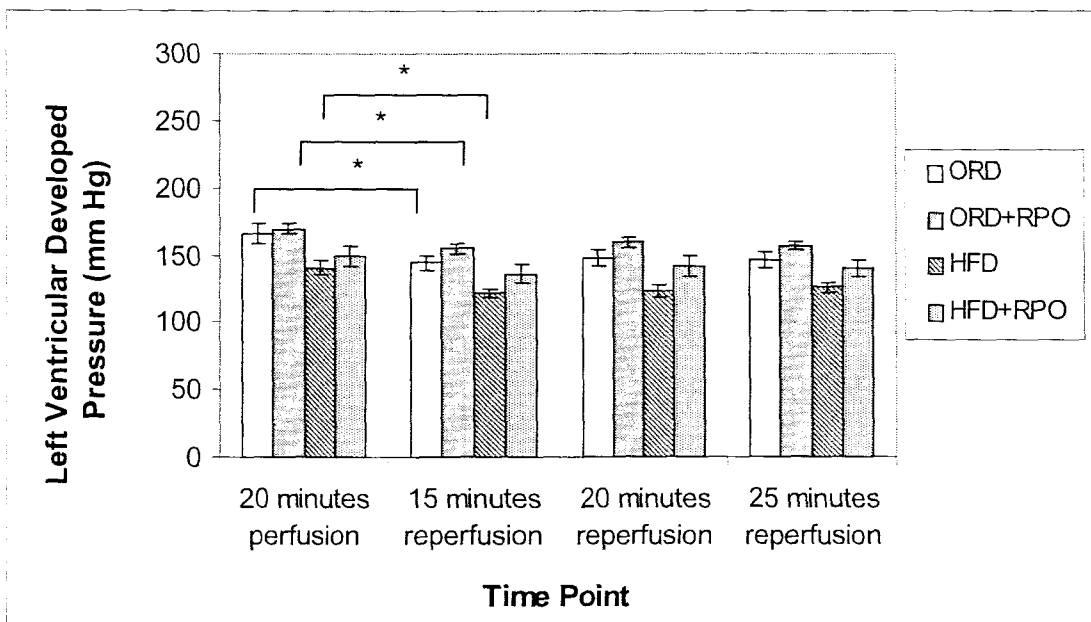
Figure 4.2. Left ventricular developed pressure before and after ischaemia of isolated perfused rat hearts fed different supplemented diets. (n=7 per group per time point) (*$P<0.05$ for the group *versus* the indicated diet group at different time points during reperfusion) (mean ± SEM)
ORD: Oxidative risk induced diet   HFD: High saturated fat diet
ORD+RPO: ORD with RPO-supplementation   HFD+RPO: HFD with RPO-supplementation

4.4 % Rate pressure product recovery

Results are presented in Figure 4.3.The RPP for the HFD was significantly decreased

when compared with the HFD+RPO at 15 minutes- and 25 minutes reperfusion (15

minutes reperfusion: 81.37 ± 2.07 % *versus* 88.41 ± 4.82 % ; 25 minutes: 92.34 ± 2.63

% versus 98.37 ± 5.20 %, P<0.05). These results showed that RPO-supplementation protected the heart of rats fed with a high saturated fat diet from ischaemia/reperfusion injury and allows for increased reperfusion function. These values may seem to be too high for recovery after a 25 minute ischaemic period, but it is important to note that the temperature during ischaemia was 34°C and not 36°C. This decrease in temperature could have a substantial protective effect. The baseline values of the RPP did not show any significant differences for any of the groups (ORD: 35706.35 ± 3010.08; ORD+RPO: 38913.13 ± 1599.00; HFD: 31393.73 ± 1691.92; HFD+RPO: 37237.37 ± 2436.33) (n=6 per group per time point).
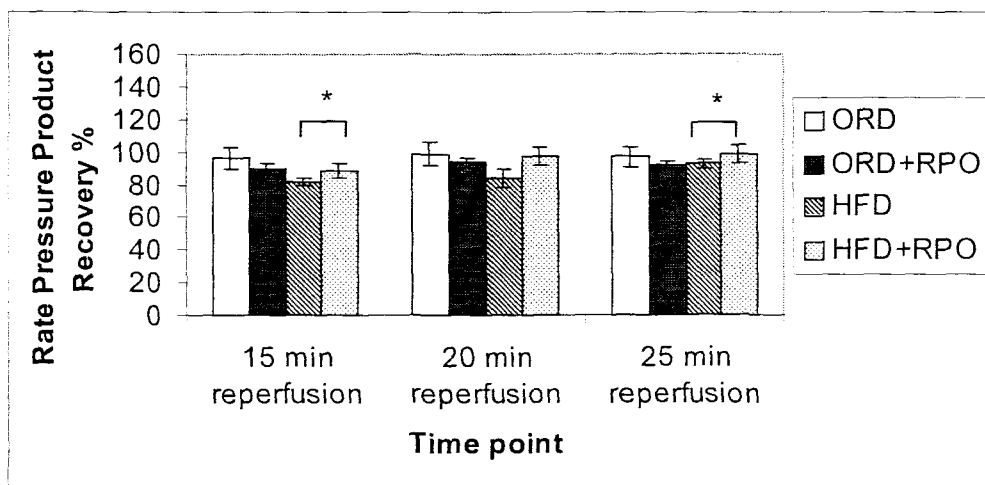
Figure 4.3. % **Rate pressure product recovery in the 4 experimental groups at 15, 20 and 25 minutes reperfusion, respectively.** (n = 7 per group per time point) (*P< 0.05 for the group *versus* RPO supplemented group) (mean ± SEM)
ORD: Oxidative risk induced diet   HFD: High saturated fat diet
ORD+RPO: ORD with RPO-supplementation   HFD+RPO: HFD with RPO-supplementation

4.5 % Aortic output recovery

The percentage aortic output recovery was significantly lower in the group that was fed with the HFD+RPO when compared with the ORD+RPO group (66.17 ± 2.85 % *versus* 83.63 ± 1.76 %, P<0.05) (Figure 4.4). RPO-supplementation caused a significantly increased aortic output recovery in both the ORD- and HFD groups (83.63 ± 1.76 %

versus 61.40 ± 3.76 % for ORD+RPO and 66.17 ± 2.85 % *versus* 50.00 ± 1.64 % for

HFD+RPO; *P*<0.05). Similar to RPP results, these values may seem to be too high for

recovery after a 25 minute ischaemic period, but the temperature during ischaemia was

34°C and not 36°C, which would have a protective effect. These findings confirm that

RPO-supplementation offers protection against ischaemic/reperfusion injury, even with

rats that are fed an unhealthy diet. (n=6 per group per time point)
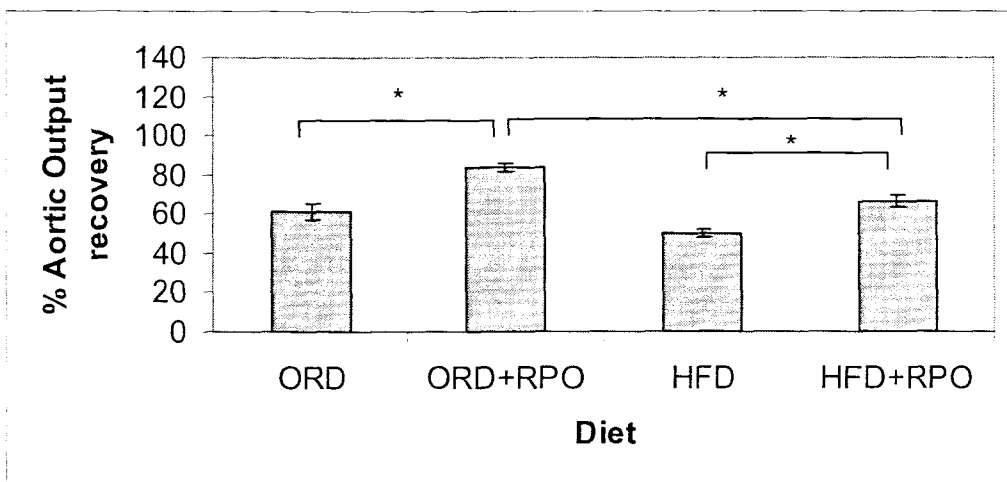
Figure 4.4. Percentage aortic output recovery of isolated perfused rat hearts in the 4 experimental groups 25 minutes into reperfusion (n = 10 per group) (*P< 0.05 for the group *versus* the indicated group) (mean ± SEM)
ORD: Oxidative risk induced diet　　　　　　　　HFD: High saturated fat diet
ORD+RPO: ORD with RPO-supplementation　　　HFD+RPO: HFD with RPO-supplementation

4.6 % Change in cGMP and cAMP

The results obtained for cGMP concentrations are presented as a percentage change

from the baseline values at 10 minutes during ischaemia and 10 minutes reperfusion.

The baseline values were as follows: 11.42 ± 2.98 pmol/g wet weight for the ORD, 8.95

± 1.03 pmol/g wet weight for the ORD+RPO, 10.63 ± 1.08 pmol/g wet weight for the

HFD and 10.41 ± 0.49 pmol/g wet weight for the HFD+RPO. The cGMP concentrations

of the RPO-supplemented groups increased significantly during ischaemia when

compared with corresponding diet groups without RPO (195.40 ± 18.26 % *versus* 90.31

± 12.78 % for the ORD groups and 132.98 ± 12.41 % *versus* 50.09 ± 10.42 % for the

HFD groups, *P*<0.05). The baseline cGMP concentrations were similar to values of

the10 minutes reperfusion time point for all the groups (n=5 per group per time point)

(Figure 4.5).

Figure 4.5. % Change in myocardial cGMP concentrations in the 4 experimental groups at 10 minutes ischaemia and 10 minutes into reperfusion (n = 5 per group per time point)(*P< 0.05 for the group versus the indicated group) (mean ± SEM)
ORD: Oxidative risk induced diet   HFD: High saturated fat diet
ORD+RPO: ORD with RPO-supplementation   HFD+RPO: HFD with RPO-supplementation

The baseline values obtained for myocardial cAMP concentrations were as follows:

253.68 ± 34.77 pmol/g wet weight for the ORD, 360.21 ± 31.55 pmol/g wet weight for the

ORD+RPO, 308.68 ± 41.04 pmol/g wet weight for the HFD and 249.89 ± 36.24 pmol/g

wet weight for the HFD+RPO. Values are presented as percentage change from the

baseline values at 10 minutes during ischaemia and 10 minutes during reperfusion.

There was a significant increase in the percentage change of the HFD+RPO when

compared with the ORD+RPO at 10 minutes during ischaemia (48.70 ± 22.11% *versus* -

32.38 ± 10.65%, *P*<0.05). At 10 minutes reperfusion cAMP concentrations were similar to baseline values for all the groups (n=5 per group per time point) (Figure 4.6).
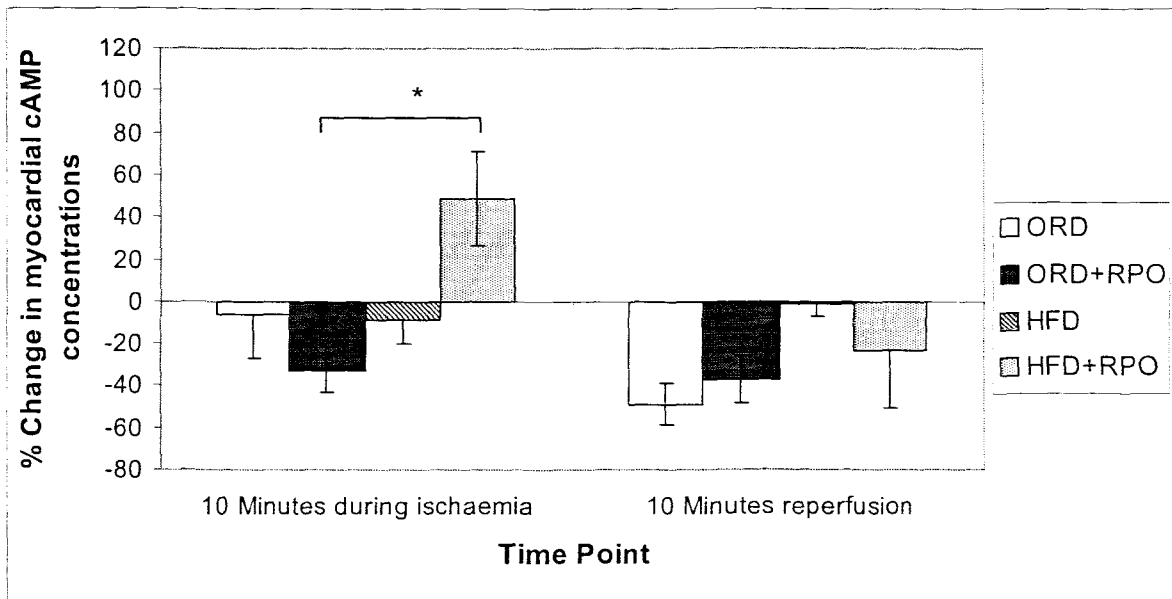
Figure 4.6. Percentage change in myocardial cAMP concentrations in the 4 experimental groups at 10 minutes ischemia and 10 minutes into reperfusion (n = 5 per group per time point) (*P<0.05 for the group versus the indicated group) (mean ± SEM)

ORD: Oxidative risk induced diet   HFD: High saturated fat diet
ORD+RPO: ORD with RPO-supplementation   HFD+RPO: HFD with RPO-supplementation

4.7 Cardiac nitric oxide concentration

Myocardial NO concentrations in hearts of the rats in the HFD group was significantly increased when compared with hearts of the ORD group at 10 minutes reperfusion (1.29 ± 0.22 µmol/g wet weight *versus* 0.19 ± 0.06 µmol/g wet weight; *P*<0.05) (n=5 per group per time point) (Figure 4.7).
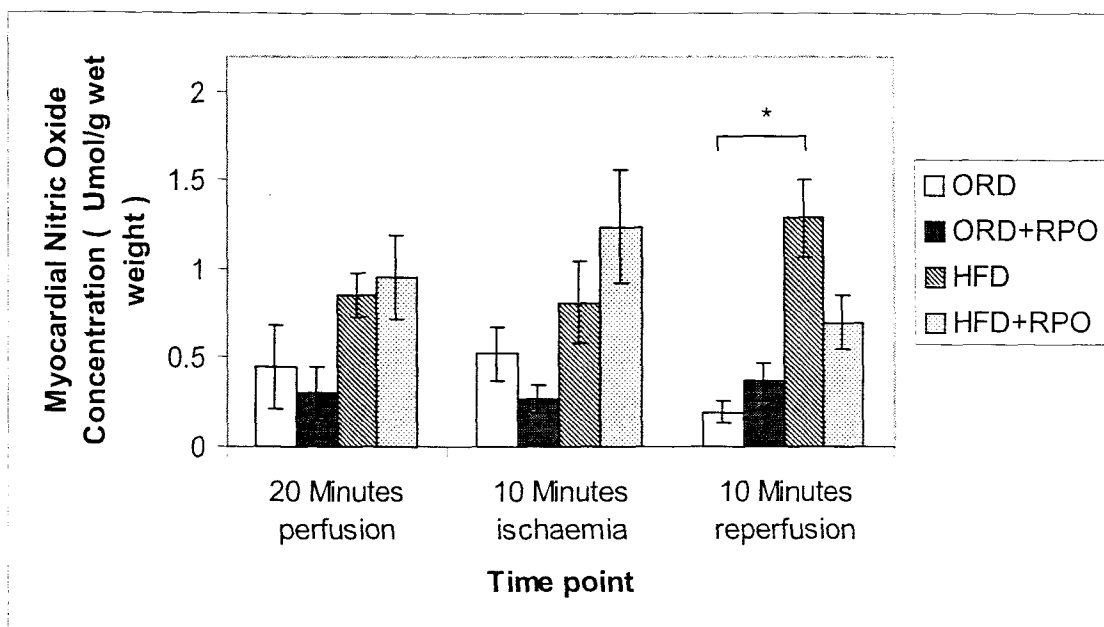
Figure 4.7. Myocardial NO concentrations of rats fed different supplemented diets for a period of fourteen weeks. (n = 5 per group per time point)(*$P<0.05$ for the group versus the indicated group) (mean ± SEM)

ORD: Oxidative risk induced diet   HFD: High saturated fat diet
ORD+RPO: ORD with RPO-supplementation   HFD+RPO: HFD with RPO-supplementation

4.8 Cardiac nitric oxide synthase activity

There were no significant differences between the 4 experimental groups at any of the time points (n=5 per group per time point) (Figure 4.8).
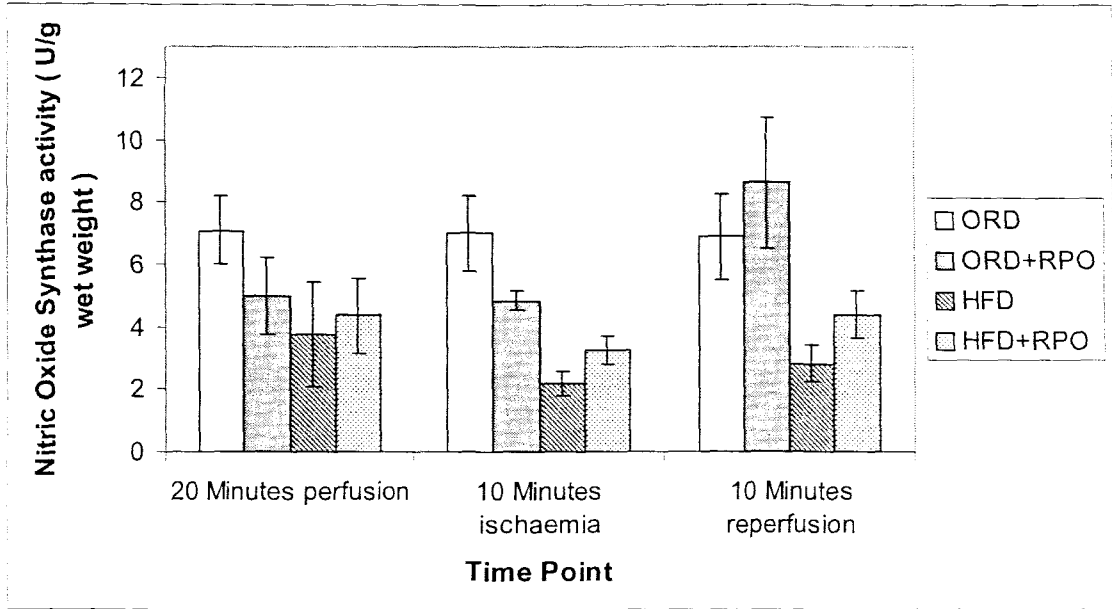
Figure 4.8. Myocardial nitric oxide synthase activity in the 4 experimental groups at 20 minutes reperfusion, 10 minutes into ischaemia and 10 minutes into reperfusion (n= 5 per group per time point) (mean ± SEM)

ORD: Oxidative risk induced diet   **HFD: High saturated fat diet**
ORD+RPO: ORD with RPO-supplementation   **HFD+RPO: HFD with RPO-supplementation**

4.9 Cardiac superoxide dismutase activity

Results are presented in Figure 4.9. The SOD activity of the HFD+RPO was significantly higher than that of the HFD at the 20 minutes perfusion time point (3.45 ± 0.12 U/g wet weight *versus* 2.74 ± 0.24 U/g wet weight, $P<0.05$). There was no other significant differences between any of the groups at any time point (n=5 per group per time point).
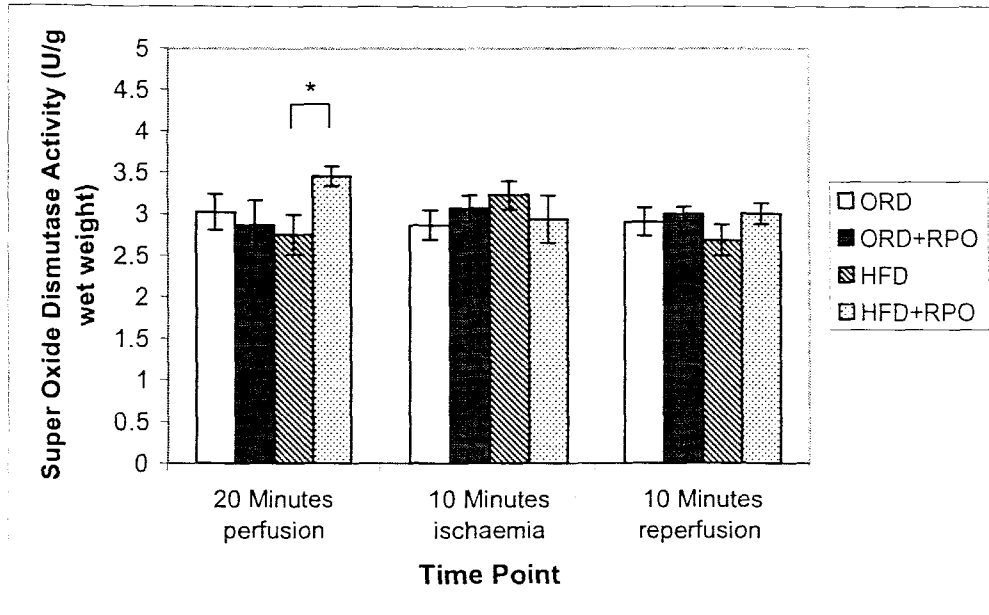
Figure 4.9. Superoxide dismutase activity in the 4 experimental groups before ischaemia, during ischaemia and in reperfusion (*P<0.05 for the group versus the indicated group) (n= 5 per group per time point) (mean ± SEM)
ORD: Oxidative risk induced diet   HFD: High saturated fat diet
ORD+RPO: ORD with RPO-supplementation   HFD+RPO: HFD with RPO-supplementation

4.10 % Change in total serum cholesterol

There were no significant differences in the percentage change in serum total cholesterol (TC) concentrations between the 4 experimental groups after the fourteen week supplementation period indicating that RPO-supplementation does not affect TC levels negatively. The baseline values for TC was: 1.28 ± 0.08 mmol/L for the ORD, 1.18 ± 0.07 mmol/L for the ORD+RPO, 1.44 ± 0.05 mmol/L for the HFD and 1.52 ± 0.04 mmol/L for the HFD+RPO. (n=9 per group per time point) (Figure 4.10).

Figure 4.10. % Change in total serum cholesterol in the 4 experimental groups after a 14- week diet period. (n = 9 per group per time point) (mean ± SEM)
ORD: Oxidative risk induced diet   HFD: High saturated fat diet
ORD+RPO: ORD with RPO-supplementation   HFD+RPO: HFD with RPO-supplementation

4.11 % Change in serum triacylglycerols

The significant differences in baseline serum triacylglycerol concentrations between the 4 experimental groups cannot be explained and requires further investigation as rats were randomly allocated to groups (ORD: 1.77 ± 0.20 mmol/L; ORD+RPO: 1.15 ± 0.08 mmol/L; HFD: 0.44 ± 0.02 mmol/L; HFD+RPO: 0.63 ± 0.06 mmol/L, $P<0.05$). The percentage change in serum triacylglycerol concentrations was similar for all groups after the diet period (n=9 per group per time point) (Figure 4.11).

Figure 4.11 % Change in serum triacylglycerols in the 4 experimental groups after a 14-week diet period (n = 9 per group per time point) (mean ± SEM)
ORD: Oxidative risk induced diet   HFD: High saturated fat diet
ORD+RPO: ORD with RPO-supplementation   HFD+RPO: HFD with RPO-supplementation

4.12 % Change in serum high density lipoprotein-cholesterol

The percentage change in serum HDL concentrations was significantly decreased for hearts of the rats of the HFD+RPO when compared to the ORD+RPO after the fourteen week diet period (-1.52 ± 4.72 % *versus* 34.77 ± 8.22 %, P<0.05). The baseline values for the HDL concentrations were: 0.73 ± 0.06 mmol/L for the ORD, 0.70 ± 0.04 mmol/L for the ORD+RPO, 1.00 ± 0.04 mmol/L for the HFD and 0.99 ± 0.03 mmol/L for the HFD+RPO. There were no other significant differences. However, the ORD and HFD appear to have opposite effects where the ORD increased HDL-cholesterol and the HFD decreased HDL (n=9 per group per time point) (Figure 4.12).

Figure 4.12. %Change in serum high density lipoprotein cholesterol in the 4 experimental groups after a 14-week diet period (n = 9 for each group at each time point)(*P< 0.05 for the group *versus* the indicated group)

ORD: Oxidative risk induced diet   HFD: High saturated fat diet
ORD+RPO: ORD with RPO-supplementation   HFD+RPO: HFD with RPO-supplementation

4.13 % Change in serum low density lipoprotein-cholesterol

There was a significant difference between the percentage change in the serum LDL-cholesterol of the ORD- and HFD groups after the fourteen week diet period (-28.9 ± 4.3 % *versus* -0.8 ± 5.7 %, P<0.05). RPO-supplementation did not affect serum LDL-cholesterol after the 14 week diet period. The baseline values for the LDL concentrations were: 0.26 ± 0.02 mmol/L for the ORD, 0.24 ± 0.04 mmol/L for the ORD+RPO, 0.23 ± 0.02 mmol/L for the HFD and 0.24 ± 0.02 mmol/L for the HFD+RPO (n=9 per group per time point, see Figure 4.13).
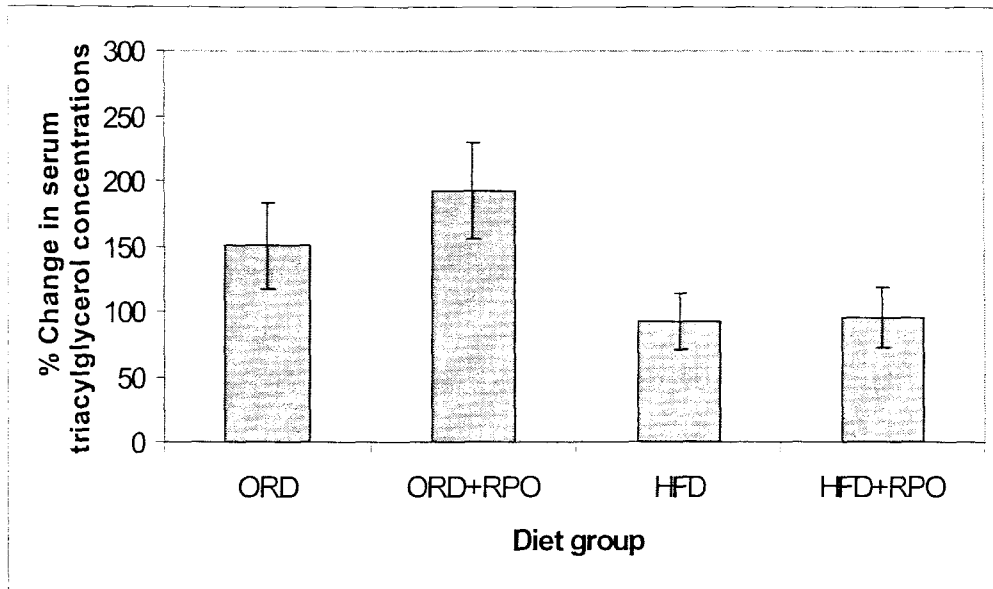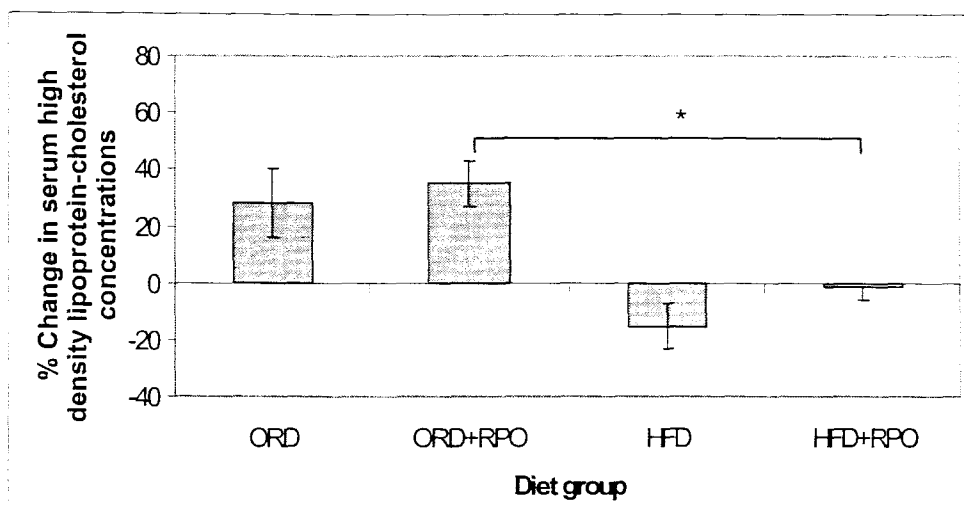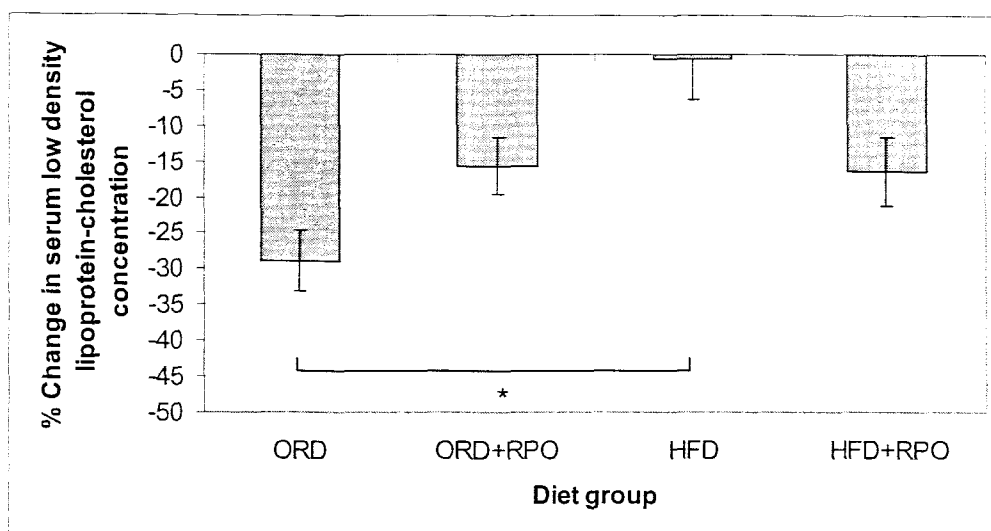
Figure 4.13. % Change in serum low density lipoprotein cholesterol in the 4 experimental groups after a 14-week diet period (n = 9 per group per time point)(*P<0.05 for the group *versus* *t*he RPO-supplemented group)(#P<0.05 for the group *versus* the indicated group)

ORD: Oxidative risk induced diet   HFD: High saturated fat diet
ORD+RPO: ORD with RPO-supplementation   HFD+RPO: HFD with RPO-supplementation

4.14 Myocardial total phospholipid fatty acid composition (%)

The major fatty acids (%) of myocardial total phospholipids before and after ischaemia in the 4 experimental groups are presented in Table 4.2. The myocardial total phospholipid fatty acid composition of the rat hearts were affected by both the diet and ischaemia. RPO-supplementation seemed to affect these levels much more in the ORD+RPO group when compared with the HFD+RPO group.

Baseline SFA- and MUFA concentrations were similar in the 4 experimental groups where baseline values refer to values at 25 minutes into perfusion before ischaemia was introduced. These values remained unchanged after the ischaemic period for the 4 experimental groups. However, the myocardial total PUFA- and n-3 PUFA composition in the ORD+RPO group were significantly increased after ischaemia (PUFA content: 49.82 ± 2.11 % before ischaemia and 55.50 ± 0.73 % after ischaemia and n-3 PUFA

content: 14.93 ± 1.12 % before ischaemia and 18.16 ± 0.26 % after ischaemia, respectively, $P<0.05$). Palmitic acid was significantly decreased in the ORD+RPO group after ischaemia (15.00 ± 0.85 % before ischaemia *versus* 12.04 ± 0.39 % after ischaemia) whereas arachidonic acid was significantly increased after ischaemia for the same group (17.05 ± 0.59 % before ischaemia *versus* 18.94 ± 0.21 % after ischaemia). The only significant differences between the ORD and ORD+RPO groups were in the n-6:n3 ratio and DHA composition after ischaemia (n-6:n-3 ratio: 2.73 ± 0.12 % *versus* 2.08 ± 0.02 % and DHA: 11.32 ± 1.56 % *versus* 15.83 ± 0.18 %, respectively).

Baseline values of linolenic acid (LA) of the HFD were significantly increased when compared with the HFD+RPO (18.82 ± 0.29 % *versus* 17.62 ± 0.51 %) Baseline AA in the ORD was significantly increased when compared with the HFD (19.40 ± 0.56 % *versus* 16.87 ± 0.27 %) and EPA was significantly decreased in the ORD+RPO when compared with the HFD+RPO (0.23 ± 0.02 % *versus* 0.37 ± 0.03 %).

The significant differences after the ischaemic period include a significantly increased PA content in the HFD when compared with the HFD+RPO (14.32 ± 1.11 % *versus* 13.12 ± 0.54 %), and a significantly lower EPA content in the ORD+RPO when compared with the HFD+RPO (0.23 ± 0.01 % *versus* 0.35 ± 0.02 %).

Table 4.2 Major fatty acids (%) of myocardial total phospholipids in the 4 experimental groups before and after ischaemia (n=5 per group per time point) (mean ± SEM)

	ORD		ORD+RPO		HFD		HFD+RPO	
	before	after	before	after	before	after	before	after
Total SFA	37.56 ± 0.50	45.03 ± 6.22	42.95 ± 2.24	37.50 ± 0.78	38.77 ± 0.91	41.96 ± 5.15	40.54 ± 1.88	39.10 ± 1.88
Total MUFA	7.40 ± 0.36	6.26 ± 0.66	7.24 ± 0.67	6.23 ± 0.21	7.23 ± 0.18	6.97 ± 0.56	7.28 ± 0.41	7.19 ± 0.32
Total PUFA	55.05 ± 0.40	48.70 ± 5.64	49.82 ± 2.11 [d]	55.50 ± 0.73	53.50 ± 0.92	51.06 ± 4.76	52.18 ± 1.81	53.71 ± 1.75
TN3	16.19 ± 0.34	13.25 ± 1.76	14.93 ± 1.12 [d]	18.16 ± 0.26	16.78 ± 0.88	15.84 ± 1.89	17.20 ± 1.05	16.97 ± 1.03
TN6	38.82 ± 0.44	35.41 ± 3.95	34.84 ± 1.79	37.76 ± 0.51	36.72 ± 0.32	35.20 ± 3.28	35.02 ± 0.90	36.75 ± 0.79
n-6:n-3 ratio	2.40 ± 0.07	2.73 ± 0.12 [a]	2.39 ± 0.22	2.08 ± 0.02	2.21 ± 0.12	2.28 ± 0.18	2.06 ± 0.10	2.20 ± 0.10
SFA:PUFA	0.68 ± 0.02	1.10 ± 0.37	0.88 ± 0.09	0.67 ± 0.02	0.73 ± 0.03	0.91 ± 0.24	0.78 ± 0.08	0.68 ± 0.03
16:0 PA	12.78 ± 0.47	13.39 ± 1.69	15.00 ± 0.85 [d]	12.04 ± 0.39	13.43 ± 0.58	14.32 ± 1.11 [a]	13.94 ± 0.60	13.12 ± 0.54
18:1n-9 OA	3.33 ± 0.24	2.80 ± 0.25	3.29 ± 0.50	2.87 ± 0.11	3.40 ± 0.07	3.05 ± 0.24	3.35 ± 0.27	3.17 ± 0.25
18:2n-6 LA	18.11 ± 0.64	17.28 ± 0.08	16.29 ± 1.54	17.53 ± 0.62	18.82 ± 0.29 [a]	17.74 ± 1.90	17.62 ± 0.51	19.10 ± 0.70
18:3n-3 ALA	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.03	0.07 ± 0.03	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.003
20:4n-6 AA	19.40 ± 0.56 [b]	16.82 ± 2.13	17.05 ± 0.59 [d]	18.94 ± 0.21	16.87 ± 0.27	16.48 ± 1.81	16.41 ± 0.73	16.60 ± 0.39
20:5n-3 EPA	0.25 ± 0.01	0.20 ± 0.03	0.23 ± 0.02 [c]	0.23 ± 0.01 [c]	0.33 ± 0.02	0.28 ± 0.03	0.37 ± 0.03	0.35 ± 0.02
22:6n-3 DHA	13.88 ± 0.37	11.32 ± 1.56 [a]	12.60 ± 1.22	15.83 ± 0.18	14.43 ± 0.78	13.68 ± 1.74	14.72 ± 0.10	14.59 ± 0.93

SFA=Saturated fatty acids
PUFA=Polyunsaturated fatty acids
MUFA=Monounsaturated fatty acids
TN6= (n-6) Polyunsaturated fatty acids
TN3= (n-3) Polyunsaturated fatty acids

a: $P<0.05$ for the group versus its RPO supplemented group of the same diet
b: $P<0.05$ for the group versus the control group of the other diet
c: $P<0.05$ for the group versus the RPO supplemented group of the other diet
d: $P<0.05$ for the group before ischaemia versus the same group after ischaemia

ORD: Oxidative risk induced diet
ORD+RPO: ORD with RPO-supplementation
HFD: High saturated fat diet
HFD+RPO: HFD with RPO-supplementation
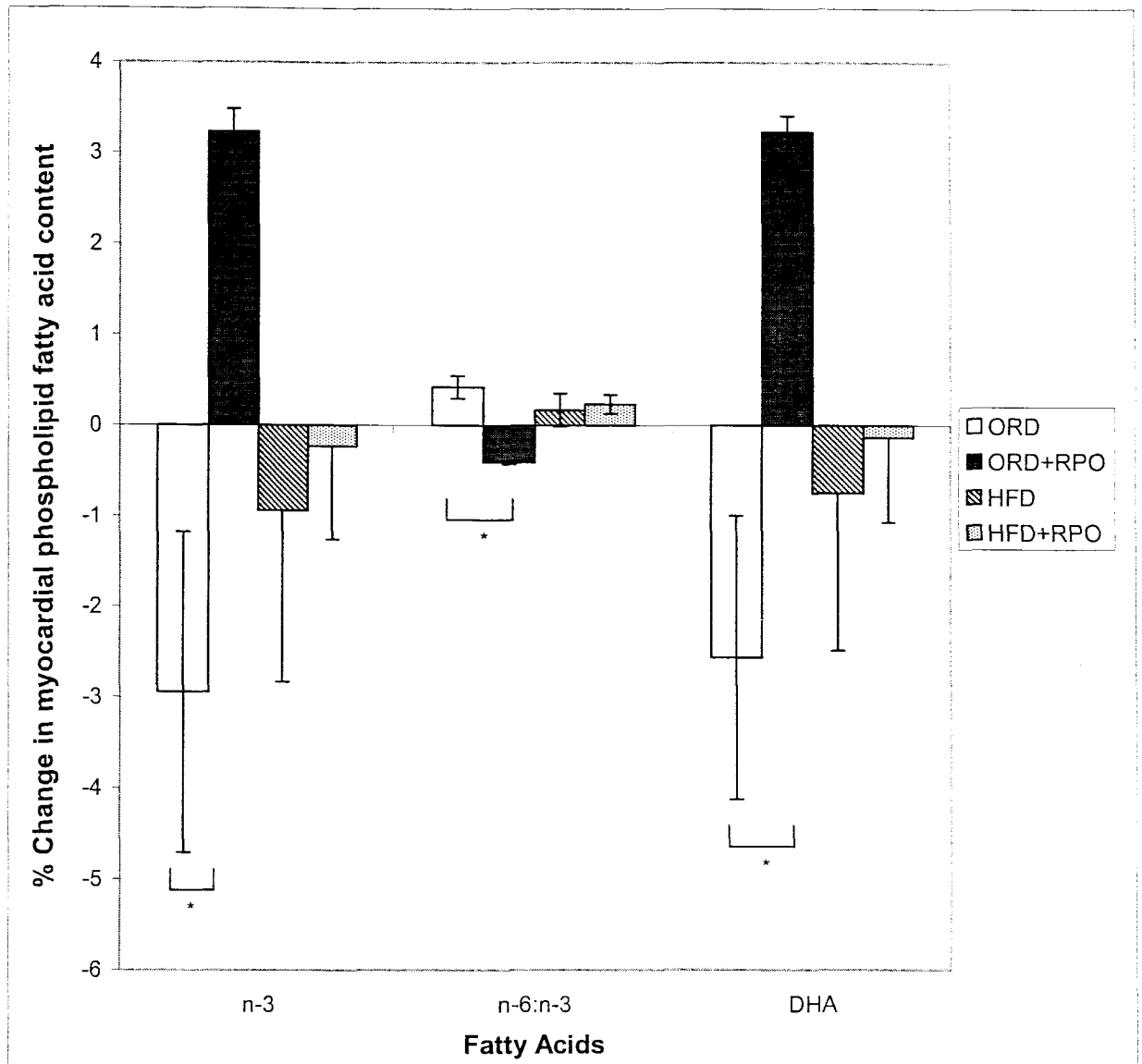
Figure 4.14. % Change in myocardial phospholipid fatty acid composition in the 4 experimental groups after an ischaemic period of 25 minutes, 10 minutes into reperfusion (n = 5 per group per time point) (*P<0.05 for the group versus the indicated group) (mean ± SEM)

ORD: Oxidative risk induced diet   HFD: High saturated fat diet
ORD+RPO: ORD with RPO-supplementation   HFD+RPO: HFD with RPO-supplementation
n-3: Omega 3 PUFAs   n-6:n-3: Omega 6- to omega 3 PUFA ratio
ALA: Alpha linolenic acid   DHA: Docosahexaenoic acid

All values in Figure 4.14 represent the percentage change in phospholipid fatty acid composition between the baseline value and the value of the corresponding experimental group at 10 minutes into reperfusion. These differences show that the fatty

acid metabolism of the ORD+RPO group was affected much more by the ischaemic period when compared with the other groups. The % change in the n-3 PUFAs of the ORD+RPO was increased when compared with the ORD (3.23 ± 0.255 % *versus* -2.94 ± 1.76 %, *P*<0.05). This change was reflected in the n-6:n-3 ratio, where the % change in ORD+RPO was significantly decreased when compared with the ORD (-0.31 ± 0.02 % *versus* 0.32 ± 0.12 %, *P*<0.05). These significant differences in % change in n-3 PUFAs is probably due to the DHA changes, where the % change was significantly increased in the ORD+RPO when compared with the ORD (3.23 ± 0.18 % *versus* -2,56 ± 1.56 %, *P*<0.05).

CHAPTER 5

Discussion

In this study we set out to investigate the effects of RPO as a supplement to diets known to have deleterious effects on the heart, and ischaemia/reperfusion injury. Diets used in this study were designed to be isocaloric and none of the differences observed between any of the groups were due to a difference in energy consumption of these groups but rather a difference in the composition of the diets. It is also interesting to note that the groups containing RPO as supplement showed very similar trends in their aortic output recovery and cGMP results, which indicate that RPO is effective in altering these parameters and therefore offers cardioprotection, irrespective of the fat content of the diet. The significant differences found in the aortic output recovery were not reflected in the rate pressure product recovery. This may be due to the fact that the rate pressure product depends only upon LVDevP and heart rate, while aortic output is a measure of all the factors that influence heart function. Rate pressure product is normally associated with the Langendorff perfusion apparatus while we used a working heart perfusion apparatus. Our results clearly indicate that the pathways involved in the protection against ischaemia/reperfusion injury of both the ORD+RPO and HFD+RPO groups are similar and that the NO-cGMP pathway seems most likely to offer this protection. Esterhuyse and co-workers (2005 b) suggested that most of the RPO-induced changes occurred during the ischaemic period with the NO-cGMP pathway being a major role player. These authors also speculated that RPO-supplementation of a high cholesterol diet probably protects the isolated rat heart against ischaemia/reperfusion injury by mechanisms independent of the NO-cGMP pathway. Engelbrecht and co-workers 2006 showed that dietary RPO-supplementation alters the activation of the MAPK and PKB in

reperfusion and may offer protection from ischaemia/reperfusion injury through these pathways.

Ong and Goh (2002) stated that RPO-supplementation had hypercholesterolaemic effects when used in conjunction with a high fat and high cholesterol diet. In our study, using the ORD and HFD as unhealthy diets with fat and/or cholesterol added failed to increase serum total cholesterol with RPO-supplementation (Figure 4.10). Esterhuyse and co-workers (2005 b) showed that RPO-supplementation in the presence of potentially harmful cholesterol did not lead to a significant increase in serum cholesterol. This may be due to the low serum cholesterol and LDL-cholesterol concentrations of rats, irrespective of high levels of dietary cholesterol, associated with a decreased cholesterylester transfer protein (CTEP) activity in their plasma (Van Tol et al., 1995; Ferdinandy et al., 1998; Diniz et al., 2004).

5.1 Cardiovascular health: oxidative risk induced diet *versus* high saturated fat diet

The improved aortic output recovery (Figure 4.4) and decreased left ventricular diastolic pressure (Table 4.1) of hearts of the ORD+RPO group when compared with hearts of the HFD+RPO group may be associated with the high concentrations of dietary PUFAs which is associated with a favorable serum lipid profile (Diniz et al., 2004). Our results indicate that compositional differences of the diets and not RPO-supplementation may have a detrimental effect on cardiovascular health.

The weight of rats in both HFD and HFD+RPO groups was significantly increased after the 14 week diet period when compared with the rats in the corresponding ORD and

ORD+RPO groups (Figure 4.1). This may be due to the high SFA content of this diet which has been shown to inhibit the hypothalamic apo A-IV system which controls the feeling of satiety. This leads to hyperphagia, which in turn may lead to increased weight, increased body fat, hyperinsulinaemia and insulin resistance, even if the animals do not become obese (Woods *et al.*, 2004). Both the weight increase and serum lipid profile of this group are serious risk factors for cardiovascular disease.

5.1.1 Effects of the ORD and HFD on the serum lipids

The major beneficial effect associated with ORD *versus* the HFD is a decrease in LDL-cholesterol. With the addition of RPO an increase in HDL-cholesterol was observed in the ORD+RPO group when compared to the HFD+RPO (Figure 4.12 and 4.13). These effects may be associated with beneficial effects of dietary PUFAs. Diniz and co-workers (2004) reported that diets rich in PUFAs showed beneficial effects on serum lipids and offer cardioprotection. It is interesting to note however, that although the HFD+RPO had significantly decreased HDL-cholesterol concentrations, when compared to the ORD+RPO, the percentage change in LDL-cholesterol was similar. Furthermore, the triacylglycerols and total cholesterol were similar in all the diet groups (Figure 4.10 and 4.11).

5.1.2 The effects of the ORD and HFD on the NO-cGMP pathway

The ORD and ORD+RPO had decreased myocardial NO concentrations at all time points when compared with the HFD and HFD+RPO according (nitrate/nitrite assay, see Figure 4.7). However, hearts of these groups showed no significant differences in NOS activity when compared with hearts of the HFD and HFD+RPO at any time points (Figure 4.8). This may be due to abundant free radicals which leads to depletion of NO

as NO will act as an antioxidant in the absence of sufficient antioxidants. The depletion of NO by ROS, rather than a reduction in production by NOS, is also suggested by the unchanged NOS activity in all groups throughout the experiment. The rate of PUFA oxidation influences depletion of antioxidants as sufficient antioxidants should be able to protect the PUFAs from oxidation (Newaz et al., 2003). It could be speculated that a high concentration of dietary PUFAs induces a high level of oxidative stress, especially during ischaemia. However, depletion of NO interfered only partially with the NO-cGMP pathway signalling, as indicated by the significant increase in cGMP concentrations during ischaemia in the ORD and ORD+RPO groups (Figure 4.5).

Increased NO concentrations in hearts of the HFD when compared with the ORD were associated with unchanged NOS activity. This may be explained by lower oxidative stress in the HFD when compared with the ORD. Esterhuyse and co-workers (2005 b) concluded that NO production is not interrupted in the presence of cholesterol, but that it may be broken down by ROS which is produced at higher concentrations in a hyperlipidaemic myocardium.

Another mechanism that may offer cardioprotection in ORD includes the NO-cGMP pathway where ORD without cholesterol-supplementation could lead to unhindered signalling via the NO-cGMP pathway and ultimately better cardioprotection. However, cGMP concentrations in hearts of the HFD group were found to be similar to those of hearts of the ORD group throughout the experiment. The percentage cGMP increase during ischaemia of the ORD+RPO appears to be significantly higher than that of the HFD+RPO (Figure 4.5). This was linked to a significantly increased cAMP in the HFD+RPO when compared to the ORD+RPO at the same time point (Figure 4.6). This

increase in cAMP which is associated with poor aortic output recovery is probably due to components in the diet other than cholesterol, such as saturated fats, or merely reflects the greater ischaemic stress that HFD hearts experienced.

5.1.3 Effects of the ORD and HFD on myocardial phospholipid fatty acid composition

Our results have shown an increased % baseline AA content in hearts of rats fed the ORD when compared with hearts of rats fed the HFD. Normally LA undergoes a series of elongations and desaturations to yield AA (Table 4.2). Myocardial phospholipid fatty acid composition in the HFD+RPO group was unaffected by the ischaemic period. However, HFD+RPO showed increased baseline EPA phospholipid fatty acids when compared with ORD+RPO which can be associated with less oxidation of PUFAs in this group. These hearts were unable to adapt to stress and aortic output recovery after ischaemia/reperfusion was poor when compared to the hearts of the rats in the ORD+RPO group. Changes in myocardial fatty acid composition could be associated with differences in dietary fatty acid composition, with the ORD containing much higher concentrations of PUFAs than the HFD. This is probably the reason for the difference in AA between the ORD and HFD and for the HFD+RPO having a significantly increased EPA content when compared to the ORD+RPO before and after ischaemia.

5.2 Effects of RPO-supplementation of the ORD

Our results indicate that dietary RPO-supplementation of the ORD diet was effective in providing protection against ischaemia/reperfusion injury as indicated by the improved aortic output recovery (Figure 4.4). Evidence in this study suggests that both antioxidants and fatty acids play a role in the cardioprotective mechanisms of dietary

RPO-supplementation. Antioxidants may offer myocardial protection by quenching ROS, and thereby allowing unhindered NO-cGMP pathway signalling (Maulik et al., 1995). Changes in total myocardial phospholipid fatty acid content induced by dietary RPO-supplementation may in part be due to the antioxidant effect of vitamin E, as it is known to protect fatty acids from peroxidation (Burton and Ingold, 1981; O'Farrel and Jackson, 1997; reviewed by Pryor, 2000). However, the increase in PUFAs, especially n-3 PUFAs, associated with dietary RPO-supplementation may ultimately lead to increased eicosanoid production, and therefore myocardial protection (reviewed by Abeywardena and Head, 2001).

Dietary RPO-supplementation of the ORD showed no weight increase after 14 weeks, when compared to the control diet. This leads to the conclusion that the SFAs which are contained in RPO (51%) do not lead to weight gain (Figure 4.1).

5.2.1 Effects of RPO-supplementation of the ORD on the serum lipids

RPO-supplementation was shown to raise TC and LDL-cholesterol when hypercholesterolaemic subjects and high fat liquid formula diets were used (Ong and Goh, 2002; Sundram and Basiron, 2004). However, our results have shown that RPO-supplementation of ORD did not lead to significant differences in serum total cholesterol, triacylglycerol or HDL-cholesterol (Figure 4.10, 4.11 and 4.12). The percentage change in the serum LDL-cholesterol concentrations of both the ORD and ORD+RPO was decreased when compared to the baseline (Figure 4.13). These data confirm the results obtained by Diniz and co-workers (2004).

The reason why RPO-supplementation does not have hypercholesterolaemic effects may be due to tocotrienols, which have a hypocholesterolaemic effect by indirectly inhibiting 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Khor *et al.*, 1995; Theriault *et al.*, 1999; O'Byrne *et al.*, 2000; Quereshi *et al.*, 2002; reviewed by Ong and Goh, 2002).

5.2.2 Effects of RPO-supplementation of the ORD on myocardial phospholipid fatty acid composition

Our results show that dietary RPO-supplementation caused significant changes in fatty acid composition of myocardial tissue measured before and after ischaemia. The major changes during this period include a significant increase in myocardial PUFA content, and more specifically an increase in total n-3 PUFAs and AA which are metabolized to form eicosanoids which are known to have a variety of beneficial cardiovascular effects (reviewed by Abeywardena and Head, 2001) (Table 4.2 and Figure 4.14). Esterhuyse and co-workers (2005 b) showed that RPO-supplementation increased total myocardial PUFAs over the 25-minute period of ischaemia in rats fed on a standard rat chow diet. Diniz and co-workers (2004) reported that changes in dietary fatty acids affect cardiac oxidative stress. They showed that diets rich in PUFAs, despite the beneficial effects on serum lipids, were deleterious when compared with SFAs in the heart by increasing cardiac susceptibility to lipid peroxidation. Their observation that SFA-fed rats had lower myocardial hydroperoxide concentrations than PUFA-fed rats, demonstrate the importance of the PUFA:SFA ratio on lipid peroxidation and the level of antioxidants. Bagchi and Puri (1998) reported that vitamin E protects PUFAs in cell membranes from peroxidation, which may confer some of the cardioprotection in the RPO-supplemented group in our study. The n-6:n-3 PUFA ratio of the ORD+RPO was lower than that of the

ORD after ischaemia and can be attributed to the significantly higher DHA in this supplemented group, when compared to the ORD. A n-6:n-3 ratio of 1:1 is thought to be optimal for normal physiological functioning, therefore, the lower ratio in this case would lead to increased functional recovery (reviewed by Simopoulos, 2002).

5.2.3 Effects of RPO-supplementation of the ORD on the cyclic nucleotides

Improved functional recovery of hearts of rats supplemented with RPO when compared with hearts of rats on an ORD diet was associated with elevated cGMP concentrations early in ischaemia (Figure 4.5). These results are in agreement with other studies which showed that dietary RPO-supplementation offered protection of hearts from rats on a standard rat chow (control) diet against ischaemia/reperfusion injury as reflected by improved aortic output recovery. This was associated with an increase in cGMP and a decrease in cAMP during ischaemia in the RPO-supplemented versus control group (Esterhuyse et al., 2005 b). Based on our results we propose that an unhealthy diet rich in PUFAs associated with oxidative stress does not interfere with the ability of RPO to increase cGMP production in the myocardium during ischaemia/reperfusion injury.

5.2.4 Effects of RPO-supplementation of the ORD on NO and other free radicals

The NO concentrations and NOS activity of the ORD+RPO group were similar to that of the ORD throughout the experiment (Figure 4.7 and 4.8). Maulik and co-workers (1995) showed that NO plays a significant role in transmembrane signalling in the ischaemic myocardium. The signalling seems to be transmitted via cGMP and they suggested that NO signalling is switched on and off due to inactivation of NO by ROS. Therefore, if dietary RPO containing vitamin E antioxidants act as free radical scavengers, elevated cGMP concentrations in early ischaemia through NO signalling could be responsible for

improved recovery in hearts of rats supplemented with RPO *versus* non-RPO fed rat hearts in the ORD group. Furthermore, by virtue of its antioxidant property, NO offers protection especially through its ability to attenuate the deleterious free radical action of O_2^- and to terminate $ONOO^-$ mediated lipid radical chain propagation (Rubbo *et al.*, 1994).

5.2.5 Possible pathways involved in the protection offered by RPO-supplementation of the ORD

The improved aortic output recovery obtained with RPO-supplementation of the ORD diet is a clear indication that it was able to limit ischaemia/reperfusion injury in the hearts of the ORD/RPO group (Figure 4.4). This improved recovery of hearts of rats supplemented with RPO when compared with hearts of rats on ORD can be associated with elevated cGMP concentrations early in ischaemia (Figure 4.5). In this regard it is possible that cGMP may attenuate ischaemia/reperfusion injury by inhibiting the cAMP-induced slow inward calcium current, thus leading to a decrease in cytosolic calcium concentrations (Du Toit *et al.*, 2001). Furthermore, based on our results, we propose that the protective effect of RPO in the ORD diet may be due to the dietary vitamin E antioxidant characteristics, which may have reduced the oxidative stress induced by the diet. Vitamin E acts as a free radical scavenger that can react with oxygen, superoxide anion radicals and hydroxyl radicals (Abudu *et al.*, 2004).

Furthermore, the increased n-3 fatty acids and AA could lead to increased eicosanoid production which may offer protection against ischaemia/reperfusion injury (reviewed by Abeywardena and Head, 2001) (Table 4.2 and Figure 4.14).

5.3 Effects of RPO-supplementation of the HFD

Our results demonstrate that RPO-supplementation offers protection against ischaemia/reperfusion injury in the isolated perfused working heart as reflected by improved aortic output recovery and RPP (Figure 4.3 and 4.4). The improved functional recovery of hearts from rats supplemented with RPO *versus* the HFD without RPO was preceded by an elevation in the cGMP concentrations early during ischaemia (Figure 4.5). This data supports the findings by Esterhuyse and co-workers (2005 a and b) that increased cGMP concentrations early in ischaemia is partially responsible for the protective effects of dietary RPO-supplementation. Baseline, ischaemic and reperfusion NO concentrations and NOS activity were similar in HFD and HFD+RPO groups at all times investigated (Figure 4.7 and 4.8).

The weight of rats of the HFD+RPO was similar to those of the HFD after dietary supplementation for 14 weeks, indicating that RPO-supplementation does not affect weight gain even when used in conjunction with a diet containing high concentrations of fat and cholesterol (Figure 4.1). It is also important to note that the percentage change in LDL-cholesterol concentrations of the HFD+RPO was decreased after the diet period, with no change in LDL-cholesterol of the HDF (Figure 4.13).

5.3.1 Effects of RPO-supplementation of the HFD on myocardial phospholipid fatty acid composition

There were no changes in myocardial phospholipid fatty acid composition in this group during the ischaemic period. However, the SFA:PUFA ratio was decreased showing that RPO was effective in preventing oxidation of PUFAs that were incorporated into the myocardial membranes. The decreased LA content in hearts in the HFD+RPO before

ischaemia when compared with hearts of the HFD can be explained by the composition of the diet. It is well known that when on a high cholesterol diet, membrane fatty acid composition shows increases in the longer chain PUFAs in order to compensate for and maintain membrane fluidity as a result of increased cholesterol molecules in the membrane. After the ischaemic period the hearts of the HFD+RPO had a significantly lower PA content than the HFD. This may be due to replacement of PA by unsaturated fatty acids provided by RPO supplementation in the HFD+RPO, as indicated by the SFA:PUFA ratio (Table 4.2).

5.3.2 Possible pathways involved in the protection offered by RPO-supplementation of the HFD

Based on our results we propose that the protective effect of RPO-supplementation in HFD may be associated with either NO-cGMP pathway activation, RPO antioxidant characteristics and/or changes in the phospholipid fatty acid composition in the myocardium during ischaemia/reperfusion.

5.4 Limitations of the study

The major limitation of this study is that it did not include a standard rat chow diet group as a control. We designed our study to focus on the effects of dietary RPO-supplementation, rather than the effects of the ORD and HFD. Thus the groups that were not supplemented with RPO served as controls to the RPO-supplemented groups in this study. Secondly, it would have been difficult to render a standard rat chow diet to be isocaloric, as both the ORD and the HFD used in this study were high energy diets. The changes that may have been observed if a standard rat chow diet was included

may then be related to differing energy intake effects and not differences in dietary composition.

CHAPTER 6

Summary and conclusions

Our results show that HFD+RPO caused decreased myocardial post-ischaemic functional recovery when compared with the ORD+RPO. However, dietary RPO-supplementation offered protection of hearts from rats on both ORD and HFD-against ischaemia/reperfusion injury as reflected by improved aortic output recovery. These results have shown that dietary RPO-supplementation is effective in protecting the myocardium from ischaemia/reperfusion injury even though the rats were fed unhealthy diets. Due to the fact that these diets were designed to be isocaloric, results are not related to differences in energy consumption between the groups, but rather with compositional differences of the diets.

Based on our results we propose that the protective effect of RPO in both ORD and HDF may be associated with an increase in cGMP early in ischaemia. We hypothesize that the palm oil vitamin E antioxidant properties may contribute to the elevated cGMP which may attenuate ischaemia/reperfusion injury. The lack of an increase in myocardial cAMP concentrations during ischaemia may be associated with increased cGMP concentrations in all groups, and also the fact that the ischaemia damage does not appear to be severe due to the low temperature during the ischaemic period. Increased cAMP concentrations of hearts of HFD when compared with ORD at 10 minutes reperfusion may be associated with poor aortic output recovery of hearts of HFD caused by compositional differences of diets.

Our data show decreased NO concentrations of the ORD+RPO when compared with HFD+RPO. This may be due to higher oxidative stress in these hearts associated with increased superoxide production which will bind to NO to form ONOO⁻, thus leading to depletion of NO. Concurrently, increased myocardial NO content for the HFD+RPO was associated with unchanged NOS activity which support the argument that oxidative stress influences the outcomes when the ORD or the HFD are supplemented with RPO. However, the quenching of NO did not seem to affect NO-cGMP signalling as cGMP was increased in both the ORD groups during ischaemia.

Although our data indicate increased n-3 fatty acids in the ORD+RPO after ischaemia, this increase could not be linked with dietary supplementations as neither the ORD, nor RPO is rich in n-3 fatty acids. It is well known that mammals (including rats) are deficient of n-3 desaturases and can therefore not produce n-3 fatty acids *in vivo*. However, the increase may be beneficial to cardiovascular health through the production of eicosanoids (reviewed by Abeywardena and Head, 2001).

It has been shown that RPO-supplementation may raise TC and LDL-cholesterol when hypercholesterolaemic subjects and high fat liquid formula diets were used (reviewed by Ong and Goh, 2002). However, our results have shown that RPO-supplementation of rats fed unhealthy diets high in fat and cholesterol did not lead to an increase in serum total cholesterol.

Therefore, our results indicate that dietary RPO-supplementation offered protection against ischaemia/reperfusion injury of rats fed unhealthy diets. We propose that the NO-cGMP pathway is the most likely pathway of protection and that fatty acid changes,

particularly n-3 fatty acids may also play a role. Based on our results we propose that the myocardial protection offered by RPO-supplementation may be associated with either its antioxidant characteristics and/or changes in the fatty acid composition of the myocardium during ischaemia reperfusion.

This study raised questions which may need further investigation: 1) We can only speculate as to the cause of the changes in the myocardial total phospholipid fatty acid composition induced by RPO, as RPO not only contributes to the fatty acid content of the diet, but it is also rich in antioxidants and trace elements, which would also exert effects on fatty acid metabolism. 2) Contrary to expected results, cAMP concentrations did not increase at all in some groups during ischaemia. Therefore, the findings of this study create opportunities for further investigations to elucidate RPO mediated mechanisms involved in cardiac protection of animals fed unhealthy diets.

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