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Effects of dietary red palm oil supplementation on myocardial infarct size in normal and hypercholesterolaemic rats: The role of matrix metalloproteinase 2, glutathione peroxidase transcription and extracellular signal regulated kinase phosphorylation.

By

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Declaration

I declare that this thesis is my own work. It is being submitted for the degree of Doctor Technologiae in Biomedical Technology to the Cape Peninsula University of Technology. It has not been previously submitted for any other degree or diploma at any other institution. The opinions and conclusions drawn are not necessarily those of the Cape Peninsula University of Technology.

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Dirk J Bester   Date

Acknowledgements

I would like to thank my wife for the sacrifices that she has made for me during this study.

Finally I would like to thank all my friends and family for their continued support throughout all my years of study.

Preface

The work presented in this thesis is written in manuscript form and consists of four reports. The first article is a review which summarizes the cardiovascular effects of five popular edible oils in order to create an understanding of the literature concerning these oils. The reader will be made aware of shortcomings in edible oil research in general and opportunities for further research in the field of edible oil associated with cardiovascular health are proposed. Additionally, this article describes the advantages and disadvantages of dietary red palm oil (RPO) supplementation, and offers the reader greater insight into the effects of RPO supplementation for further research. The first article has been placed at the end of the literature review, as it replaces a large part of the review by nature of its content. The effects of dietary RPO supplementation on the cardiovascular system will only be discussed in this document as part of the literature review.

The second article deals with the effects of dietary RPO supplementation on myocardial infarct size in an isolated perfused rat heart model. This document also describes the effects of dietary RPO supplementation on matrix metalloproteinase 2 activity in the heart.

The last two articles report on the result of follow-up studies from previous research performed in our laboratory. The third article included in this thesis describes the effects of dietary RPO supplementation on myocardial infarct size and matrix metalloproteinase 2 activity in cholesterol fed rats. Previous studies

have reported that cholesterol feeding may inhibit protective mechanisms in the heart. However, it has also been reported that dietary RPO supplementation is able to offer protection against ischaemia/reperfusion injury as was demonstrated by functional recovery. The final article in this dissertation describes the effects of dietary RPO supplementation on genetic transcription of glutathione peroxidase. Additionally, this document reports on the effects of RPO supplementation on extra cellular signal regulated kinases.

Chapters describing materials and methods used, a discussion of the results obtained and a conclusion have also been included in this thesis in order to summarize all the work performed in order to produce this thesis.

The work performed towards the first two original work articles was performed in the laboratory of Prof. Peter Ferdinandy at the University of Szeged. The candidate however played a major role (60 – 70 %) in all experiments performed towards these manuscripts. The final manuscript contains work performed at the Experimental Anitioxidant Research Division at the Cape Peninsula University of Technology, the laboratory of Prof. Amanda Lochner at the University of Stellenbosch and the laboratory of Prof. Anne Jonassen at the University of Bergen. All the work in this study was performed by the candidate, with the exception of the glutathione peroxidase gene experiments; witch was performed by a staff member of Prof. Anne Jonassen.

Abstract

Cardiovascular disease remains one of the leading causes of death globally. Many pharmacotherapeutic strategies are constantly being developed in order to effectively reduce the prevalence, as well as the morbidity and mortality associated with cardiovascular disease. In recent years, studies in our laboratory were able to demonstrate that dietary red palm oil (RPO) supplementation could offer protection against myocardial ischaemia/reperfusion injury. Several possible mechanisms for this protection were proposed, including: 1) upregulation of nitric oxide (NO), 2) cyclic guanylyl-mono-phosphate (cGMP) signaling, 3) scavenging of harmful free radicals associated with ischaemia/reperfusion injury and 4) the modulation of mitogen activated protein kinase (MAPK) signaling pathways which inhibit myocyte apoptosis. These findings created opportunities for further investigations to be conducted in order to elucidate RPO mediated pathways involved in cardiac protection.

Matrix metalloproteinase 2 (MMP2) is an endogenous protease which is normally associated with the digestion of gelatin. This protease has recently become the focus of many cardiovascular studies, as it was found to be a mediator of ischaemia/reperfusion injury. Activation of MMP2 by free radicals leads to induction of myocyte death through degradation of intracellular targets.

Research has shown that dietary RPO supplementation is able to increase activity of glutathione peroxidase (GPX) which is an endogenous antioxidant

expressed in most mammalian tissue, including the heart. Increased antioxidant activity in the heart may lead to a reduction in ischaemia/reperfusion injury. However, the mechanism by which RPO supplementation was able to increase GPX activity is not known.

The aims of the study were: 1) To investigate whether dietary RPO supplementation can reduce myocardial infarct size of rats fed a standard rat chow diet and rats fed a cholesterol supplemented diet 2) To investigate the whether inhibition of MMP2 plays a role in RPO mediated protection against ischaemia/reperfusion injury 3) To determine whether dietary RPO supplementation regulates GPX activity through gene transcription.

Three study designs are described in this thesis where use was made of a male Wistar rat model being fed RPO supplemented diets for a 5 to 9 week period. Study design 1 compared rats placed on RPO supplemented diets with sunflower oil (SFO) fed rats and standard rat chow (SRC) fed rats while Study design 2 compared rats fed cholesterol supplemented diets with rats placed on a cholesterol+RPO supplemented diet. Study design 3 makes use of similar groups as Study design 1. After the supplementation period, rats were sacrificed and the excised hearts perfused on either a Langendorff perfusion apparatus or a working heart perfusion apparatus. Myocardial infarct size and aortic output recovery were measured in order to determine whether RPO does offer protection against ischaemia/reperfusion injury. MMP2 activity was measured in

coronary effluent samples of the hearts, in order to determine its level of activity in RPO supplemented hearts. GPX1, -3 and -4 gene transcription were also measured by quantitative real time polymerase chain reaction (qrtPCR), in hearts of RPO supplemented rats, and compared to controls after supplementation. Other assays performed include ELISA (enzyme linked immunosorbent assay) for the determination of serum cholesterol, serum triglyceride and myocardial 3-nitrotyrosine levels as well as western blots to determine protein kinase B (also known as Akt (PKB/Akt)) and extracellular signal-regulated kinases (ERK) in myocardial tissue.

Our results showed that dietary RPO supplementation was able to reduce myocardial infarct size in SRC fed rats (9.17 ± 1.03% in the RPO group, *versus* 30.20 ± 3.97% in the SRC group), as well as rats fed a cholesterol supplemented diet 26,87 ± 2,96% in the HCRPO group, *versus* 37.16 ± 3.58% in rats fed a cholesterol supplemented diet). Dietary RPO supplementation was also able to increase aortic output recovery in SRC fed rats (47.16 ± 5.46 % *versus* 13.44 ± 6.34 %). The decreased infarct size was associated with decreased MMP2 activity during reperfusion in the SRC fed rats (1389.27 ± 124.34 arbitrary units *versus* 1724.42 ± 69.77 arbitrary units; 72kDa isoform: 2635.03 ± 163.02 arbitrary units *versus* 3201.63 ± 104.97 arbitrary units). Dietary SFO supplementation reduced MMP activity at the same time point, with no reduction in infarct size. Dietary supplementation of RPO and cholesterol to rats, led to decreased MMP2 activity before ischaemia (228.43 ± 28.06 arbitrary units *versus*

450.83 ± 33.62 arbitrary units). The MMP2 activity was significantly increased in the cholesterol+RPO supplemented group when compared to the cholesterol fed rats after ischaemia (2107.06 ± 50.99 arbitrary units *versus* 1821.90 ± 56.92 arbitrary units). RPO supplementation did not show any significant differences in GPX transcription.

We therefore conclude that dietary RPO supplementation reduced myocardial infarct size in SRC fed rats and rats fed a cholesterol supplemented diet. Inhibition of MMP2 activity was also shown not to be a major pathway of protection involved in RPO mediated protection against ischaemia/reperfusion injury. Furthermore, our results are in agreement with previous studies which show that dietary RPO supplementation is able to improve aortic output recovery. RPO supplementation does however, not increase GPX activity through upregulation of GPX gene transcription.

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Abbreviations

ATP	Adenosine-triphosphate
BHT	Butylated hydroxytoluene
cGMP	Cyclic guanlylyl-mono-phosphate
COX-2	Cyclooxygenase-2
DMSO	Dimethyl sulfoxide
ERK	Extracellular signal regulated kinases
GPX	Glutathione peroxidase
GSSH	Reduced glutathione
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
JNK	c-Jun N-terminal kinase
LDL	Low density lipoprotein
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
MMP	Matrix metalloproteinase
MUFA	Mono-unsaturated fatty acid
NCEP	National Cholesterol Education Program
NO	Nitric oxide
$ONOO^-$	Peroxynitrite
O_2^-	Superoxide
PDK	Phosphoinositide-dependent protein kinases

PKB/Akt	Protein kinase B, also known as Akt
PO	Palm oil
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
RPO	Red palm oil
SFA	Saturated fatty acid
SFO	Sunflower oil
SRC	Standard rat chow
TBARS	Thiobarbituric acid-reactive substance
TIMP	Tissue inhibitors of metalloproteinase
TRF	Tocotrienol rich fraction
TTC	2,3,5-Triphenyltetrazolium chloride

CHAPTER 1

INTRODUCTION

Myocardial disease is one of the leading causes of death world wide (Braunwald and Kloner 1985; Banegas *et al.,* 2003; Jemal *et al.,* 2005). This necessitates the development of treatment strategies to reduce the mortality rate associated with myocardial infarction. Several pathological mechanisms have been suggested for myocardial ischaemia/reperfusion injury. Hearse and co-workers (1973) found that the reinstatement of oxygenation during reperfusion was associated with a rapid increase in cell death. This led to the conclusion that the development of free radicals plays a key role in the pathogenesis of ishaemia/reperfusion injury. This finding was confirmed by the work of other investigators, who found that overexpression of reactive oxygen species (ROS) in the ishaemic/reperfused myocardium can be associated with increased cell death (Ferrari *et al*., 1985; Ceconi *et al.,* 2003; Saotome *et al.,* 2009). These studies created an opportunity for investigation of the protective effects of antioxidants in the ishaemic/reperfused myocardium.

Red palm oil (RPO) is an antioxidant rich oil, which contains 51% saturated fatty acids (SFAs), 38% mono-unsaturated fatty acids (MUFAs) and 11% polyunsaturated fatty acids (PUFAs). Antioxidants within RPO include 500ppm carotenes of which 60% is β-carotene and 25% α-carotene. It also contains a minimum of 500ppm vitamin E of which 75% is tocotrienols and 25% tocopherols

(Nagendran *et al.,* 2000; Sundram *et al.,* 2003). The antioxidants present in palm oil were described by Serbinova and co-workers (1992) to be effective in offering protection against ischaemia/reperfusion injury. They used a tocotrienol-rich fraction of palm oil (TRF) which was added to the perfusion buffer in an isolated perfused rat heart model. The addition of TRF in the perfusion buffer was associated with improved reperfusion functional recovery. Esterhuyse and co-workers (2005) showed that dietary RPO supplementation protects against ischaemia/reperfusion injury in the isolated perfused rat heart. Another study by Esterhuyse and co-workers (2005b) demonstrated that dietary RPO supplementation is also able to improve reperfusion functional recovery in rats fed a cholesterol supplemented diet. From their work it is evident that dietary RPO supplementation protected standard rat chow (SRC) fed rats and rats fed a cholesterol supplemented diet through different mechanisms. These studies demonstrated that the NO-cGMP signaling pathway was involved in RPO mediated protection of the SRC fed rat, where dietary RPO supplementation increased myocardial NO levels through the scavenging of superoxide. This would by implication mean that RPO supplementation decreases peroxynitrite levels, as the product of NO and superoxide binding is peroxynitrite (Deliconstantinos *et al.*, 1995). Peroxynitrite is a cytotoxic radical which have been shown to cause cardiac contractile failure (Ferdinandy *et al.,* 2000) and MMP2 activation (Siwik *et al.,* 2001; Viappiani *et al.,* 2006).

Previous studies with RPO supplementation used functional parameters to determine whether RPO could offer protection against ischaemia/reperfusion injury (Esterhuyse *et al.,* 2005; Esterhuyse *et al.,* 2005b; Engelbrecht *et al.,* 2006; Esterhuyse *et al.,* 2006; Bester *et al.,* 2006; Kruger *et al.,* 2007). However, the measurement of functional recovery may be influenced by temporary contractile failure during the early phases of reperfusion (Yang *et al.,* 2004). Contractile failure early in reperfusion is the result of calcium overload and oxidation of contractile proteins which results due to ischaemic changes (Bolli and Marban, 1999). Therefore, measurement of infarct size, as an independent parameter of myocardial damage sustained during ischaemia/reperfusion injury, could explain possible protective mechanisms involved. Little is known about the effects of dietary RPO supplementation on myocardial infarct size.

Matrix metalloproteinase 2 (MMP2) activation through peroxynitrite has been shown to lead to increased infarct size and decreased functional recovery in the ischaemic/reperfused myocardium (Cheung *et al.,* 2000; Hayashidani *et al.,* 2003; Menon *et al.,* 2004; Bergman *et al.,* 2007; Fert-Bober *et al.,* 2008). This form of MMP2 activation does not take place through the conventional cleavage of the regulatory subunit of MMP2, but rather *via* a redox modification of this subunit (Okamoto *et al.,* 2001; Viappiani *et al.,* 2006). MMP2 activation in this manner has been associated with degradation of intracellular targets within the myocyte, rather than the conventional extracellular matrix proteins. Intracellular targets that have been identified for MMP2 activity are the regulatory contractile

subunit, troponin I, and other structural and cytoskeletal proteins (Hein *et al.,* 1995; Matsumura *et al.,* 1996; Gao *et* al., 1997; Bolli and Marban 1999; McDonough *et al.,* 1999; Wang *et al.,* 2002).

A study by Narang and co-workers (2004) showed that dietary RPO supplementation increases glutathione peroxidase (GPX) activity, although the study did not investigate the mechanism by which RPO increases GPX activity. This increase in GPX activity may well play a role in RPO mediated protection against ischaemia/reperfusion injury, as GPX reduces oxidative stress through the reduction of hydroperoxides (Brigelius-Flohe, 1999). An increase in the endogenous antioxidant defense system together with the addition of dietary antioxidants present in RPO may attenuate the oxidative imbalance in conditions such as ischaemia/reperfusion more effectively than the addition of dietary antioxidants alone.

RPO supplementation is also known to modulate mitogen activated protein kinase (MAPK) signaling when supplemented to the diet (Engelbrecht *et al.,* 2006; Kruger *et al.,* 2007; Engelbrecht *et al.,* 2009). This modulation of MAPK signaling is also influenced by cholesterol supplementation, as demonstrated by Kruger and co-workers (2007). Dietary RPO supplementation of SRC fed rats was found to be associated with increased phosphorylation of PKB/Akt and p38, while phosphorylation of JNK was decreased (Engelbrecht *et al.,* 2006; Engelbrecht *et al.,* 2009). With added cholesterol, dietary RPO supplementation

caused decreased phosphorylation of p38 and JNK and increased phosphorylation of ERK (Kruger *et al.,* 2007). These results demonstrate that RPO supplementation has the potential to inhibit apoptosis through modulation of different MAPK signaling pathways in different models of ischaemia/reperfusion injury. Das and co-workers (2008) have shown that dietary supplementation of TRF or individiual fractions of tocotrienol are able to offer protection against ischaemia/reperfusion injury. This protection was considered to be associated with increased phosphorylation of PKB/Akt, which shows that the PKB/Akt signaling pathway may be one of the pathways of protection involved in RPO mediated protection against ischaemia/reperfusion injury in the non-hypercholesterolaemic heart. The role of ERK in RPO mediated protection of the non-hypercholesterolaemic heart is not clear.

To our knowledge no studies have measured myocardial infarct size in RPO supplemented subjects or animals. As studies were able to show that dietary RPO supplementation was able to improve reperfusion functional recovery (Esterhuyse *et al.,* 2005b; Esterhuyse *et al.,* 2006; Bester *et al.,* 2006), infarct size measurement was a logical extension of our investigations.

The aims of this study were therefore to:
   1) Investigate the effects of dietary RPO supplementation on myocardial infarct size in SRC fed rats and cholesterol fed rats.

2) Determine the role of MMP2 activity in the protection offered by RPO supplementation.

3) Investigate whether upregulation of GPX activity by RPO supplementation is an effect of genetic transcription.

4) Determine whether ERK phosphorylation plays a role in RPO mediated protection of the heart against ischaemia/reperfusion injury.

CHAPTER 2

LITERATURE REVIEW

2.1 Myocardial ischaemia/reperfusion injury and infarct size measurement

Interest in myocardial infarct size measurement started as result of studies performed by Maroko and co-workers (1971). They proposed strategies by which myocardial necrosis can be limited in the setting of myocardial infarction. The reason for this need to limit myocardial cell death is that the mortality and morbidity associated with myocardial infarction seem to be proportional to the amount of myocardium which is destroyed or infarcted (Pfeffer *et al.,* 1979; Chareonthaitawee *et al.,* 1995; Wu *et al.,* 2009). The exact trigger mechanism for myocyte death associated with myocardial infarction is still unknown. It is however important to salvage as much myocardium as possible, as it is known not to regenerate (Tam *et al.,* 1995).

One theory proposed by Hearse and co-workers (1973), is known as the "oxygen paradox". They found that when a rat heart is reperfused with oxygenated buffer, immediate cell death occurs. There is, however, little consequence if unoxygenated buffer is used. This has led researchers to believe that cell death in the presence of oxygen is due to infiltration of leukocytes into the myocardium with subsequent destruction of the hypoxic area due to the inflammatory response. Alternatively the reinstatement of oxygenation to the hypoxic area is believed to lead to an increased production of ROS. In both these scenarios,

ROS is thought to be responsible for the rapid cell death upon reperfusion (Reimer *et al.,* 1989). This type of cell death has become known as "immediate lethal reperfusion injury", as it occurs between the first few minutes to hours of reperfusion. This form of reperfusion injury is normally associated with necrotic cell death which is demonstrated by the release of intracardiac enzymes, such as lactate dehydrogenase, creatine kinase and troponin-T (Ravkilde *et al.,* 1995; Ishikawa *et al.,* 1997). Necrotic cell death in these conditions is dependant on several factors, which lead to disruption of the sarcolemma due to hypercontracture of myofibrils. Possible mechanisms that have been suggested include: 1) re-energisation of the myocardium, which is coupled to loss of calcium control and generation of ROS, 2) normalization of tissue pH, 3) oxygen radical generation and 4) normalization of tissue osmolality (Babbs, 1988; Becker *et al.,* 1988; Piper *et al.,* 1998). Necrosis is not the only method of cell death in reperfusion. Signs of myocyte apoptosis appear in the ischaemic myocardium even after short periods of ischaemia (Bartling *et al.,* 1998; Maulik *et al.,* 2000). It has been proposed that this form of cell death may be caused by a redox shift in the cell, which may be due to the oxidative stress associated with ischaemia/reperfusion injury (Hearse *et al.,* 1991; Bartling *et al.,* 1998; Maulik and Yoshida 2000)

Myocardial ischaemia, if sustained for long periods of time, may also lead to cell death (or infarction) in myocardial tissue (Hearse *et al.,* 1988; Hearse *et al.,* 1991; Poston and Parenteau 1992). This is achieved through the metabolic

changes in the heart muscle due to decreased oxygen supply, and is associated with: 1) a switch from aerobic fatty acid metabolism to anaerobic glycolysis, 2) decreased removal of waste products such as lactate and protons, due to reduced blood supply, 3) decrease in pH due to the generation of protons, 4) disruption of glycolysis due to inhibition of enzymes by decreased pH, 5) depression of contractile function through the alteration of ionic homeostasis, 6) accumulation of the adenosine catabolites, inosine and hypoxanthine, which may contribute to development of ROS, 7) calcium overload due to failure of the sodium/potasium ATPase pump, which leads to exchange of sodium for calcium (Hearse *et al.,* 1988; Hearse *et al.,* 1991; Host *et al.,* 1992; Poston and Parenteau 1992; Deboer *et al.,* 1993; Janier *et al.,* 1994; Pierce and Czubryt 1995; Knight *et al.,* 1996; Ferrari *et al.,* 1998; Hassinen *et al.,* 1998; King and Opie 1998; Kuzmin *et al.,* 1998; Piper *et al.,* 1998; Taegtmeyer *et al.,* 1998; Xiao and Allen 2000). Additionally, increased cathecholamine levels associated with ischaemia lead to accumulation of potassium in the extracellular space. This may contribute to electrophysiological disturbances and eventually ventricular arrhythmia (Wilde *et al.,* 1988; Poston *et al.,* 1992). Release of catecholamines may further complicate myocardial recovery during reperfusion through the induction of platelet aggregation and ensuing β-adrenergic stimulation, which is associated with vasoconstriction (Simpson and Lucchesi 1987).

In order to determine the extent of damage (or infarction) associated with an ischaemic episode of the heart, two protocols could be followed. Firstly, the

functional recovery of the heart can be measured by comparing reperfusion function to the function of the heart before ischaemia. Alternatively, the amount of infarcted tissue may be measured and expressed as a percentage of the ischaemic area of the myocardium (also known as "area at risk"). Some evidence has emerged that contractile dysfunction in non-infarcted myocardial tissue may play a role in cardiac function during early reperfusion (Yang *et al.,* 2004). This suggests that the measurement of myocardial infarct size to determine the extent of damage in the myocardium may be a more reliable measurement.

2.2 Matrix Metalloproteinase 2 and its involvement in myocardial ischaemia/reperfusion injury

Matrix metalloproteinases (MMPs) are a group of zinc-containing, calcium-dependant, endopeptidases that are structurally similar, and are related in function (Bode and Maskos 2003). MMPs are proteins secreted in the form of pro-MMPs, called zymogens. These zymogens are inactive and require an activation step before they become active (Bode and Maskos 2003). MMPs may be found in the extracellular matrix, or in a complex with their inhibitors (tissue inhibitors of metalloproteinases, referred to as TIMPs) at the cell surface (Baker *et al.,* 2002). Under normal physiological conditions they facilitate cell migration and tissue remodeling, such as wound healing or growth (Nagase and Woessner 1999; Steffensen *et al.,* 2001). A balance between TIMPs and MMPs are important to control MMP activity (Bode and Maskos 2003). An inbalance of TIMP and MMP is found in many pathological conditions, including acute and

chronic cardiovascular disease (Konttinen *et al.,* 1999; Cheung *et al.,* 2000; Tetlow *et al.,* 2001; Baker *et al.,* 2002).

MMPs are classified according to their target molecules e.g. the main target protein of MMP2 and MMP9 is known to be gelatin (Steffensen *et al.,* 2001; Visse and Nagase 2003). MMP2 is considered to be the main MMP implicated in ischaemia/reperfusion injury.

Zymogens contain a propeptide domain which is attached to the MMP molecule through a cysteine residue (Cys[73]). This cysteine residue is bound to a zinc atom which is present in the catalytic domain of the molecule. As long as the zinc atom remains bound to the cysteine molecule, it is unable to perform its catalytic function, rendering the enzyme inactive (Springman and Angleton 1990; Van Wart and Birkedal-Hansen 1990). The propeptide domain may be cleaved from zymogens by autolytic cleavage once the cysteine bond has been disrupted. *In vivo*, MMPs are normally activated through other proteinases (Springman and Angleton 1990; Nagase and Woessner 1999; Visse and Nagase 2003). Activation of MMPs are normally associated with a decrease in molecular weight and in the case of MMP2 this reduction is from 72kDa or 75kDa to 64kDa (Park *et al.,* 1991).

A second mode of activation of MMP2 that has become an area of research in cardiovascular disease is where MMP2 may be activated by ROS, formed in

oxidative stress conditions (Rajagopalan *et al.,* 1996). More recent studies have shown that ONOO⁻ specifically is involved in this mode of MMP2 activation (Siwik *et al.,* 2001; Viappiani *et al.,* 2006). During this mode of MMP2 activation the propeptide domain of the molecule is not cleaved. The cysteine bond to the zinc atom is disrupted through redox modification of the propeptide domain (Okamoto *et al.,* 2001; Viappiani *et al.,* 2006). This form of MMP2 activity can thus be distinguished from the classic form, as this will lead to MMP molecules with a molecular weight of 72kDa and 75kDa which display activity.

In the area of myocardial ischaemia/reperfusion research, activation of MMP2 is associated with poor reperfusion recovery and larger infarct size in the heart (Cheung *et al.,* 2000; Hayashidani *et al.,* 2003; Menon *et al.,* 2004; Bergman *et al.,* 2006; Fert-Bober *et al.,* 2008). Attempts to inhibit MMP2 activation have been associated with improved myocardial recovery after ischaemia/reperfusion injury (Clark *et al.,* 1997; Cursio *et al.,* 2002; Roach *et al.,* 2002; Bendeck *et al.,* 2003; Krishnamurthy *et al.,* 2009). However, acute myocardial damage through the action of a matrix metalloproteinase with gelatin as its main target is met with scepticism by many investigators (as gelatin is not considered a critical target for ischaemia/reperfusion induced myocardial damage). This has led to new investigations being undertaken to study the effect of MMP2 on myocardial ischaemia/reperfusion injury. Novel intracellular targets are being researched for MMP2 in the setting of myocardial ischaemia/reperfusion injury. Several studies have shown that MMP2 activation leads to cleavage of troponin I, which is the

regulatory element of contractile proteins within the myocyte (Gao *et* al., 1997; Bolli and Marban 1999; McDonough *et al.,* 1999; Wang *et al.,* 2002). Cleavage of troponin I causes decreased functional recovery of hearts, as it will lead to contractile failure. Other possible targets for MMP2 include structural and cytoskeletal proteins of the myocyte (Hein *et al.,* 1995; Matsumura *et al.,* 1996). Degradation of sufficient levels of intracellular protein may lead to necrosis or apoptosis, which explains the increased infarct size and decreased function of the heart.

MMP2 is rapidly washed out of the heart after activation and therefore, the activity of this molecule is best measured in coronary flow early in reperfusion (Cheung *et al.,* 2000). This MMP2 release rapidly leads to depletion of tissue MMP2 levels in an ischaemia/reperfusion setting and the amount of active MMP2 released into the coronary flow early in reperfusion is normally proportional to the amount of myocardial damage (Cheung *et al.,* 2000).

2.3 Serum lipids

Fatty acids are transported in the blood 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 high density lipoprotein cholesterol (HDL), low density lipoprotein cholesterol (LDL), and triacylglycerol.

The serum lipid profile is considered to be a good indicator of risk for developing cardiovascular disease. Increased LDL is regarded as a major risk factor for the development of cardiovascular disease (Van Tol *et al.*, 1995; Hu *et al.*, 2000; Quereshi *et al.*, 2002). The National Cholesterol Education Program (NCEP) has recommended that a reduction in LDL should be used as a treatment of choice for heart disease and that increased LDL levels be used for the early detection of potential heart disease (NCEP, 2001). Research has shown that increased triglycerides may also be considered to be a risk factor for the development of heart disease (Assman *et al.,*1996; Jeppesen *et al.,*1998; Austin *et al.,* 1998; Cullen , 2000). More recently it has been discovered that oxidation of LDL plays an important role in the development of cardiovascular disease (Chen *et al.,* 2003; Kontush *et al.*, 2005). A reduction in HDL concentrations has also been associated with cardiovascular risk (Abeywaredna, 2003; Kontush *et al.*, 2005) and it has been suggested that decreased HDL may lead to the development of oxidative stress, as HDL molecules transport antioxidative enzymes (Frias *et al.,* 2009).

The effects of dietary supplementation of edible oils on serum lipids will be discussed in Chapter 3.

2.4 Effects of a hypercholesterolaemic diet in the rat heart

Hypercholesterolaemic diets lead to the development of hyperlipidaemia and atherosclerosis which is regarded as an important factor in the development of

cardiovascular disease (Puskas *et al.,* 2004). The development of hyperlipidaemia is normally associated with increased serum cholesterol or triglyceride levels. In the rat model however, serum cholesterol levels may only increase after long periods of moderate dietary cholesterol supplementation, as this species is particularly resistant to changes in the serum lipid profile (Ferdinandy *et al.,* 1997; Onody *et al.,* 2003). Despite the lack of changes in the serum lipid profile, many studies have been performed that used the rat model successfully in dietary cholesterol supplementation investigations (Ferdinandy *et al.,* 1997; Szekeres *et al.,* 1997; Ferdinandy *et al.,* 1998; Csont *et al.,* 2002; Chen *et al.,* 2003; Girics *et al.,* 2003; Onody *et al.,* 2003; Esterhuyse *et al.,* 2005b; Girics *et al.,* 2006).

The major pathological effect of hyperlipidaemia seems to be an increased susceptibility of the heart to ischaemia/reperfusion injury. The mechanisms by which hyperlipidaemia exerts this effect on the myocardium are not fully understood. However, several mechanisms have been found to be associated with the pathology of cardiac hyperlipidaemia.

In the presence of excess exogenous cholesterol, the mevalonate pathway may be downregulated by the inhibition of 3-hydroxy-3-methyl-glutatyl-CoA (HMG-CoA) reductase. This enzyme is the rate-limiting enzyme for the mevalonate pathway (Roberts, 1995). By inhibition of this pathway polyprenyl derivatives such as ubiqiunone, is downregulated and protein prenylation is reduced

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(Goldstein and Brown 1990; Cassey, 1992). Decreased prenylation of GTP-binding regulatory proteins and the nuclear protein lamin may interfere with their normal function (Finegold *et al.,* 1990). Ubiqiunone is an endogenous antioxidant that has been associated with protection of the rat myocardium against ischaemia/reperfusion injury. Additionally, it plays an important role in mitochondrial electron transport (Hano *et al.,* 1994). A decrease in NO bioavailability and ensuing decreased cGMP metabolism has also been observed in the hyperlipidaemic rat model (Ferdinandy *et al.,* 1997; Szekeres *et al.,* 1997). This decrease in NO and cGMP levels was shown to be associated with unchanged NOS activity (Girics *et al.,* 2003). Girics and co-workers (2003) speculated that NO may have been quenched by ROS, as the unchanged NOS activity indicates that NO production is not decreased. It has indeed been shown that increased ROS formation and especially that of the superoxide radical (O_2^-), is associated with the hyperlipidaemic heart (Kojda and Harrison, 1999; Warnholtz *et al.,* 1999). Onody and co-workers (2003) found that dietary cholesterol supplementation increased peroxynitrite ($ONOO^-$) levels in the myocardium and this supports the speculation by Girics and co-workers (2003) that NO may have been quenched by ROS, as $ONOO^-$ is the product of NO and O_2^-. Onody and co-workers (2003) was also able to demonstrate that $ONOO^-$ is responsible for an increase in left ventricular end diastolic pressure in hyperlipidaemic hearts. Clear evidence exists that peroxynitrite may activate matrix metalloproteinase 2 (MMP2) (Rajagopalan *et al.,* 1996; Okamoto *et al.,* 2001; Siwik *et al.,* 2001; Viappiani *et al.,* 2006). Furthermore, Girics and co-

workers (2006) demonstrated that MMP2 activity is increased in the hyperlipidaemic heart. This increase in MMP2 activity in hyperlipidaemic rat hearts is supported by the finding of Onody and co-workers (2003) that peroxynitrite is increased in this model.

Dietary cholesterol supplementation has also been shown to inhibit the heat shock response in the rat myocardium (Csont *et al.,* 2002), as well as expression of oxidized low-density lipoprotein receptors which induce apoptosis (Chen *et al.,* 2003).

2.5 Myocardial glutathione peroxidase (GPX)

Glutathione peroxidase is a group of antioxidant enzymes which reduces hydrogen peroxide and alkyl hydrogen peroxides (Brigelius-Flohe, 1999). There are five different isoforms of GPX named GPX1 to GPX5. GPX1 reduces mostly soluble hydroperoxides such as H_2O_2, cumene hydroperoxide and t-butyl hydroperoxide. GPX3 and GPX 4 reduce more complex hydroperoxides, such as phosphatidylcholine hydroperoxide. GPX4 also reduces the hydroperoxide group of thymine, lipoproteins and cholesterol esters. Furthermore, GPX4 is the only isoform of GPX that may act on hydroperoxides which are integrated in membranes (Brigelius-Flohe, 1999; Bao *et al.,* 1997; Sattler *et al.,* 1994; Thomas *et al.,* 1990; Ursini and Bindoli 1987)

GPX1 is known to be expressed in organs with increased hydrogen peroxide production, such as the liver, lung, kidney and erythrocytes (Flohe, 1989; Brigelius-Flohe, 1999). It has been observed the GPX1 expression may increase in the presence of increased oxidative stress (Brigelius-Flohe, 1999; Clerch and Massaro 1993). Increased GPX1 activity has also been associated with a decreased incidence of apoptosis. GPX2 is mainly expressed in the epithelium of the gastrointestinal tract and the liver (Chu *et al.,* 1993; Brigelius-Flohe, 1999). GPX3 is expressed in the extracellular fluid of especially the kidney, the ciliary body and the fetal/maternal interface (Brigelius-Flohe, 1999). Although GPX4 is expressed in most organs, with the highest concentrations found in the testes, it is found in lower concentrations than GPX1 (Brigelius-Flohe, 1999). GPX5 is expressed only in the epididymis of mice (Brigelius-Flohe, 1999).

The mechanism by which GPX reduces hydroperoxides, leads to oxidation of the active site (selenolate) of the GPX molecule. Oxidized GPX then requires glutathione to restore it to the reduced state in order for it to be functional. Glutathione is oxidized in this process to glutathione disulfate, which is reduced by glutathione reductase. Glutathione reductase makes use of electrons from NADPH which is replenished through the hexose monophosphate shunt (Ferrari *et al.,* 1991; Dolphin *et al.,* 1985; Griffith, 1999; Bjornstedt *et al.,* 1994; Grant, 2001; Moran *et al.,* 2001). Reduced glutathione (GSSH) may therefore be used as a marker for oxidative stress (Ferrari *et al.,* 1991).

A study using rats fed a high fat diet showed that dietary antioxidant supplementation may reduce transcription of GPX1 in rat muscle (Sreekumar *et al.,* 2002). However, research has shown that antioxidant rich RPO supplementation may in fact increase glutathione peroxidase (GPX) activity in the isolated rat heart (Narang *et al.,* 2004).

2.6 The role of the reoxygenation induced salvage kinase (RISK) pathway in the ischaemic/reperfused myocardium

The RISK pathway is important in myocardial ischaemia/reperfusion injury and consists of two sub-pathways, namely the PKB/Akt pathway and the ERK pathway.

2.6.1 The Protein kinase B (PKB/Akt) pathway

The serine/threonine protein kinase, protein kinase B or AKT (PKB/Akt), is an important regulator of apoptosis, cellular proliferation and differentiation. PI-3-kinase by the phosphoinisitide-dependant protein kinases (PDK) PDK-1 and PDK-2 are the major upstream mediators of PKB/Akt activation (Anderson *et al.,* 1998). When phosphorylated, PKB/Akt may activate or inhibit a number of downstream targets which play various roles in cell survival. One target of PKB/Akt is the pro-apoptotic Bcl-2 family member BAD (Del Peso *et al.,* 1997). Phosphorylation of BAD on Ser^{136} by PKB/Akt inhibits its pro-apoptotic function, thus promoting cell survival (Datta *et al.,* 1997).

PKB/Akt has been associated with survival of cardiomyocytes *in vitro* and has been demonstrated to protect against ischaemia/reperfusion injury in the mouse heart (Fujio *et al.,* 2000). Overexpression of PKB/Akt has been shown to reduce cellular apoptosis, while the absence of PKB/AKT in a knockout mouse model showed a reduced cell survival, despite stimulation by growth factors (Kauffmann-Zeh *et al.,* 1997; Kennedy *et al.,* 1997).

2.6.2 Extracellular signal-regulated kinases (ERK)

ERK is normally referred to as ERK 1/2 as it consists of two isoforms which is also known as p44 and p42 MAPKs. ERK 1/2 may be activated through a host of stimuli, including growth factors, hypertrophic agents and ROS (Bogoyevitch, 2000; Samavati *et al.,* 2002). This activation of ERK may take place in all the major cardiac cell lines, including myocytes, fibroblasts, vascular smooth muscle cells and endothelial cells.

Activation of ERK during myocardial ischaemia/reperfusion is associated with decreased apoptosis (Yue *et al.,* 2000). ERK activation may lead to cell survival despite the activation of pro-apoptotic molecules such as JNK (Omuro *et al.,* 1999). Inhibition of ERK has been shown to lead to increased apoptotic changes in an *in vitro* model of neonatal cardiomyocytes after hypoxia. Activation of ERK in this same model was associated with increased cell survival (Punn *et al.,* 2000; Yue *et al,* 2000). ERK may be activated by the opening of the

mitochondrial K_{ATP} pore which is associated with increased superoxide production (Samavati *et al.,* 2002).

ERK mediated cell survival is not well understood. However, some downstream mediators of ERK activity have been proposed. Cyclooxygenase-2 (COX-2) is one possible downstream mediator of protection in association with ERK 1/2 signalling in cardiomyocytes (Adderley and Fritzgerald 1999). ERK is also suggested to exert its anti-apoptotic effects through the phosphorylation of p90RSK and BAD (Robinson and Cobb 1997; Bonni *et al.,* 1999; Abe *et al.,* 2000; Bueno and Molkentin 2002).

ERK may however be involved in cardiac hypertrophy and heart failure (Haq *et al.,* 2001; Bueno *et al.,* 2002), but it is not known whether ERK plays a causative or protective role. It was suggested by Badrian and co-workers (2006) that ERK mediated protection may depend largely upon the duration of ERK activity and also the type of insult that leads to ERK activation.

Chapter 3 describes cardiovascular effects of edible oils and is part of the literature review. It has been written as a review article to be submitted to Nutritional Research Reviews for publication.

CHAPTER 3

Review Article

Cardiovascular effects of edible oils: A comparison between four popular edible oils.

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3.1 Abstract

Edible oils form an essential part of the modern diet. These oils play a role as an energy source, and provide the diet with many beneficial micronutrients. Although a popular conception may be that fat should be avoided, certain edible oils as a dietary supplement may play an important role in the improvement of cardiovascular health.

Cardiovascular disease has become one of the leading causes of death world wide. Dietary supplementation with different oils may have beneficial effects in cardiovascular health. While olive oil and sunflower oil are known to reduce serum cholesterol, fish oil has become well known for reducing potentially fatal cardiac arrhythmias. Recently, red palm oil research showed beneficial effects on cardiac recovery from ischaemia/reperfusion injury.

It is clear that dietary supplementation with edible oils may play a vital role in reducing the mortality rate due to heart disease. The specific benefits and disadvantages of these oils should however be explored in greater depth.

This review will attempt to identify the benefits and shortcomings of four popular edible oils, namely olive oil, sunflower oil, fish oil and palm oil. Additionally this review will aim to reveal potential areas of research which could further enhance our understanding of the effects of edible oils on cardiovascular health.

3.2 Introduction

In the last decade or two, research on the consumption of dietary fats and oils has become an important topic. High fat content, together with the type of fat in the diet, has been blamed for causing conditions such as obesity, insulin resistance and metabolic syndrome X. Therefore, life threatening conditions such as stroke and acute myocardial infarction can be directly related to either the fat content or fat type in the diet (Elson and Quereshi 1995; Schrauwen *et al.,* 2000; Diniz *et al.*, 2004; Puskas *et al.,* 2004). However, the inclusion of oils and fats in the diet are necessary, as the lack thereof could lead to other potentially life shortening disorders, like glomerulonephritis, hypertension, diabetes mellitus, metabolic syndrome X, psoriasis, Alzheimer's disease, schizophrenia, depression, coronary heart disease, atherosclerosis and cancer (Horrobin, 1990; Das UN, 1990; Das UN, 2004; Das UN, 2005; Suresh Y *et al.*, 2006; Das UN, 2007). Above- mentioned diseases are not only a result of decreased fat intake, but also due to a shortage of fat soluble vitamins and essential fatty acids (EFAs) which are normally contained in oils and fats.

Many studies have been done on edible oils and their effects on cardiovascular health. However, few of these studies compared the effects of more than two of these oils to each other. There also seem to be considerable gaps in the literature concerning certain areas of cardiovascular health with respect to many oils. The need to revisit the literature has thus arisen.

Heart disease is still one of the major causes of mortality in the developed world (Banegas *et al.,* 2003; Jemal *et al.,* 2005). The National Cholesterol Education Program (NCEP) has recommended that a reduction in low density lipoprotein cholesterol (LDL) should be used as a treatment of choice for heart disease and that increased LDL cholesterol be used for the early detection of potential heart disease (NCEP, 2001). Previous studies have shown that increased triglycerides may also be considered to be a risk factor for the development of heart disease (Assman *et al.,*1996; Jeppesen *et al.,*1998; Austin *et al.,* 1998; Cullen P, 2000). Research has also shown that the attenuation of inflammation may offer protection against ischaemia/reperfusion injury. (Mullane *et al.*, 1984; Braunwald *et al.*, 1985; Jones *et al.*, 2000; Carnieto *et al.*, 2009). Carnieto and co-workers (2009) demonstrated in an *in vivo* dog model, that a reduced cyclooxygenase-2 related inflammatory response was able to limit tissue damage associated with ischaemia/reperfusion injury. Cyclooxygenase-2 is an enzyme that is responsible for converting free arachidonic acid to pro-inflammatory prostaglandins. These authors conclude that a cyclooxygenase-2 inhibitor may offer some protection against a myocardial ischaemia/reperfusion injury.

Apart from the fatty acids which are present in these oils, they also provide a variety of micronutrients which may have beneficial effects on amongst other, the cardiovascular system. Both the fatty acid component and micronutrient component of an oil may influence cardiovascular health. Researchers often focus on a particular component of a product and call it the "active ingredient". In

the case of natural products, the oil may be viewed as a cocktail of active ingredients which often have a synergistic effect on health (Palozza *et al.,* 1992; Böhm *et al.,* 1998; Stahl *et al.,* 1998; AREDS, 2001; Stahl and Sies, 2005; Schroeder *et al.,* 2006).

Better understanding of the physiological control, mechanisms of lipid metabolism and the associated risk factors for heart disease and stroke have increased markedly *via* research carried out in recent times. Many of these studies have shown that fatty acids and combinations of fatty acids are beneficial to cardiovascular health (Elson and Quereshi 1995; Jones *et al.*, 2000; Das, 2005; Carnieto *et al.,* 2009).

This review will focus on the beneficial effects of dietary edible oils and fat on the cardiovascular system. We will focus on four well known oils, namely olive oil, sunflower oil, fish oil and palm oil.

3.3 Olive Oil

3.3.1 Refining of olive oil

Olive oil is a rich tasty oil produced from the fruit of *Olea europaea* which grows naturally in the Mediterranean region. This oil is produced from olives by: 1) washing, 2) crushing, 3) kneading and 4) centrifugation of olives (Owen *et al.*, 2000; Brenes *et al.,* 2004). When the pH of the virgin olive oil is below 3.3, the oil is refined to produce a common olive oil (Owen *et al.*, 2000). Refining of olive

pomace (a crude oil produced by centrifugation of olives in water) can take place through secondary physical extraction and centrifugation or chemical refining (Brenes *et al.,* 2004). Olive oil has a rich medical history and has traditionally been used to treat colic, alopecia, paralysis, rheumatic pain, sciatica, and hypertension (Gilani *et al.,* 2005).

3.3.2 Composition of olive oil

Olive oil is a yellow coloured oil which consists of mainly monounsaturated fatty acids (MUFAs), of which oleic acid comprizes 72-79% (Newmark *et al.,* 1997; Owen *et al.,* 2000; Quiles *et al.,* 2004). In comparison to polyunsaturated fatty acids (PUFAs), MUFAs are less susceptible to oxidation. This in turn leads to increased availability of antioxidants in the active form and better stability of olive oil (O'Farrel and Jackson, 1997; Turpeinen *et al.,* 1998; Owen *et al.,* 2000; Newas *et al.,* 2003; Diniz *et al.,* 2004; Wahle *et al.,* 2004; Eder *et al.,*2006).

Olive oil also contains some antioxidant micronutrients, namely polyphenols and squaline (Newmark *et al.,* 1997; Lercker *et al.,* 2000; Owen *et al.,* 2000; Quiles *et al.,* 2004; Caravaca *et al.,* 2005). More than 80% of the olive oil phenolic compounds are lost during the refinery process. Therefore, the phenolic compound content of virgin olive oil (about 230 mg/kg, common range 130-350 mg/kg) is higher than in common olive oil (Owen *et al.,* 2000; Tuck *et al.,* 2002). However, in another study Owen and co-workers (2000) found that the total phenolic content of olive oil may be as high as 500mg/kg. The major phenols are

tyrosol, hydroxytyrosol, oleuropein and ligstroside (Owen *et al.,* 2000; Caravaca *et al.,* 2005; Perona *et al.,* 2006). Of these polyphenols, tyrosol and hydroxytyrosol are the most abundant and represent 30% of the total polyphenol content of olive oil (Owen *et al.,* 2000). Formation of tyrosol and hydroxytyrosol take place through the hydrolysis of oleuropein and ligstroside during storage of the olive oil (Romero *et al.,* 2007). Tyrosol, hydroytyrosol and oleuropein all contain a catechol group, which have been shown to have antioxidant activity (Montedoro *et al.,* 1993; Owen *et al.,* 2000; Visioli *et al.,* 2002; Caravaca *et al.,* 2005).

Research has shown that hydroxytyrosol and oleuropein are the most effective antioxidants in olive oil and may exert better antioxidant activity than vitamin E (Owen *et al.,* 2000; Perona *et al.,* 2006).

Olive oil is considered to be extremely rich in squalene and contains approximately 0.7% of this hydrocarbon (Newmark *et al.,* 1997; Owen *et al.,* 2000). Other foods and oils may contain squalene at a level between 0.002-0.03%. Squalene is an antioxidant by virtue of its ability to quench singlet oxygen radicals, and inhibits cholesterol synthesis through HMG-CoA reductase inhibition (Newmark *et al.,* 1997; Relas *et al.,* 2000). Apart from the inhibition of HMG-CoA reductase by olive oil, its fatty acid composition may play a role to improve the serum lipid profile. This however, has not been proven conclusively (Heyden 1994).

3.3.3 Cardiovascular studies with olive oil

A study by Keys and co-workers (1986) suggest that olive oil consumption decreases the incidence of degenerative diseases such as coronary heart disease and cancer. This study used surveys to measure the general diet of a population, and calculate the fat content of the diet. The mortality rate of men between the ages of 40-59 years was then monitored over a 15 year period for these populations. One of the major findings of this study was a negative correlation between MUFAs in the diet and death due to coronary heart disease.

The high polyphenol content of olive oil suggests that it can be an important antioxidant *in vivo*, although no conclusions can be made as to its effectiveness. One study showed that even when these polyphenol levels were increased in the blood, it failed to protect LDL from oxidation (Vissers *et al.,* 2001). Volunteers in this study consumed both a high and low polyphenol diet respectively for three weeks, with a two week washout period between the two diets. The high polyphenol diet contained 308mg/kg polyphenols, while the low polyphenol diet contained 43mg/kg. The mean difference in polyphenol intake between the diets per day was 18mg per day. It is possible that the difference in polyphenol content of the diets was too small to achieve significant protection against HDL and LDL oxidation. However, the authors designed the high polyphenol diet to contain the highest amount of polyphenols that would be possible to consume in a daily diet. A study by Eder and co-workers (2006) investigated the effect of supplementation of edible oils on lipid peroxidation in the liver. Female rats were

fed a standard rat chow diet (SRC), or a SRC plus sunflower-, rapeseed-, olive- or coconut oils for four weeks. They found that olive oil neither contributes to lipid peroxidation, nor prevents it and concluded that there is no antioxidative protection offered by olive oil supplementation. However, chemical analysis by Briante and co-workers (2004), have shown that polyphenols present in olive oil may have an antioxidant or pro-oxidant effect. They concluded, firstly, that low concentrations of olive oil polyphenols (lower than 23 μg/mg oleuropein and 18.2 μg/mg hydroxytyrosol) have a pro-oxidant effect on LDL, while higher concentrations have an antioxidant effect. Secondly, they found that metal ions play a major role in LDL oxidation *in vivo*, and that olive oil polyphenols may reduce copper ions and thus prevent LDL oxidation. In a study by Aguilera and co-workers (2004), virgin olive oil was compared to α-tocopherol enriched sunflower oil, and was found to offer better protection against LDL oxidation. Male volunteers with peripheral vascular disease were fed a diet supplemented with olive oil or α-tocopherol enriched sunflower oil for four months. In this study the antioxidant content in the blood of the olive oil supplemented group was found to be lower than that of the sunflower oil group. However, olive oil still offered better protection against oxidative stress, as shown by LDL oxidation. The authors of this study suggested that the high levels of antioxidants in the sunflower oil group could not protect LDL from oxidation due to its high level of unsaturated fat. The results of this study may alternatively argue that the polyphenols in olive oil were more protective than the added α-tocopherol in sunflower oil. The fact that the antioxidant content in the blood was also lower in

the olive oil fed group compared to the sunflower oil group may suggest that more antioxidants are not necessarily better. These results may thus argue that natural oils are better than artificially manipulated oils.

Hydroxytyrosol and oleuropein have been shown to have a dose dependant effect by which they can inhibit LDL oxidation (Owen *et al.,* 2000; Visioli *et al.,* 2002; Aguilera *et al.,* 2004). The study by Owen and co-workers (2000), showed these molecules to be more effective antioxidants than vitamin E, dimethyl sulfoxide (DMSO) and butylated hydroxytoluene (BHT). Rietjens and co-workers (2007) argued that hydroxytyrosol offers effective protection against LDL oxidation in *vivo* and in *vitro*, but that *ex vivo* experiments lead to a false negative result. This false negative result is achieved by the removal of hydroxytyrosol from LDL during the isolation of the LDL and the subsequent oxidation thereof. Furthermore, hydroxytyrosol has also been shown to reduce cyclooxygenase and also reduces platelet aggregation (Fabiani *et al.,* 2002; Visioli *et al.,* 2002). Both these effects are associated with a decreased inflammatory response.

It is generally accepted that olive oil decreases oxidative stress (Theriault *et al.,* 1999; Krichefsky *et al.*, 2000; Diniz *et al.,* 2004; Quiles *et al.,* 2004). Quiles and co-workers (2004) compared olive oil to sunflower oil and its ability to reduce the amount of DNA double strand breakage. In this study rats were fed for 6 and 24 months, respectively, with diets containing either olive oil or sunflower oil as a fat source. Some rats continued on this diet after 24 months, in order to determine

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the mean survival rate on each diet. The beneficial effects of MUFAs in olive oil are clear, as it contributes to an increased lifespan of rats and a decrease in ROS associated DNA damage. Furthermore, higher concentrations of plasma retinol and coenzyme Q_{10} was found to be present in the olive oil treated group when compared with a sunflower oil group. This suggests that there was less oxidative stress in rats supplemented with olive oil than in those supplemented with sunflower oil. Fabiani and co-workers (2008) showed that an extract consisting of a mixture of olive oil antioxidants was able to protect peripheral blood mononuclear cells and promyelocytic leukemia cells from hydrogen peroxide induced DNA damage. Cell cultures were incubated with various concentrations of antioxidants and 40 μmol/L hydrogen peroxide for 30 minutes. Antioxidant doses as low as 1 μmol/L was still able to offer significant protection against DNA damage, as measured by the Comet assay.

A study by Briante and co-workers (2004) showed that olive leaf extracts added to olive oil, increased the efficacy of the oil's antioxidant effect. This study showed that oleuropein (derived from olive leaf extract) and hydroxytyrosol worked synergistically to prevent LDL oxidation and thus atherosclerosis. This corresponds with the comment by Stahl and co-workers (1998). The addition of these polyphenols to sunflower oil improved the immune status when supplemented to the diet of volunteers for eight weeks (Baeza *et al.,* 2008; Diaz *et al.,* 2008). Baeza and co-workers (2008) showed that leukocytes had increased glutathione peroxidase activity after eight weeks of supplementation,

while in the study by Diaz and co-workers (2008) no significant changes were seen after three weeks of enriched oil supplementation. The authors suggested that further studies should be done to determine an optimal supplementation time.

Studies done by Demonty and co-workers (2006), report on hypercholesterolaemic subjects whose diets were supplemented with fish oil, olive oil or enriched oils for four weeks. Olive oil supplementation was found to significantly lower LDL concentrations when compared to fish oil supplementation and also led to higher levels of serum triacylglycerol than fish oil supplementation whereas fish oil enriched with plant sterols led to the lowest total cholesterol, LDL and triacylglycerol levels. Although olive oil showed a hypocholesterolaemic effect on the serum lipid profile, there is some evidence to suggest that it does not significantly affect the phospholipid profile of the heart or erythrocyte (Metcalf *et al.,* 2007). These authors compared the changes in atrium and erythrocyte fatty acid composition of volunteers after different periods of olive oil, flaxseed oil and fish oil supplementation. The olive oil group showed no significant difference from the control group in either the erythrocyte or atrium fatty acid composition. Heyden (1994) concluded that MUFAs do not play a significant role in reducing cholesterol or LDL cholesterol. However, squaline present in olive oil has been shown to downregulate HMG-CoA reductase activity and therefore reduce cholesterol synthesis (Newmark *et al.,* 1997; Relas *et al.,* 2000).

Some studies have shown that olive oil has a beneficial effect on blood pressure. Giliani and co-workers (2005) found that intravenous administration of olive oil extracts reduced both systolic and diastolic blood pressure in normotensive rats. Ferrara and co-workers (2000) showed that patients on an olive oil supplemented diet were able to reduce their antihypertensive medication, in contrast with patients on a sunflower oil supplemented diet.

3.3.4 Olive oil in the ischaemia/reperfusion model

To our knowledge little is known about the effects of dietary olive oil supplementation on cardiac ischaemia and reperfusion. Manna and co-workers (2004) performed a study to investigate the effectiveness of oleuropein in offering protection against oxidative stress associated with global myocardial ischaemia and reperfusion. Rat hearts were isolated and perfused with a Langendorff perfusion apparatus. Control hearts were stabilized for 20 minutes, after which they were subjected to 30 minutes of normothermic global ischaemia and 60 minutes of reperfusion. Experimental hearts were perfused with oleuropein at a concentration of 50 µmol/L for 15 minutes before the induction of ischaemia. Perfusion with oleuropein was able to significantly reduce creatinine kinase release during reperfusion, suggesting that it offered protection against tissue damage. Decreased glutathione and reduced glutathione released in coronary effluent, along with the decreased thiobarbituric acid-reactive substance (TBARS) in heart tissue indicate that oxidative stress was significantly decreased

by oleuropein. Further studies need to be done to confirm cardioprotection by dietary olive oil supplementation and on cardiac health in general.

3.3.5 Benefits of olive oil supplementation

Even though olive oil contains some micronutrients like polyphenols and squalene, the monounsaturated fatty acids in the oil have been thought to be the major active component. MUFAs are known to have a beneficial effect on the serum lipid profile and thus decrease the risk of cardiovascular disease (Heyden 1994; NCEP 2001; Demonty *et al.,* 2006; Metcalf *et al.,* 2007). Furthermore, these fatty acids are stable in oxidative stress conditions and are less likely to react with ROS when compared to PUFAs (Owen *et al.,* 2000; Diniz *et al.,* 2004). MUFAs may thus be seen as beneficial to cardiovascular health, as it does not cause negative serum lipid profiles, which is normally associated with SFAs. Furthermore, MUFAs do not increase oxidative stress by the formation of lipid hydroperoxides, as is the case with PUFAs (Theriault *et al.,* 1999; Krichefsky *et al.*, 2000; Newas *et al.,* 2003; Diniz *et al.,* 2004; Quiles *et al.,* 2004; Wahle *et al.,* 2004; Eder *et al.,*2006).

Olive oil polyphenols have an antioxidative effect which inhibits LDL oxidation and thus reduces atherosclerosis (Owen *et al.,* 2000; Manna *et al.,* 2004; Perona *et al.,* 2006). Squalene also exerts an antioxidant effect and inhibits cholesterol synthesis (Newmark *et al.,* 1997; Relas *et al.,* 2000). Together these micronutrients may affect atherosclerosis by inhibition of LDL oxidation and

reduction of total cholesterol (Owen *et al.,* 2000; Patrick *et al.,* 2001; Visioli *et al.,* 2002; Manna *et al.,* 2004; Fabiani *et al.,* 2008). Hydroxytyrosol also reduces inflammation by reduction of pro-inflammatory cyclooxygenase and platelet aggregation (Fabiani *et al.,* 2002; Visioli *et al.,* 2002). It is also clear that olive oil offers more effective protection against oxidative stress in the blood than in the liver (Owen *et al.,* 2000; Manna *et al.,* 2004; Eder *et al.,* 2006; Perona *et al.,* 2006).

A number of studies have shown that olive oil supplementation decreases hypertension in humans through mechanisms not yet resolved (Keys *et al.,* 1985; Ruiz-Gutierrez *et al.,* 1997; Ferrara *et al.,* 2000; Giliani *et al.,* 2005; Alonso *et al.,* 2006). Reduction of hypertension has been shown in human studies and animal models with varying supplementation times, or modes of application. In many studies this was not the main aim of the study but rather an additional observation noted. Giliani and co-workers (2005) suggested that olive oil may be a calcium channel agonist and thereby reduce systolic and diastolic blood pressure. Others suggest that olive oil improve endothelial function by inhibiting the formation of ROS, leading to a nitric oxide mediated vasorelaxation (Ruiz-Gutierrez *et al.,* 1997; Alonso *et al.,* 2006; Perona *et al.,* 2006). Other authors suggest decreased vascular tone and changes in the fatty acid composition of the aorta as a possible mechanism (Ferrara *et al.,* 2000).

It is evident that olive oil, due to its micronutrient content and fatty acid composition can play a vital role in maintaining beneficial serum lipid profiles. Together with its ability to reduce systemic oxidative stress and inflammation, it becomes an appropriate dietary supplement for lowering the risk of coronary heart disease.

3.4 Sunflower oil

3.4.1 Refining of sunflower oil

Sunflower oil (SFO) is produced by refining of the seeds of the sunflower. The refinery process includes the following steps: 1) pressing to yield crude oil; 2) acidification and neutralization; 3) pre-gumming by centrifugation; 4) washing; 5) bleaching; 6) gumming by filtration; and 7) deodorization (Zitouni *et al.,* 2000).

3.4.2 Components of sunflower oil

This refinery process yields a yellow oil, which is rich in polyunsaturated fatty acids of which the major polyunsaturated fatty acid is linoleic acid (60-70%). Oleic acid and stearic acid are the major mono-unsaturated and saturated fatty acids, respectively. The levels of MUFA and SFA present in sunflower oil may vary, depending on the desired product being manufactured (Navorro *et al.*, 1992; Larsen *et al.,* 1999; Aguilera *et al.,* 2004; Eder *et al.,* 2006; AbuGhazaleh *et al.*, 2007). Commercially there are three main forms of SFO available. The first is a high PUFA-SFO which consists of up to 75% PUFAs. The second form is a high MUFA-SFO which contains up to 45% MUFAs, whilst the third is a high

stearic acid SFO which may contain up to 14% stearic acid. These three forms of SFO have very different effects on the serum lipid profile. The most commonly used form of SFO is the high PUFA-SFO. Whilst the high stearic acid SFO is mainly used in industrial processes, high MUFA-SFO is used for general cooking. Sunflower oil contains some natural vitamin E in the form of tocopherol (Aguilera *et al.,* 2004; Eder *et al.,* 2006; AbuGhazaleh *et al.*, 2007).

It has been shown that sunflower oil can modulate the serum lipid profile (Girardet *et al.,* 1977; Nydahl *et al.,* 1994; Lambert *et al.,* 2007) and the major finding was shown to be a decrease in total cholesterol and LDL-cholesterol. Lambert and co-workers (2007) supplemented the diets of regularly exercising individuals with 3.90 g of high-MUFA sunflower oil for 12 weeks. A reduction in total cholesterol and LDL-cholesterol of male and female volunteers were found after the supplementation period (men: total cholesterol decreased 6.4% while LDL cholesterol decreased 11.1%; women: total cholesterol decreased 12.8% while LDL cholesterol decreased 8.33%). There was also a significant reduction of 12.5% in HDL-cholesterol in women after the 12 week supplementation period. The decreased HDL-cholesterol was ascribed to weight loss or increased dietary consciousness of the participants. Similar findings were made by Nydahl and co-workers (1994) when they fed a sunflower oil supplemented diet to 101 volunteers for three weeks. During the supplementation period all other oils in the diet were replaced with sunflower oil. A decrease of 4% percent in total cholesterol, 5-7% in LDL cholesterol and 5% in apolipoprotein B was observed

after the three weeks. There was virtually no difference in other molecules of the serum lipid profile. Girardet and co-workers (1977) showed a decrease in total serum cholesterol, cholesterol esters and triglycerides in rats fed a 12% sunflower oil supplemented diet for one year. However, in a study done by Aguilera and co-workers (2004), four months of dietary supplementation with high PUFA-sunflower oil and virgin olive oil in patients with peripheral vascular disease, sunflower oil supplementation did not affect the serum lipid profile significantly. In this study all meals were cooked in sunflower oil and patients received additional supplements (15,14 g of the oil per day). The fat content of the diet in this study may have been excessive, as supplementation with virgin olive oil in this study led to similar results. This pattern is also seen in a study by Quiles and co-workers (2004) where all the fat in the diet was replaced by sunflower oil for 6-24 months.

Omega-6 PUFAs provided by sunflower oil in the diet, may play a vital role in regulation of inflammation, as it is used to produce pro-inflammatory prostaglandins (Simopilous *et al.,* 1991; Navarro *et al.,* 1992; Das *et al.,* 2008).

3.4.3 Cardiovascular studies with sunflower oil

Only a few studies have been done with SFO to determine its influence on cardiovascular health. SFO has been used as a control in many studies when compared with other oils (Charnock *et al.,* 1991; Larsen *et al.,* 1999; Aguilera *et al.,* 2004; Quiles *et al.,* 2004; De Roos *et al.,* 2008). The choice of SFO as a

control may be due to the fact that SFO showed no effect on the cardiovascular system apart from its beneficial effect on the serum lipid profile (Charnock *et al.,* 1991; Larsen *et al.,* 1999; Aguilera *et al.,* 2004; Quiles *et al.,* 2004; De Roos *et al.,* 2008). Sunflower oil had little effect on myocardial arrhythmia after 44 weeks of 12% supplementation in the diet of rats (Charnock *et al.,* 1991). Sunflower oil supplementation (19% of total diet) could also not induce an antithrombotic effect after being supplemented for 22 days to volunteers (Larsen *et al.,* 1999). Aguilera and co-workers (2004) found little protection against oxidative stress after 4 months of supplementation with sunflower oil. This was supported by similar findings made by Quiles and co-workers (2004) after 6 and 24 months of sunflower oil supplementation (as sole fat supply) in the diet of rats. Little effect was seen on acute phase inflammatory markers and apolipoprotein levels after 12 weeks of 3.5g sunflower oil supplementation (De Roos *et al.,* 2008).

Due to the relative lack of micronutrients in SFO, hydroxytyrosol is often added to commercial blends to increase the antioxidant content. Hydroxytyrosol is a polyphenol which has been shown to improve immunity by increasing leukocyte glutathione peroxidase activity (Baeza *et al.*, 2008; Díaz *et al.,* 2008).

Having large amounts of PUFAs in the diet may be worse than large amounts of SFAs, even despite its positive impact on the serum lipid profile. A study by Diniz and co-workers (2004) showed that a five week diet containing 47.0 g of linoleic acid (the major omega 6 PUFA in most edible oils) per 100g of fat in the diet,

leads to higher oxidative stress than a diet containing 81.7 g of saturated fat per 100g fat in the diet. In this study, the high PUFA group showed significantly increased tissue hydoperoxide and lipoperoxide concentrations when compared to a high SFA group and the control group. This increase in ROS was accompanied by a significant decrease in SOD-, catalase- and citrate synthase activity when compared to a high SFA group and the control group. The high PUFA diet also contained significantly less glycogen than the other diet groups. Diniz and co workers (2004) suggest that the increased susceptibility to lipoperoxidation and metabolic shifting is associated with high levels of PUFAs in the diet. These negative effects of PUFA supplementation may be so detrimental that they outweigh the positive effects that PUFAs have on the serum lipid profile. In another study it was found that sunflower oil supplementation led to statistically significant higher adduct (DNA double strand breakage) levels in the kidneys, lungs, glandular mucosa, small intestine mucosa and colon mucosa of rats (Eder *et al.,* 2008). DNA adduct levels were measured after a four week diet containing 169g of sunflower oil, rapeseed oil, coconut oil or olive oil per kilogram diet. Eder and co-workers (2008) suggest that the increased DNA adduct levels in dietary sunflower oil supplemented rats may be associated with increased genotoxic cancer risk. They concluded that the vitamin E content of the sunflower oil evidently did not significantly contribute to a reduction in DNA-adduct levels. It would normally be expected that vitamin E reduces DNA-adduct levels, as DNA adducts are the product of lipid peroxidation. However, they speculated that the high linoleic content in sunflower oil had a higher lipid peroxidation-inducing

effect than the inhibitory effect of the vitamin E contained in the oil. In another study, these authors also postulated that the vitamin-E content of sunflower oil is not high enough to compensate for the high concentrations of omega-6 PUFAs in this oil (Eder *et al.,* 2006). In this study a similar supplementation was used as that of Eder and co-workers (2008). However, the focus of this study was to determine the effects of the oils on lipid peroxidation in the liver. Aguilera and co-workers (2004) showed that four months of dietary sunflower oil supplementation in patients with peripheral vascular disease could not protect LDL from oxidation. The sunflower oil used in this study was enriched with tocopherol (1200mg/kg in total). Despite the increased antioxidants added to the oil, the thiobarbituric acid-reactive substance (TBARS) was still found to be significantly higher in the blood of subjects receiving sunflower oil than those receiving unaltered olive oil. These results argue that the unsaturated fatty acids in sunflower oil lead to high levels of oxidative stress, despite the high levels of antioxidants present in the oil. It can also be concluded that artificial addition of antioxidants to oils may not yield desirable results.

A study by Tabatabaei and co-workers (2008) reports on the use of fresh sunflower oil and sunflower oil heated for 48 hours to 180ºC. These oils were fed to rats together or without selenium supplements (1ppm) for 43 days. The heated oil groups showed increased serum malondialdehyde (MDA) levels when compared to the fresh oil group, indicating higher fat oxidation rates in this group. Even though the selenium supplemented group showed increased liver

glutathione peroxidase activity, the selenium supplemented heated oil still showed increased MDA levels when compared to the control.

3.4.4 Sunflower oil in the ischaemia/reperfusion model

To our knowledge no studies have been done to determine whether sunflower oil supplementation protects against ischaemia/reperfusion injury. Judging from studies with sunflower oil in other experimental models, the effects on reperfusion recovery would be minimal (Charnock *et al.,* 1991; Larsen *et al.,* 1999; Aguilera *et al.,* 2004; Quiles *et al.,* 2004; De Roos *et al.,* 2008).

3.4.5 Benefits of sunflower oil supplementation

Despite modulation of the serum lipid profile and the provision of essential fatty acids which may help in the regulation of inflammation, sunflower oil has a neutral effect on the cardiovascular system. This makes sunflower oil an excellent choice as a control group in cardiovascular research, as most other oils would influence more parameters (Charnock *et al.,* 1991; Larsen *et al.,* 1999; Aguilera *et al.,* 2004; Quiles *et al.,* 2004; De Roos *et al.,* 2008). The beneficial effect of sunflower oil in ischaemia/reperfusion injury can only be clarified with additional studies.

Due to the positive effects on the serum lipid profile, this oil may lead to a decrease in atherosclerosis and therefore should be considered to be beneficial

to cardiovascular health (Girardet *et al.,* 1977; Nydahl *et al.,* 1994; Lambert *et al.,* 2007).

3.5 Fish Oil

3.5.1 Refining of fish oil

Fish oil is derived from the tissues of oily fish. There is very little refining or chemical alteration which takes place after the recovery of the oil from these fish species (Moghadasian, 2008). Fish oil supplementation in the diet is known to offer protection against various pathological conditions, such as cardiovascular diseases, respiratory diseases, diabetes, depression, cancers, inflammatory and immune renal disorders (Simopoulos, 1991; De Caterina *et al.,* 1994; Priyamvada *et al.,* 2008).

3.5.2 Components of fish oil

Fish is known to be a good source of protein, vitamin B12, vitamin D, selenium, iodine and long chain n-3 fatty acids. The n-3 fatty acids supplied by fish oil are mostly eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6) (Dahl *et al.,* 2006; Conde *et al.,* 2007; Metcalf *et al.,* 2007). In the past many studies focused on the effects of EPA only, as it is provided by fish oil in large amounts. However, recently the focus has shifted to the beneficial effects of DHA on health (Mozaffarian *et al.,* 2006; Conde *et al.,* 2007; Theobald *et al.,* 2007). A study by McLennan and co-workers (1996) suggests that DHA may be responsible for most of the beneficial cardiovascular effects of dietary fish oil

supplementation. In this study spontaneously hypertensive (SHR) rats were fed either DHA or EPA or both in low doses (0.4 to 1% of energy intake). DHA proved to be more effective than EPA in reducing ischaemia induced myocardial arrhythmias, hypertension, thromboxane-like vasoconstrictor responses (in the aorta) and the development of proteinuria after salt loading of the diet in the SHR rats. The question was subsequently raised whether it is not DHA that may be the principal active component of fish oil in offering cardiovascular protection.

It is of great importance to include sufficient amounts of EPA and DHA in the diet, as the human body can only convert small amounts of α-linolenic acid (ALA; 18:3) to EPA (5%) and DHA (less than 5%) (Plourde *et al.,* 2007; Lee *et al.,* 2008). It has been shown that fish oil supplementation is able to significantly increase myocardial levels of DHA and EPA in one week of supplementation (Metcalf *et al.,* 2007). Volunteers in this study received 10ml of fish oil concentrate per day for either 7, 14 or 21 days. Control groups in this study received either flax seed oil, olive oil, or no supplementation. Therefore, the increase in DHA and EPA could play a vital role in preventing life-threatening arrhythmias in supplemented individuals. Furthermore, the increase in DHA and EPA levels in the myocardium was found to be inversely proportional to arachidonic acid (AA) levels when fish oil was supplemented in the diet. This indicates that AA is replaced by EPA and DHA in cell membranes.

3.5.3 Cardiovascular studies with fish oil

McLennan and co-workers (1996) showed that dietary supplementation with purified DHA (3.9-10% of daily energy intake) delayed the onset of hypertension in spontaneously hypertensive rats (SHR), while EPA supplementation could not render similar results. They also showed that DHA can inhibit thromoxane-like vasoconstrictor responses in SHR aortas. Furthermore, DHA supplementation delayed the onset of salt loading induced proteinuria in these SHRs with already established hypertension. However, dietary supplementation with EPA did not show similar results in this study. Another study showed that fish oil supplementation was able to decrease diastolic blood pressure by the provision of DHA (Theobald *et al.,* 2007). Purified DHA was tested against a placebo control group in this study and exhibited a decreased diastolic blood pressure. Healthy volunteers were recruited and supplemented with capsules containing 500mg of either DHA or olive oil for at least 3 months. The antioxidant levels in the two capsules were adjusted to similar levels. Together with a decreased blood pressure, it was also shown that a decrease in heart rate occurred and it was speculated that the decrease in blood pressure was caused by the decreased heart rate rather than a decrease in arterial stiffness.

In a study on hypercholesterolaemic mice, Chiu and co-workers (2008) showed that fish oil supplementation decreased adhesion molecule expression and some inflammatory markers in early stage sepsis. Fish oil supplementation was performed in this study over a three week period, following a high fat diet. Late

stage inflammatory markers were not affected and myeloperoxidase activity at the sepsis site was similar in an olive oil fed group. Chiu and co-workers (2008) concluded that even though fish oil decreases adhesion molecule expression and early stage inflammatory markers when fed to hypercholesterolaemic mice, it did not cause immuno-suppression when sepsis was induced in these animals. As inflammation plays an important role in the pathophysiology of myocardial infarction, reduction in inflammatory markers may prevent the development of heart disease (Mullane *et al.*, 1984; Braunwald *et al.*, 1985; Jones *et al.*, 2000; Carnieto *et al.*, 2009).

Charnock and co workers (1991) showed that fish oil mixed in a 1:1 ratio with sheep fat significantly reduced cardiac arrhythmias during coronary occlusion and reperfusion, when compared to rats fed sheep fat only. Diets of rats were supplemented with 12% of either sheep fat, sheep fat/fish oil blend, physically refined palm oil, chemically refined palm oil or sunflower seed oil for 12 months. In this study, neither palm oil, nor sunflower oil could decrease arrhythmias significantly when compared to the sheep fat fed control group. In another study it was shown that fish oil was more effective in reducing cardiac arrhythmia during or after ischaemia when compared to olive oil and sheep fat and to a lesser extent sunflower seed oil (McLennan *et al.,* 1993). In this study, 30 week old rats received 12% olive oil, sunflower seed oil, fish oil or sheep fat supplementation for 12 weeks. Sunflower seed oil and fish oil supplementation led to significantly lower arrhythmia scores during and after ischaemia when

compared to sheep fat. Fish oil, however, led to a much lower incidence of arrhythmia than sunflower seed oil. These results suggest that PUFAs do not only decrease arrhythmia by replacing SFAs in the membrane, but that they play an active role in reducing arrhythmia. If it is accepted that PUFAs reduce arrhythmia only by replacement of SFAs in the membrane, one would expect that olive oil supplementation would also be able to reduce arrhythmia. However, olive oil was not shown to be able to reduce arrhythmia in this study and therefore the authors suggest another mechanism of protection by PUFAs. Furthermore, these results also indicate that n-3 PUFAs are more effective in reducing arrhythmia than n-6 PUFAs. It can therefore be accepted that an unknown mechanism of protection can be associated with n-3 PUFAs but not n-6 PUFAs. McLennan and co-workers (1996) showed that low doses of purified DHA intake (0.4% to 1.1% of daily energy intake) could reduce post ischaemic cardiac arrhythmias in spontaneously hypertensive rats. Dietary supplementation with a similar dosage of EPA did not have the same effect as DHA. McLennan and Abeywardena (2005) concluded that fish oil is a powerful modulator of arrhythmia. These authors ascribe the ability of fish oil to decrease arrhythmias, especially ventricular fibrillation, to the incorporation of long chain PUFAs (especially DHA) into the myocardium. They suggest that DHA is the main long chain PUFA responsible for fish oil cardioprotection, as it is selectively incorporated into myocardial cell membranes. Several possible mechanisms have been suggested for the anti-arrhythmic action of long chain PUFAs. This includes altered sympathetic innervation to the conduction system and coronary

vessels, increased fluidity of the lipid environment of cardiac membranes, different temperature-activity relationships of key membrane enzymes and altered handling of intracellular Ca^{++} (Nielsen and Owman, 1968; Wolowyk *et al.,* 1990; Wang *et al.,* 2002). Abdukeyum and co-workers (2008) compared ischaemic preconditioning and several edible oils in their ability to exert anti-arrhytmogenic effects during myocardial ischaemia and reperfusion in an isolated perfused rat heart model. Rats of the fish oil group received 10% fat (of which 7% was tuna fish oil) in their diet for six weeks. These authors found that the antiarrhythmogenic effects of fish oil supplementation were comparable to that of ischaemic preconditioning. A study by Hlavackova and co-workers (2007) demonstrated significant decreases in ischaemia/reperfusion induced arrhythmia in rats fed a n-3 rich diet when compared to rats fed a SFA or n-6 rich diet. After 10 weeks of supplementation, one group of each diet was exposed to hyperbaric conditions for five to six weeks, after which hearts were perfused. Pre-ischaemic hypoxic incidents was shown to increase resistance to ischaemia/reperfusion induced arrhythmia in the n-3 fed group.

Other studies suggest that fish oil or n-3 PUFA supplementation do not always offer protection against cardiac arrhythmias. In patients with an implantable cardioverter defibrillator, fish oil supplementation was found to exhibit inconsistent effects. In some cases it was antiarrhythmic, in others proarrhythmic, whilst in some it had no effect (Burr *et al.,* 2003; Leaf *et al.,* 2005; Brouwer *et al.,* 2006; Den Ruijter *et al.,* 2007; Wilhelm *et al.,* 2008). Burr and co-workers (2003)

found that n-3 PUFA supplementation led to increased mortality and sudden cardiac death in patients with angina pectoris. Men with chest pain were asked to supplement their diet with two portions of oily fish or 3g of fish oil per week for 6 months. The control group in this study did not modify their diet. The amount of cardiac and sudden arrhythmic deaths recorded in the fish oil group was significantly higher than those in the control group. The authors could not explain these findings. Den Ruijter and co-workers (2007) demonstrated that n-3 PUFAs have different electrophysiologic effects when it is incorporated into the membranes than when it is in the blood. These authors speculated that this difference in electrophysiologic effects may play a role in the seemingly contradictory effects of n-3 PUFA supplementation on cardiac arrhythmia. Results of a study by Wilhelm and co-workers (2008) are in agreement with the aforementioned speculation. This study focused on patients with structural heart disease, as most sudden deaths are caused by ventricular arrhythmias in patients with structural heart disease or impaired left ventricular function (Huikuri *et al.,* 2001). Wilhelm and co-workers (2008) found that patients with heart failure or structural heart disease showed altered red blood cell fatty acid profiles. Their results suggest that higher levels of n-3 PUFAs in red blood cells may have caused increased cardiac arrhythmia. This in turn suggests that red blood cell n-3 PUFA levels may be seen as an independent risk factor for occuring cardiac arrhythmias.

In a study by Demonty and co-workers (2006) it was shown that supplementing 7,6g fish oil per day for 29 days was able to decrease serum triacylglycerols when compared to olive oil supplementation in hypercholesterolaemic volunteers. This study also demonstrated that plant sterols esterified to fish oil is even more effective in reducing triacylglycerols. Plant sterol esters were also able to decrease LDL cholesterol levels when compared to an olive oil supplemented control group. Fish oil has beneficial effects on the serum lipid profile. It is known to reduce cholesterol and triglycerides. Reducing triglycerides is of great importance to diabetics or metabolic syndrome-X patients, as the increased free fatty acids associated with these conditions may interfere with cardiac function (D'Allesandro *et al.,* 2008). A study done by D'Allesandro and co-workers (2008) used a sucrose rich diet to induce lipotoxicity in rats after eight months of feeding. Changing the fat source in the diet from corn oil to fish oil for the last 2 months of feeding was able to reduce lipotoxicity. Another study showed that n-3 PUFA or fish supplementation may reduce subclinical atherosclerosis (He *et al.,* 2008). These authors used coronary artery calcium score, common carotid intima-media thickness, internal carotid intima-media thickness and ankle brachial index to measure the level of atherosclerosis in 5488 individuals after evaluating their diets by questionnaire. They concluded that dietary supplementation with n-3 PUFAs or fish reduced the incidence of subclinical atherosclerosis.

3.5.4 Fish oil in the ischaemia/reperfusion model

While many studies focused on the effects of fish oil on myocardial arrhythmias caused by ischaemia/reperfusion injury, only a few studies investigated the effects of this oil on functional recovery. Logic would dictate that inhibition of myocardial arrhythmia would ultimately lead to increased functional recovery. However, to our knowledge, no evidence exists to substantiate whether fish oil supplementation may lead to myocardial protection from ischaemia/reperfusion injury.

3.5.5 Benefits of fish oil supplementation

Dietary fish oil supplementation may play a role in preventing heart disease, heart attacks and atherosclerosis, by reducing serum triacylglycerols and LDL cholesterol (Demonty *et al.,* 2006; D'Allisandro *et al.,* 2007; Liu *et al.*, 2008). Dietary supplementation of the individual fatty acids, EPA and DHA, showed similar effects as fish oil supplementation. It has been suggested that DHA is the main active component of fish oil and that supplementation of DHA would have similar effects as in fish oil supplementation (McLennan and Abeywardena, 2005). Evidence also suggests that fish oil can reduce diastolic blood pressure (McLennan *et al.,* 1996; Theobald *et al.,* 2006) and it is known that a reduction in blood pressure is normally associated with better long term cardiac health (De Marco *et al.,* 2009).

Fish oil may also reduce mortality after a cardiovascular incident, as it plays a role in reducing potentially fatal arrhythmias (Charnock *et al.,* 1991; McLennan *et al.,* 1993; McLennan *et al.,* 1996; McLennan and Abeywardena, 2005; Hlavackova *et al.,* 2007). Little is however known about the effects of fish oil on infarct size and post ischaemic functional recovery of the heart.

3.6 Palm Oil

3.6.1 Refining of palm oil

Palm oil is produced by extraction of the oil from the mesocarp of the fruit of the *Ealius genesis* plant, commonly known as the oil palm. As for most oils to be retailed as edible oil it also has to undergo certain refinery processes. Refining of palm oil requires bleaching and deodorization which may destroy some of the important micronutrients contained in the oil (Wilson *et al.,* 2005).

A novel process has been developed by which palm oil can be refined in order to preserve more micronutrients. Carotenoids and vitamins which would normally be destroyed by the deodorization and bleaching steps of the refining of palm oil are thus retained (Nagendran *et al.,* 2000; Sundram *et al.,* 2003) and these micronutrients, particularly the carotenoids give red palm oil (RPO) its distinctive red colour.

3.6.2 Components of palm oil

Palm oil has a balanced fat composition of saturated fatty acids (SFAs) and unsaturated fatty acids. Of these fats, 51% are SFAs, 39% MUFAs and 10% PUFAs (mostly linoleic acid) (Ong and Goh, 2002; Wilson *et al.,* 2005). Palmitic acid is the major SFA in palm oil making up 44% of the total fatty acids, with the other 7% SFAs being made up by myristic acid and stearic acid. Most of the MUFAs in palm oil are oleic acid. The vitamin E content of palm oil is approximately 62mg/g of which 16mg/g is made up by α-tocopherol with the balance being made up mostly by tocotrienols (70%)(Ooi, 1999; Sundram *et al.,* 2003; Wilson *et al.,* 2005). Very little of the carotenoids present in crude palm oil survives the conventional refinery process. The carotene levels in refined, bleached and deodorized palm oil may be as low as 1.2mg/g (Wilson *et al.,* 2005).

RPO has a similar fatty acid profile as refined palm oil, with 51% saturated fatty acids, 38% monounsaturated fatty acids and 11% polyunsaturated fatty acids. (Nagendran *et al.,* 2000; Ong and Goh, 2002; Sundram *et al.,* 2003). In addition to the fatty acids contained in RPO there are also an abundance of micronutrients naturally occurring in this oil. The colour of the oil is obtained from its high concentrations of carotenoids. RPO contains at least 500 parts per million (ppm) of carotenoids which include α-, β- and γ-carotene along with some lycopene and xanthophyls. Of these carotenoids, 80-90% are α- or β-carotene which occurs in a ratio of 2:1 in the favour of β-carotene (375mg/g) (Lehninger *et*

al., 1993; Ping, 2006). RPO is also unique among oils due to its high Vitamin E content. It contains 560 to 1000 ppm of Vitamin E, of which there are approximately 18-22% tocopherols and 78-82% tocotrienols (Nagendran *et al.,* 2000; Sundram *et al.,* 2003; Schroeder *et al.,* 2006). This makes RPO the oil with the highest content of tocotrienols of all vegetable oils. The most abundant tocotrienol in RPO is γ-tocotrienol, which is a potent antioxidant that reduces cholesterol production and platelet aggregation (Steiner *et al.,* 1975; Chan *et al.,* 1981; Helub *et al.,* 1989; Quereshi *et al.,* 1991; Pearce *et al.,* 2002). Serbinova and co-workers (1992) showed that tocotrienols are 40-60 times more potent as an antioxidant than tocopherol in a study done on palm oil vitamin E in an isolated perfused rat heart model. RPO also contains squalene, phytosterols and co-enzyme Q10 (Goh *et al.,* 1985; Nagendran *et al.,* 2000; Sundram *et al.,* 2003). RPO has been the focus of many studies aimed at improving vitamin A status of certain communities (Leitz *et al.,* 2001; Van Stuijvenberg *et al.,* 2001; Radhika *et al.,* 2003). The main reason for RPO being the focus of these studies is the high carotene content of the oil and the major finding was that RPO is effective in combating vitamin A deffiency by the provision of ample carotene in the diet. RPO may thus prevent complications associated with vitamin A deficiency.

3.6.3 Cardiovascular studies with palm oil

Most of the studies done on palm oil focused on its effect on the serum lipid profile (Hornstra, 1988; Ng *et al.,* 1991; Qureshi *et al.,* 1991; Quereshi *et al.,* 1995; Sundram 1997; Chandrasekharan, 1999; Krichevsky *et* al., 1999; Theriault

et al., 1999; Kritchevsky, 2000). Historically the perception of dieticians and clinicians was that palm oil would have a negative effect on the serum lipid profile, due to the relatively high levels of saturated fatty acids in the oil. However, research has proven this perception to be wrong, and suggested possible mechanisms by which palm oil may in fact reduce serum cholesterol. Firstly, palmitic acid does not impact negatively on the serum lipid profile (Khosla *et al.,* 1997; Krichefsky *et al.,* 2000; Ong and Goh, 2002). Secondly, the triglyceride conformation in palm oil has saturated fatty acids on the sn-1 and sn-3 positions of the glycerol backbone, and unsaturated fatty acids on the sn-2 postion in 75-87% of these molecules. This triglyceride conformation leads to absorption of more unsaturated fats than saturated fats, as the fatty acid on the sn-2 position is absorbed preferentially to those in the sn-1 and sn-3 positions (Renaud *et al.,* 1995; Krichefsky *et al.,* 1999; Krichefsky *et al.,* 2000). Furthermore, tocotrienols contained within palm oil has been shown to inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme in cholesterol biosynthesis (Quereshi 1991; Khor *et al.,* 1995; Quereshi 1995; Theriault *et al.,* 1999). RPO has a similar fatty acid composition as refined palm oil, and thus it also has a neutral effect on the serum lipid profile. It also has a similar tocotrienol content to refined palm oil. It may therefore be accepted that RPO has similar effects on the serum lipid profile than palm oil.

In a study done on rabbits, Hornstra and co-workers (1988) found that palm oil was significantly less atherogenic than fish oil, linseed oil and olive oil. These

results were obtained after the diet of these rabbits were supplemented with the different oils for 18 months. Wilson and co-workers (2005) investigated the effect of palm oil on cholesterol concentrations and aortic cholesterol accumulation in a hypercholesterolaemic hamster model. They found that supplementation of 10% palm oil to a standard hamster diet for 10 weeks had reduced total cholesterol, non-HDL cholesterol and triglycerides when compared to a coconut

supplemented group. In addition they demonstrated that palm oil supplementation can be associated with significantly lower lipid hydroperoxide formation than coconut oil supplementation. The authors suggest that this may be due to different antioxidant pathways associated with the oils, or an intrinsic effect of dietary fats. In the same study, RPO also reduced total cholesterol and non-HDL cholesterol when compared to a coconut oil supplemented group. In addition, the RPO led to a significant increase in HDL cholesterol in this study. Results showed that RPO had the highest free cholesterol to cholesterol ester ratio, indicating that it was the least atherogenic of all the oil preparations used. Zhang and co-workers (2003) showed that red palm oil did not change the serum lipid profile of volunteers fed a RPO supplemented diet (60% of fat was RPO) for 42 days. They also found that the carotenoid and tocopherol levels of the volunteers were significantly increased after the diet period. Qureshi *et al.,* (1991) supplemented 15 hypercholesterolaemic subjects with 800mg of tocotrienol rich fraction (extracted from palm oil) for four weeks. There was a 20% reduction in total cholesterol and a 27% reduction in LDL cholesterol in these subjects after the four weeks. In another study, the same authors showed that supplementation of a blend of tocotrienols for four weeks could reduce the total cholesterol levels of hypercholesterolaemic individuals by 10%. In a study by Salinas and co-workers (2008) it was found that a 35 day supplementation of a deodorized and bleached form of palm oil could significantly reduce total cholesterol and increase HDL-cholesterol in rats with induced hyperlipidaemia by the addition of 5% egg yolk powder to the diet. It was concluded that the high concentrations of

micronutrients and mono-unsaturated fatty acids in palm oil altered the serum lipid profile favorably in hyperlipidaemic rats. In a study by Girardet and co-workers (1977), rats were fed a diet containing 12% palm oil for a year and found that palm oil caused increased total serum cholesterol, but that it was accompanied with a lower aortic accumulation of cholesterol esters when compared with sunflower oil, rapeseed oil, soybean oil and butter. In the light of previous studies the palm oil associated increase in cholesterol in this study may be due to the longer feeding period.

Research has shown that palm oil may have some anti-arrhythmogenic properties. Charnock and co workers (1991) demonstrated that palm oil had some efficacy in reducing arrhythmia during reperfusion. These authors supplemented rats with 12% fat in the diet for one year, before inducing ischaemia and reperfusion *in vivo*. They also noted that the incidence of ventricular fibrillation decreased in the palm oil group when compared to a control group which was supplemented with sheep fat. Abeywardena and co-workers (1995) found that that the anti-arrhytmogenic effect of palm oil was significantly lower than that of sunflower oil. In this study rat diets were supplemented for 9 months with 12% of the oils before induction of ischaemia/reperfusion.

3.6.4 Palm oil in the ischaemia/reperfusion model

The tocotrienols present in palm oil have been shown to offer protection from myocardial ischaemia/reperfusion injury in the isolated perfused rat heart model

(Serbinova *et al.,* 1992; Das *et al.,* 2008). In both studies, Vitamin E components of palm oil were fractionated from the oil. Serbinova and co-workers (1992), perfused isolated rat hearts with tocotrienol rich fraction (TRF). They found that TRF could increase post ischaemic functional recovery and suggested that the antioxidant activity of the TRF was responsible for the protection. In the study by Das and co-workers (2008), individual isoforms of tocotrienols (alpha, gamma and delta) were fed to rats by gavage. Three concentrations of tocotrienols (0.35%, 1% or 3.5%) were fed to the rats for two or four weeks. Das and co-workers (2008) demonstrated a decreased infarct size with tocotrienol supplementation when compared to a control group. Similar decreases in infarct size could be achieved with α-tocotrienol and γ-tocotrienol supplementation. They proposed that the antioxidant activity of tocotrienols may play a role in the protection offered against ischaemia/reperfusion injury, but emphasised the ability of tocotrienols to alter protein kinase signaling. In this study, decreased infarct size was accompanied by increased phosphorylation of PKB/Akt.

Palm oil supplementation may also reduce oxidative stress associated with ischaemia/reperfusion injury. Narang and co-workers (2004) supplemented 5 or 10% palm oil to the diet of rats for 30 days. They found an increase in glutathione peroxidase, catalase and super oxide dismutase activities in hearts of rats supplemented with palm oil. This was associated with lower thiobarbituric acid-reactive substance (TBARS) measurements. The authors conclude that palm oil

augments the activities of endogenous antioxidant enzymes, and thus reduce oxidative stress.

To our knowledge only a few studies have been conducted on dietary RPO supplementation related to cardiovascular health. Most studies on RPO dealt with vitamin A deficiencies. However, in recent studies the effect of RPO in the ischaemic/reperfusion model was investigated. The group of Van Rooyen and co-workers published several papers on the protective effect of RPO against ischaemia/reperfusion injury (Esterhuyse *et al.,* 2005; Esterhuyse *et al.,* 2005b; Bester *et al.,* 2006; Engelbrecht *et al.*, 2006; Esterhuyse *et al.,* 2006; Kruger *et al.*, 2007; Van Rooyen *et al.,* 2008; Engelbrecht *et al.,* 2009).

Esterhuyse and co-workers (2005) demonstrated that dietary RPO supplementation could offer protection against ischaemia/reperfusion injury in the isolated perfused heart. These authors fed rats 200µl RPO per day for five to six weeks before perfusions were performed. Recent studies published on RPO have mostly used the ischaemic/reperfused isolated rat heart model (Esterhuyse *et al.,* 2005b; Bester *et al.,* 2006). A follow up study showed that dietary RPO supplementation could offer similar protection in cholesterol fed rats where rats were placed on a diet that included 2% synthetic cholesterol with or without 200µl RPO (Esterhuyse *et al.,* 2005b). The cholesterol supplemented group showed decreased functional recovery when compared to standard rat chow fed controls. RPO supplementation could however significantly increase functional recovery in

the hearts of cholesterol supplemented animals. Esterhuyse *et al.* (2005b) suggested that protection was offered in cholesterol fed rats *via* a different pathway when compared to rats fed a standard rat chow diet based on the finding that the NO-cGMP signaling pathway was not affected by RPO supplementation in cholesterol fed rats.

Bester and co-workers (2006) showed that dietary RPO supplementation could offer protection against ischaemia/reperfusion injury, irrespective of the fat content of the diet. In this study the diets were designed to be isocaloric to investigate whether increased energy in the RPO group may have played a role in previous studies. The one diet contained high levels of saturated fatty acids (SFAs), while the other contained high levels of polyunsaturated fatty acids (PUFAs). RPO supplementation was able to offer protection against ischaemia/reperfusion injury in both diets when compared to controls. These authors suggested several mechanisms for RPO mediated protection against ischaemia/reperfusion injury. Amongst the proposed mechanisms are the nitric oxide-cGMP pathway, phosphorylation of mitogen activated protein kinases (MAPK's) and scavenging of deleterious reactive oxygen species (ROS) by RPO (Esterhuyse *et al.*, 2005b; Esterhuyse *et al.*, 2006; Engelbrecht *et al.*, 2006; Bester *et al.*, 2006; Kruger *et al.,* 2007). In the study by Engelbrecht and co-workers (2006), the RPO supplementation caused increased phosphorylation of PKB/Akt and p38, and decreased phosphorylation of JNK and ERK in rats fed a standard rat chow diet supplemented with 200µl of RPO for 5 to 6 weeks. These

findings were accompanied by decreased caspase activation and PARP cleavage, as well as improved aortic output recovery, indicating a protective effect. The MAPK activation was markedly different when a similar diet was supplemented with 2% cholesterol (Kruger *et al.,* 2007). These authors supplemented cholesterol and RPO simultaneously for 5 to 6 weeks in order to determine whether cholesterol supplementation altered the effects of RPO on MAPK signaling. They found increased caspase activation and decreased PARP cleavage with RPO supplementation. This was accompanied by increased ERK phosphorylation together with decreased JNK and p38 phosphorylation in the RPO supplemented group when compared to the cholesterol fed controls. This indicates that RPO supplementation activated survival kinase pathways and inhibited apoptotic pathways. These changes led to a significantly greater aortic output recovery in the RPO supplemented group than in the cholesterol fed controls. Engelbrecht and co-workers (2009) found in a similar model that dietary RPO supplementation increases PKB/Akt phosphorylation *via* a PI3K independent pathway. They perfused hearts in the presence of wortmanin which is a known inhibitor of PI3K. Despite inhibition of PI3K in this study, RPO could still induce increased phosphorylation of PKB/Akt, and induce increased reperfusion functional recovery.

Above mentioned studies showed that hearts of dietary RPO supplemented rats have significantly increased cGMP levels early in ischaemia (Esterhuyse *et al.,* 2005; Esterhuyse *et al.,* 2005b; Esterhuyse *et al.,* 2006; Bester *et al.,* 2006).

Hearts of dietary RPO-supplemented rats showed increased levels of nitric oxide within the myocytes. These findings suggest that dietary RPO-supplementation leads to an increased NO conservation in the myocytes, and therefore stronger NO-cGMP signaling. Both these signaling molecules are antagonists of cAMP and would thus offer protection against ischaemia/reperfusion injury by blocking intracellular calcium overload (Du Toit *et al.,* 2001). Khairallah and co-workers (2008) suggest that increased levels of cGMP may prevent intracellular triglyceride accumulation in cardiomyocytes and that this may offer further protection. Van Rooyen and co-workers (2008), argued that not one, but a few mechanisms may be involved in the protective effect of RPO against ischaemia/reperfusion injury. Therefore, it is not conclusive that the pro-survival Akt pathway, the anti-apoptotic pathway or the NO-cGMP pathway alone is responsible for this protection. The complexity of the composition of the oil, including 50% unsaturated fatty acids and several highly potent antioxidants, may well be the strong point of this oil.

3.6.5 Benefits of palm oil supplementation

Palm oil may be able to offer protection against ischaemia/reperfusion injury, through the TRF contained in it (Serbinova *et al.,* 1992; Das *et al.,* 2008). Palm oil may also lead to the reduction of oxidative stress, and this could play a role in the reduction of ischaemia/reperfusion injury (Narang *et al.,* 2004).

It has also been suggested that palm oil may have some antiarrhythmogenic effects, which may reduce sudden death after ischaemic incidents (Charnock *et al.,* 1991).

Palm oil may also exert a neutral or positive effect on the serum lipid profile through the effects of its fatty acid composition and tocotrienols (Hornstra, 1988; Ng *et al.,* 1991; Qureshi *et al.,* 1991; Quereshi *et al.,* 1995; Sundram, 1997; Chandrasekharan, 1999; Krichevsky *et* al., 1999; Theriault *et al.,* 1999; Kritchevsky, 2000).

Few studies were performed using red palm oil that focused on the baseline effects of this oil on cardiovascular health. Red palm oil supplementation does however, offer protection against myocardial ischaemia/reperfusion injury *via* several suggested mechanisms (Esterhuyse *et al.*, 2005b; Esterhuyse *et al.*, 2006; Engelbrecht *et al.*, 2006; Bester *et al.*, 2006; Kruger *et al.,* 2007; Van Rooyen *et al.,* 2008). The mechanisms suggested for the aforementioned protection includes upregulation of the NO-cGMP signaling pathway and increased phosphorylation of PKB/Akt.

This oil has positive effects on the serum lipid profile and reduces oxidative stress (Zhang *et al.,* 2003; Wilson *et al.,* 2005; Das *et al.,* 2007; Salinas *et al.,* 2008). Furthermore, as refined palm oil and RPO have comparable contents except for the higher micronutrient (mainly carotenoids) content of RPO, RPO

may exibit similar antiarrhythmogenic effects, as is suggested of palm oil (Charnock *et al.,* 1991).

RPO has been shown to be effective in reducing vitamin A deficiency and associated ocular disorders. This is due to the pro-vitamin A provided by RPO (Leitz *et al.,* 2001; Van Stuijvenberg *et al.,* 2001; Radhika *et al.,* 2003).

The refinery process of RPO, which retains more of the micronutrients, makes it unnecessary to add more antioxidants to the oil. Addition of antioxidants to palm oil does not offer similar protection as natural red palm oil (Wilson *et al.,* 2005). RPO contains high concentrations of tocotrienols and carotenoids and research has shown that different carotenes may not only work synergistically with each other, but also with tocotrienols to achieve greater antioxidant effects (Stahl and Sies, 2005; Schroeder *et al.,* 2006).

3.8 Discussion and Conclusion

More research needs to be done on edible oil products. Many studies focus on only certain components of the oils that are normally employed during research. There also seems to be a trend that each oil is only used in studies to obtain specific types of endpoints which have previously been associated with that oil. These approaches frustrate any attempt to speculate what the effects of dietary supplementation of edible oils on the cardiovascular health would be. In our opinion, the best way to determine the effects of dietary edible oil

supplementation on the cardiovascular system would be, to supplement the whole oil (unmodified) to the diet of research subjects and subsequently measuring a broad spectrum of endpoints, irrespective of the oil used.

From the studies that have been done however, it is clear that the oils that have been reviewed in this article all may form part of a healthy diet. Each oil seems to have some beneficial effects. It may be that some undesirable effects are still to be elucidated in these oils and there is still controversy about the beneficial effects of some of these oils. However, from the current literature, these oils all have more beneficial effects on cardiovascular health than detrimental effects, if supplemented to the diet in acceptable proportions.

Most studies performed with olive oil focused on the anti-oxidative effects of the polyphenols in this oil and many researchers used only extracts of the antioxidants in the olive oil to perform their studies. The inhibition of oxidative stress along with the reduction of serum cholesterol, which is associated with olive oil, would lead to reduced risk of ischaemic heart disease. However, it is difficult to predict if this oil would have any protective effects against ischaemia/reperfusion injury. It is also unclear if this oil would have anti-arrhythmogenic effects. More research needs to be done using this oil as a dietary supplement, with the focus of elucidating its effects on ischaemia/reperfusion injury.

Sunflower oil has mostly been used as a control to compare other oils to. The only clear effects of this oil on cardiovascular health are reduction of serum cholesterol, and some mild anti-arrhythmogenic effects.

Dietary fish oil supplementation inhibits arrhythmia in healthy individuals. This finding could not be repeated with regularity in individuals with a history of heart disease. More research needs to be done in order to elucidate the reasons for the variations in this effect by fish oil. Little is known of other effects that fish oil may have on the cardiovascular system. The effects of dietary fish oil supplementation on the risk factors for ischaemic heart disease, and its effects on ischaemia/reperfusion injury should be investigated.

Palm oil has mostly been used in studies to determine its effects on the serum lipid profile. This oil has a neutral effect on the serum lipid profile and may even reduce atherosclerosis and prevent ischaemic heart disease. The few studies done with palm oil on arrhythmogenesis are non conclusive. However, palm oil may have some minor anti-arrhythmogenic effects. Furthermore, the studies performed with TRF-extracts from palm oil suggest that palm oil may offer protection against ischaemia/reperfusion injury. These effects however, should be subjected to further study in a dietary supplementation model.

An advantage of doing research on the effects of red palm oil is that it contains the same components as refined palm oil, except for the presence of more

micronutrients. We can therefore assume investigations performed using palm oil would also apply to red palm oil. Studies performed with red palm oil confirm this assumption, and additionally the presence of more micronutrients often lead to enhanced effects due to their synergistic action. RPO was shown to have a neutral or slightly lowering effect on the serum lipid profile. Furthermore, our research has shown that RPO offers protection against ischaemia/reperfusion injury when supplemented to the diet. More research should be done with RPO to confirm its effects on risk factors for ischaemic heart disease and cardiac arrhythmia.

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CHAPTER 4

MATERIALS AND METHODS

This chapter will summarize the materials and methods used in all the studies included in this dissertation. The methods will be discussed as three separate study designs in this chapter. Where applicable reference is made to the first instance where the method was described. All experiments in this dissertation has been approved by the ethics comity of the Health and Wellness Faculty of the Cape Penisnula University of Technology (Ref: CPUT/HW – REC 2008/009)

4.1 Study design 1 (Chapter 5)

5 week diet period

SRC	10 min perfusion	30 min ischaemia	120 min reperfusion
SFO			
RPO			

SRC: Standard rat chow diet
SFO: SRC plus 200µl sunflower oil per day
RPO: SRC plus 200µl red palm oil per day

Blood was collected for serum lipid analysis

Hearts were snap frozen for biochemical analysis

* 5 and 10 minute coronary effluent samples were collected upon reperfusion

Hearts were frozen for infarct size determination

Figure 4.1. Experimental model for Study design 1.

4.1.1 Dietary supplementation

Male Wistar rats weighing approximately 150g (six weeks old) were randomly divided into three groups and fed the following diets over a five week period:

Control Group 1: standard rat chow (SRC)

Control Group 2: SRC plus 200μl/day of sunflower oil (SFO)

Experimental Group: SRC plus 200μl/day of red palm oil (RPO)

The rats were individually housed to ensure that each animal received equal amounts of supplements, which were prepared on a daily basis in order to prevent spoiling. All rats were allowed *ad libitum* access to food and water. The dosage used for RPO supplementation was similar to previous studies from our laboratory (Esterhuyse *et al.,* 2006), while the dosage used for SFO was matched to that of the RPO group (this would equal 0.58 mg/kg/day in humans). Supplements were given to rats mixed into rat chow, and willingly consumed.

Table 4.1: A breakdown of the major components of the oils used as supplements in the diets of this study. Tocotrienols were expressed as a percentage of the vitamin E content in the oil.

	Sunflower oil	Red palm oil
SFA (%)	12	51
MUFA (%)	26	38
PUFA (%)	62	11
Carotenoids (ppm)	-	500
Vitamin E (ppm)	±500	500-1100
Tocotrienols (%)	0	75

4.1.2 Langendorff perfusion

After the feeding period, rats were anaesthetized using diethyl ether. Hearts were rapidly excised, then mounted on a Langendorff perfusion apparatus and perfused at 37°C using a Krebs Henseleit buffer solution which was constantly gassed with 5% carbon dioxide, balanced oxygen. The Krebs Henseleit buffer contained the following: 118,4mM NaCl, 25mM $NaHCO_3$, 4,3mM KCl, 1,2mM KH_2PO_4, 11,1mM glucose-mono-hidrate, 1,2mM $MgSO_4$, 1.5mM $CaCl_2 . 2H_2O$. A perfusion pressure of 100mmHg was maintained throughout the perfusion period. After mounting, hearts were subjected to a 10 minute stabilization period. Following stabilization, hearts were subjected to a 30 minute period of normothermic global ischaemia. Hearts were subsequently reperfused for 120 minutes after which ventricular tissue was frozen at -20°C overnight. Hearts for biochemical analysis were snapfrozen after the 10 minute stabilization period and also after 10 minutes reperfusion using a Wollenberger clamp which was pre-cooled in liquid nitrogen.

4.1.3 Determination of infarct size

Frozen hearts were removed from the freezer 12 hours after their initial freezing and cut into 3mm thick cross-sectional slices. These slices were stained in 2,3,5-triphenyltetrazolium chloride (TTC) for 10 minutes at 37°C. After TTC staining, the slices were transferred to a formalin solution for ten minutes and then placed in phosphate buffer (pH 7.4).

Heart slices were then placed between two sheets of glass and scanned and analyzed using infarct size planimetry software (Infarct size). Infarct size was represented as percentage of the area at risk.

4.1.4 Lactate dehydrogenase

Lactate dehydrogenase (LDH) release from hearts was measured in coronary effluent samples that were collected for the first 5 min of reperfusion, using a LDH-P kit (Diagnosticum, Budapest, Hungary). The kit makes use of spectrophotometric measurement at a wavelength of 340 nm to determine the absorbance of substrate catalyzed by the enzyme. Temperature was measured after each reading, and results were adjusted accordingly. LDH release was expressed as milliunits per minute per gram wet heart weight.

4.1.5 Matrix Metalloproteinase-2 Zymography

Coronary effluent was collected for the first 10 minutes of reperfusion and concentrated by ultrafiltration using Milipore Amicon 30kDa ultrafiltration tubes. The concentrated coronary effluent was then subjected to gelatin zymography.

Gelatinolytic activities of MMPs were determined as previously described (Cheung et al., 2000). Briefly, 8 % polyacrylamide gels were copolymerized with gelatin (2 mg/ml, type A gelatin from porcine skin; Sigma-Aldrich), and a constant amount of protein per lane (coronary perfusate, 40 µg) was loaded. Following electrophoresis (150 V, 1.5 h), gels were washed with 2.5% Triton X-100 for 3

times 15 min and incubated for 24 to 48 h at 37°C in incubation buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl2, and 0.05% NaN3, pH 7.4). Gels were then stained with 0.05% Coomassie Brilliant Blue (G-250; Sigma-Aldrich) in a mixture of methanol/acetic acid/water (2.5:1:6.5, v/v) and destained in aqueous 4% methanol/8% acetic acid (v/v). Gelatinolytic activities were detected as transparent bands against the dark-blue background. Zymograms were digitally scanned, and band intensities were quantified using Quantity One software (Bio-Rad, Hercules, CA) and expressed as a ratio to the internal standard. Band density was expressed as arbitrary units per milligram of protein to exclude a possible nonspecific release of MMP into the perfusate during ischemia.

4.1.6 Nitrotyrosine

Myocardial nitrotyrosine levels were measured by ELISA test kit (supplied by Cayman Chemical). This assay is based on competition between nitrotyrosine in the sample and an actylcholinesterase-linked tracer for limited binding sites on antibodies. After incubation with sample and tracer, unbound analytes were removed by washing. Addition of the enzyme substrate to the wells leads to the development of a distinct yellow color in proportion to the amount of tracer present in each well. Spectrophotometrical measurement at a wavelength of 412nm yields results which are inversely proportional to the levels of nitrotyrosine present in the sample.

4.1.7 Western Blot

Snap frozen heart tissue was ground into a fine powder under liquid nitrogen using a pestle and mortar. The tissue was homogenized by adding 600µl homogenization buffer and 7.5µl phenylmethanesulphonylfluoride (PMSF) to 150mg of the sample. For phosphoprotein determination, 3µl of a NaF, Na_3VO_4 solution was added per 150mg of the sample after which samples were sonicated for 3 x 10 seconds. Samples were then centrifuged at 5000rpm for 10 minutes in a refrigerated centrifuge cooled to 4°C. The supernatant was then collected and the pellet discarded. Protein concentration was determined by the biconcic acid test kit method. This assay depends on the conversion of alkaline Cu^{2+} to Cu^+ in a concentration dependant manner by an unknown protein. An colour can then be quantitated from the reaction of bicinchonic acid-copper sulfate complex and the protein.

Samples were diluted with Laemmli sample buffer and boiled for five minutes, after which 40 µg of protein was separated by SDS-PAGE electrophoresis. Gels were prepared at a concentration of 10% acrylamide. After electrophoresis the proteins were transferred to polyvinylidene fluoride (PVDF) membranes which were stained with Ponceau Red to visualize protein. Membranes were then washed with Tris- buffered saline - 0.1% Tween 20 (TBST). Non specific binding was blocked by overnight incubation in 5% fat free milk in TBST. Membranes were then incubated with primary antibodies that recognize Erk p42/44 (Thr^{202}/Thr^{204}) and PKB (Thr^{308}). Membranes were subsequently washed and

incubated with secondary antibody. After thorough washing with TBST, membranes were covered with ECL detection reagents and exposed to autoradiography films which were densitometrically analyzed. (All primary antibodies were used at an concentration of 1 in an thousand while the secondary antibody was used at an concentration of 1 in 500. Antibodies were purchased from Cell Signalling Techonology)

4.1.8 Serum Cholesterol

Whole blood obtained from the thoracic cavity of rats after the removal of the heart, was centrifuged to obtain serum. Serum cholesterol levels were determined using a test kit supplied by Diagnosticum Zrt. (Budapest, Hungary). This test kit makes use of cholesterol esterase and cholesterol oxidase in order to produce hydrogen peroxide from cholesterol present in the sample. Hydrogen peroxide together with phenol and 4-aminoantipyrine then forms a red quonone in the presence of peroxidase. This red quonone can be measured spectrophotometrically at a wavelength of 505nm.

4.1.9 Serum Triglycerides

Whole blood obtained from the thoracic cavity of rats (without fasting) after removal of the heart was centrifuged to obtain serum. Serum triglyceride levels were measured using a test kit supplied by Diagnosticum Zrt. (Budapest, Hungary). Triglycerydes present in the sample are degraded to dihydroxiacetone-phosphate and hydrogen peroxide in the presence of lipoprotein lipase, glycerol

kinase and glycerol-3-phosphate oxidase. Hydrogen peroxide together with 4-aminoantipyrine and P-chlorophenol forms a red quonone in the presence of peroxidase. This red quonone is then measured spectrophotometrically at a wavelength of 505nm.

4.1.10 Statistical Methods

All values in this study are presented as mean plus or minus standard error of the mean. Significance between groups was determined with one way ANOVA with Tukey post hoc test. *P* was considered significant if it was less than 0.05.
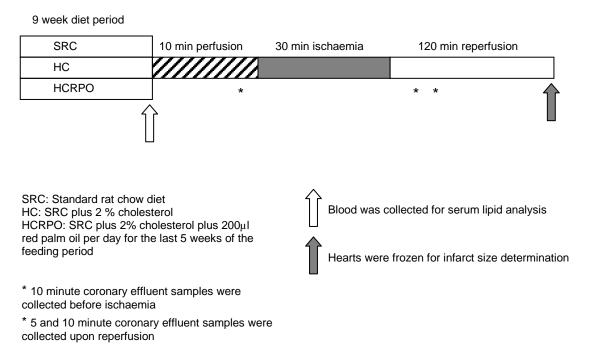
4.2 Study design 2 (Chapter 6)

9 week diet period

SRC	10 min perfusion	30 min ischaemia	120 min reperfusion
HC			
HCRPO	*	* *	

SRC: Standard rat chow diet
HC: SRC plus 2 % cholesterol
HCRPO: SRC plus 2% cholesterol plus 200µl
red palm oil per day for the last 5 weeks of the
feeding period

Blood was collected for serum lipid analysis

Hearts were frozen for infarct size determination

* 10 minute coronary effluent samples were
collected before ischaemia

* 5 and 10 minute coronary effluent samples were
collected upon reperfusion

Figure 4.2. Experimental model for Study design 2.

4.2.1 Dietary supplementation:

Male Wistar rats weighing approximately 150g (6 weeks of age) were divided into

three groups. Rats in these groups were placed on the following diets,

respectively:

Control group 1: Standard rat chow diet (SRC) for nine weeks

Control group 2: SRC diet plus 2% cholesterol for nine weeks

Experimental group: SRC diet plus 2% cholesterol for nine weeks, plus 200µl red

palm oil (RPO) for the last five weeks of the nine week period

Rats were individually housed to ensure that each animal received equal amounts of supplements. RPO and supplements were prepared on a daily basis in order to prevent spoiling. Rats were allowed *ad libitum* access to food and water. The dosage of RPO used in this study was similar than that of previous studies in our laboratory (Esterhuyse *et al.,* 2006) (this would equal 0.58 mg/kg/day in humans). Supplements were given to rats mixed into rat chow, and willingly consumed. Cholesterol dosage was also obtained from a previous study performed with the intension of mild cholesterol supplementation (Giricz *et al.,* 2003; Esterhuyse *et al.,* 2006).

4.2.2 Langendorff perfusions

Langendorff perfusions were performed employing the same method used in Study design 1 (see section 4.1.2).

4.2.3 Determination of infarct size

Infarct size determination was performed using the same method as in Study design 1 (see section 4.1.3).

4.2.4 Lactate dehydrogenase

The same methods were employed as described in Study design 1 (see section 4.1.4).

4.2.5 MMP2 zymography

MMP2 zymography was performed using the same method as described in Study design 1 (see section 4.1.5).

4.2.6 Serum cholesterol

Serum cholesterol was measured using the same method as described in Study design 1 (see section 4.1.8).

4.2.7 Serum triglycerides

Serum triglyceride levels were determined using the same method as described in Study design 1 (see section 4.1.9).

4.2.8 Statistical methods

All values are presented as mean plus or minus standard error of the mean. Significance between groups was determined with one way ANOVA with Tukey post hoc test. *P* was considered significant if it was less than 0.05.
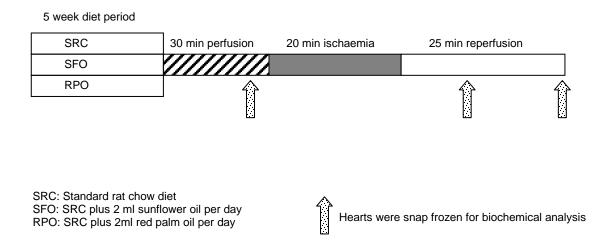
4.3 Study design 3 (Chapter 7)

Figure 4.3. Experimental model for Study design 3.

4.3.1 Dietary supplementation

Male Wistar rats weighing approximately 150g (6weeks of age) were divided into three diet groups at random. These groups were placed on the folowing diets for a 5 week period:

Control group 1: Standard rat chow diet (SRC)

Control group 2: SRC plus 2ml sunflower oil (SFO) per day

Experimental group: SRC plus 2ml red palm oil (RPO) per day

Rats were individually caged in order to ensure that they consume similar amounts of supplements. The sunflower oil used in this study was similar to that of Study design 1 (see table 4.1). The RPO used in this study is RPO oleïen (Carotino Palm Oil was supplied by Carotino SDN BHD, Johar-Bahru, Malaysia). Antioxidant levels in the RPO oleïen is approximately 10 times less than in RPO

baking fat (used in Study design 1). The dosage of RPO oleïen is thus 10 times greater than the dosage used for RPO baking fat in Study design 1 (this would equal 5.8 mg/kg/day in humans). Rats were allowed *ad libitum* access to rat chow and water, after supplements were consumed. Supplements were given to rats mixed into rat chow, and willingly consumed.

4.3.2 Working heart perfusion

At the end of the feeding period rats were sacrificed by injection with Euthenase (sodium pentabarbitol). Hearts were rapidly excised, placed in ice cold Krebs Henseleit buffer and mounted on the working heart perfusion apparatus by canulating the aorta and pulmonary artery. Once the aorta was canulated retrograde perfusion was initiated and sustained for the 10 minute stabilization period. Hearts were perfused against a perfusion pressure of 100 cm water. Once the stabilization period elapsed, the heart was switched to the working heart mode for 20 minutes. Hearts were then subjected to 20 minutes of normothermic global ischaemia. After ischaemia, hearts were reperfused for 25 minutes of which the first 10 minutes was retrograde perfusion and the last 15 minutes working heart perfusion.

During the working heart perfusion period, aortic output and coronary flow was measured every five minutes. Hearts were freeze clamped for biochemical analysis at the following time points: 20 minutes perfusion, 10 minutes ischaemia, 10 minutes reperfusion and 25 minutes reperfusion.

4.3.3 Western blot

This was performed using the same method as in Study design 1 (see section 4.1.7).

4.3.4 Quantitative real-time polymerase chain reaction

Total RNA was isolated from snap frozen left ventricular tissue by homogenisation in Trizol (1ml/100 mg tissue) using a bead mill homogenizer (Precellys 24, Bertin Technologies). Insoluble material was removed from the homogenate by centrifugation (12000xg, 4°C, 10 min), and the supernatant plus 200 µl chloroform was transferred to a phase lock gel (Eppendorf, Hamburg, Germany) for separation of total RNA from genomic DNA. After DNase treatment and ammonium acetate precipitation, total RNA was purified using RNeasy Mini Kit (Qiagen), and the concentration measured on a NanoDrop Spectrophotometer (ND 1000). First strand cDNA was synthesised from 1µg total RNA primed with 4 µl of oligo(dT) using qScript cDNA SuperMix (Quanta Biosences) in a total volume of 20 µl and subjected to PCR. 2,5µl cDNA synthesis template were mixed with 5µl PerfeCta SYBR Green 2x FastMix (Quanta Biosences), 0.5uM forward and reverse primers (Gpx1, Gpx3 and Gpx4; qPCR primers assays from SA Biosences) and RNase free water to a total volume of 10µl. The qPCR was performed on a Roche Light Cycler 480. The level of each Gpx mRNA was analysed and expressed relative to the housekeeping gene GAPDH using the Light Cycler$_R$ 480 SW software (f´CCAAGGTCATCCATGACAACTT, ´AGGGGCCATCCACAGTCTT; Invitrogen).

4.2.5 Statistical methods

All values are presented as mean plus or minus standard error of the mean. Significance between groups was determined with one way ANOVA with Tukey post hoc test. P was considered significant if it was less than 0.05.

CHAPTER 5

Dietary Red Palm Oil supplementation reduces myocardial infarct size in an isolated perfused rat heart model.

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5.1 Abstract:

Background and Aims: Recent studies have shown that dietary red palm oil (RPO) supplementation offers protection against ischaemia/reperfusion injury. The studies performed report increased aortic output and left ventricular developed pressure recovery. Mechanisms of protection include NO-cGMP signaling, survival kinase pathways and inhibition of apoptotic pathways.

The main aim of this study was to investigate the effects of dietary red palm oil (RPO) supplementation on myocardial infarct size after ischaemia/reperfusion injury. The effects of dietary RPO supplementation on matrix metalloproteinase-2 (MMP2) activation and PKB/Akt phosphorylation were also investigated.

Materials and Methods: Male Wistar rats were divided into three groups and fed a standard rat chow diet (SRC), a SRC supplemented with RPO, or a SRC supplemented with sunflower oil (SFO), for a five week period, respectively. After the feeding period, hearts were excised and perfused on a Langendorff perfusion apparatus. Hearts were subjected to thirty minutes of normothermic global ischaemia and two hours of reperfusion. Infarct size was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining and the volume of the infarct analyzed, using infarct size analysis software. Coronary effluent was collected for the first ten minutes of reperfusion. This coronary effluent was used to measure lactate dehydrogenase (LDH) activity by spectrophotometry and MMP2 activity by gelatin zymography.

Results: Dietary RPO-supplementation was found to decrease myocardial infarct size significantly when compared to the SRC-group and the SFO-supplemented group (9.17 ± 1.03% *versus* 30.20 ± 3.97% and 27.19 ± 2.43% respectively). Both dietary RPO- and SFO-supplementation were able to decrease MMP2 activity when compared to the SRC fed group (75kDa isoform: 1389,27 ± 124,34 arbitrary units and 1433.61 ± 103.65 arbitrary units *versus* 1724,42 ± 69,77 arbitrary units; 72kDa isoform: 2635,03 ± 163,02 arbitrary units and 2597,92 ± 158,5 arbitrary units *versus* 3201,63 ± 104,97 arbitrary units). PKB/Akt phosphorylation (Thr 308) was found to be significantly higher in the dietary RPO supplemented group when compared to the SFO supplemented group at 10 minutes into reperfusion (457.29 ± 29.53 arbitrary units *versus* 207.67 ± 18.59). There was, however, no significant changes observed in ERK phosphorylation.

Conclusions: Dietary RPO-supplementation was found to be more effective than SFO-supplementation in reducing myocardial infarct size after ischaemia/reperfusion injury. Both dietary RPO and SFO were able to reduce MMP2 activity, which suggests that MMP2 activity does not play a major role in protection offered by RPO. PKB/Akt phosphorylation may, however, have been involved in RPO mediated protection.

Key Words: Red Palm Oil, Ischaemia, Reperfusion, Infarct Size, Matrix Metalloproteinase, Heart, PKB/Akt

5.2 Introduction:

To our knowledge, little is known about the effects of dietary edible oil supplementation on myocardial infarct size. Previous studies demonstrated that dietary RPO supplementation offers protection against ischaemia/reperfusion injury by improved aortic output recovery (Esterhuyse *et al.,* 2005b; Engelbrecht *et al.,* 2006; Esterhuyse *et al.,* 2006). Esterhuyse and co-workers (2005), showed that 6 weeks of dietary RPO-supplementation can significantly improve reperfusion aortic output when compared to standard rat chow fed controls. This improvement in post ischaemic function was also observed when RPO was supplemented together with a potentially harmful cholesterol diet (Esterhuyse *et al.,* 2005b). They suggested that the NO-cGMP pathway may be a mechanism of RPO mediated protection, but that other mechanisms of protection should also be considered. Engelbrecht and co-workers (2006) found that 6 weeks of dietary RPO supplementation was associated with increased PKB/Akt and p38 phosphorylation and decreased phosphorylation of JNK, when compared to standard rat chow fed controls. This suggests that RPO may inhibit apoptosis through MAPK and PKB/Akt signaling pathways. Other MAPK signaling effects was found to be induced by RPO supplementation in cholesterol fed rats (Kruger *et al.,* 2007). In this study it was found that RPO induced protection in cholesterol fed could be associated with reduced phosphorylation of p38 and JNK, and increased phosphorylation of ERK. This suggests that RPO can induce reduced apoptosis through other signaling pathways in this model. RPO-supplementation was shown to increase cGMP levels early in ischaemia (Esterhuyse *et al.,* 2005b;

Esterhuyse *et al.,* 2006). This increase in cGMP was associated with increased NO levels in myocytes after hypoxic conditions and suggests that RPO-supplementation upregulate NO-cGMP signaling. These authors speculated that this was achieved through the antioxidant effects of RPO. In subsequent studies by Bester and co-workers (2006), RPO was used as a component in isocaloric diets containing either high levels of PUFAs, or SFAs. They showed that RPO offers protection against ischaemia/reperfusion in both these diets. They concluded that the NO-cGMP signaling pathway is most likely the main pathway of protection and that changes in the fatty acid profile of the heart may play a supplementary role in protection. Van Rooyen and co-workers (2008) summarized these results in a review and proposed several possible protective pathways. These pathways include the NO-cGMP pathway during ischaemia, and MAPK signaling- and anti-apoptotic pathways during reperfusion. Engelbrecht and co-workers (2009) also found that PI3K signaling may play an important role in RPO mediated protection against ischaemia/reperfusion. The inhibition of the PI3K pathway by pharmaceutical drug, wortmannin (a specific PI3K inhibitor) led to a loss of the improved functional recovery after ischaemia in RPO supplemented groups. The inhibition of PI3K by wortmannin could only reduce functional recovery in RPO supplemented groups partially. This suggests that there may be more than one pathway of RPO-mediated protection involved. These studies have shown that dietary RPO supplementation is able to increase reperfusion function. However, there is some evidence that contractile dysfunction in non-infarcted myocardial tissue may play a role in cardiac function

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early after ischaemia (Yang *et al.,* 2004). This necessitates the measurement of myocardial infarct size to determine the efficacy of dietary RPO-supplementation in offering protection against ischaemia/reperfusion injury.

RPO is considered to be a balanced oil and contains 51% SFAs, 38% MUFAs and 11% PUFAs. This oil also contains various beneficial micronutrients including, 500ppm carotenes of which 60% is β-carotene and 25% α-carotene. It also contains a minimum of 500ppm vitamin E of which 75% is tocotrienols and 25% tocopherols (Nagendran *et al.,* 2000; Sundram *et al.,* 2003). The combination of micronutrients contained naturally in RPO provides better protection than any individual compound contained therein (Wilson *et al.,* 2005; Schroeder *et al.,* 2006). In previous studies, dietary RPO-supplemented rats were compared to standard rat chow (SRC) fed controls and cholesterol fed rats (Esterhuyse *et al.,* 2005; Esterhuyse *et al.,* 2005b; Engelbrecht *et al.,* 2006; Bester *et al.,* 2006; Kruger *et al.,* 2007). None of these studies compared RPO-supplementation to other edible oils. Sunflower oil has mostly been used as a control group in investigations which studied the effects of edible oils (Charnock *et al.,* 1991; Larsen *et al.,* 1999; Aguilera *et al.,* 2004; Quiles *et al.,* 2004; De Roos *et al.,* 2008). The commercial sunflower oil used in this study contained 92% fat, of which 12% was SFAs, 26% MUFAs and 62% PUFAs. The oil contained no cholesterol, proteins, carbohydrates or added antioxidants and micronutrients.

Myocardial infarction is one of the leading causes of mortality globally (Braunwald and Kloner 1985; Banegas *et al.,* 2003; Jemal *et al.,* 2005). Maroko and co-workers (1971) proposed strategies for reducing necrosis in myocardial infarction which has caused much interest in infarct size research. The hypothesis is that the mortality and morbidity due to myocardial infarction is directly related to the amount of myocardium which is destroyed or infarcted (Pfeffer *et al.,* 1979; Chareonthaitawee *et al.,* 1995; Wu *et al.,* 2009). These studies demonstrated in different models that a decrease in infarct size leads to improved ventricular function. Research has shown that function was better in hearts with small infarcts a year after the myocardial incident, when compared to functional measurements at initial discharge from hospital (Chareonthaitawee *et al.,* 1995). This hypothesis is supported by the fact that regeneration of myocardial tissue is negligible. There is however, uncertainty as to the reason why ischaemic myocytes are killed and this complicates the search for agents that may reduce myocardial infarct size.

Reactive oxygen species (ROS) are produced in the body as a waste product of normal metabolism, and may even lead to myocardial protection through a phenomenon termed "pre-conditioning" (Saotome *et al.,* 2009). However, overproduction of ROS during pathological conditions such as myocardial infarction may be considered as one of the major causes of tissue death in myocardial infarction (Ferrari *et al.*, 1985; Ceconi *et al.,* 2003; Saotome *et al.,* 2009). Hearse and co-workers (1973) found that reperfusion of the ischaemic

heart with oxygenated buffer led to rapid cell death. This suggests that ROS is responsible for cell death during reperfusion. Although this phenomenon is not yet fully understood, it does lead to opportunities for the development of treatment strategies against ischaemia/reperfusion injury. As RPO contains several antioxidant micronutrients, we hypothesize that the antioxidative action of RPO may indeed contribute to the protection offered by RPO against ischaemia/reperfusion injury.

Matrix metalloproteinases (MMPs) are calcium and zinc dependant, endopeptidases. Under normal physiological conditions they facilitate cell migration and tissue remodelling (Nagase *et al.,* 1999). Recently it was found that MMP2 plays a role in ischaemia/reperfusion injury to the heart (Cheung *et al.,* 2000). Activation of MMP2 during an ischaemic insult is associated with poor recovery and larger infarct size of the heart (Cheung *et al.,* 2000; Hayashidani *et al.,* 2003; Menon *et al.,* 2004; Bergman *et al.,* 2006; Fert-Bober *et al.,* 2008). However, inhibition of MMP2 may lead to improved myocardial recovery after ischaemia/reperfusion injury (Clark *et al.,* 1997; Cursio *et al.,* 2002; Roach *et al.,* 2002; Bendeck *et al.,* 2003; Krishnamurthy *et al.,* 2009). MMP2 may be activated by reactive oxygen species (ROS), which is associated with oxidative stress conditions, like myocardial infarction (Rajagopalan *et al.,* 1996). The major free radical molecule implicated in activating MMP2 is peroxynitrite ($ONOO^-$) (Siwik *et al.,* 2001; Viappiani *et al.,* 2006). This activation is achieved through redox modification of the regulatory region of the pro-MMP molecule (Okamoto *et al.,*

2001; Viappiani *et al.,* 2006). This redox modification leads to the demonstration of activity in 72 kDa and 75 kDa isoforms of MMP-2, which is normally pro-MMP isoforms. Activated MMP2 damages cardiomyocytes during reperfusion by cleaving the contractile protein regulatory element, troponin I (Gao *et* al., 1997; Bolli *et al.,* 1999; McDonough *et al.,* 1999; Wang *et al.,* 2002). Other possible novel targets of MMP2, such as structural and cytoskeletal proteins may also be involved in myocardial ischaemia/reperfusion injury (Hein *et al.,* 1995; Matsumura *et al.,* 1996). MMP2 activity may be best measured in coronary effluent early in reperfusion, as it is released from the myocytes in proportion to myocardial damage (Cheung *et al.,* 2000). This MMP2 release rapidly leads to depletion of tissue MMP2 levels in an ischaemia/reperfusion model.

Our aims for this study were: 1) to measure infarct size in dietary RPO-supplemented rat hearts which have been exposed to ischaemia/reperfusion injury and 2) to determine the effects of dietary RPO-supplementation on MMP2 activation and PKB/Akt phosphorylation.

5.3 Materials and Methods

For full description of the materials and methods, see chapter 4.

All rats received humane animal care in accordance with the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH publication 8523, revised 1985). Ethical aproval was obtained from the Ethics comitee of the Faculty of Health and Wellness Sciences at the Cape Peninsula University of Technology on the 26th of January 2007.

5.3.1 Experimental design

Male Wistar rats were randomly divided into three groups and placed on the following diets for a five week period:

Control Group 1: standard rat chow (SRC)

Control Group 2: SRC plus 200μl/day of sunflower oil (SFO)

Experimental Group: SRC plus 200μl/day of red palm oil (RPO)

The rats were individually housed to ensure that each animal received equal amounts of supplements, which were prepared on a daily basis in order to prevent spoiling. All rats were allowed *ad libitum* access to food and water. The RPO dosage was obtained from previous studys which found cardioprotective effects with RPO supplementation (Esterhuyse *et al.,* 2005). The dosage of SFO was chosen so as to provide equal amounts of oil supplements to each supplemented diet.

5.3.2 Whole heart perfusion

After the feeding period, rats were anaesthetized using diethyl ether. Hearts were rapidly excised, then mounted on a Langendorff perfusion apparatus and were perfused at 37°C using a Krebs Henseleit buffer solution which was constantly gassed with 5% carbon dioxide, balance oxygen and a perfusion pressure of 100mmHg was maintained. After mounting, hearts were subjected to 10 minutes of stabilization, followed by 30 minutes of normothermic global ischaemia and 120 minutes of reperfusion. At the end of the protocol ventricular tissue was frozen at -20°C overnight. Hearts for biochemical analysis were snap frozen after the 10 minute stabilization period and also after 10 minutes reperfusion.

5.3.3 Infarct size determination

Frozen hearts and cut into 3mm thick cross-sectional slices. These slices were stained in 2,3,5-triphenyltetrazolium chloride (TTC) for 10 minutes at 37°C. After TTC staining, the slices were transferred to a formalin solution for ten minutes and then placed in phosphate buffer (pH 7.4).

Heart slices were then placed between two sheets of glass and scanned into a computer and analyzed using infarct size planimetry software (Infarct size). Infarct size was represented as percentage of the area at risk.

5.3.4 LDH measurement

Lactate dehydrogenase (LDH) release from hearts was measured in coronary effluent samples that were collected for the first 5 min of reperfusion, using a LDH-P kit (Diagnosticum, Budapest, Hungary). The kit makes use of spectrophotometric measurement to determine the absorbance of substrate catalyzed by the enzyme.

5.3.5 MMP2 zymography

Coronary effluent collected for the first 10 minutes of reperfusion was concentrated by ultra filtration using Amicon ultra filtration tubes. The concentrated coronary flow was then subjected to gelatin zymography.

Gelatinolytic activities of MMPs were examined as previously described (Cheung et al., 2000). Briefly, polyacrylamide gels were copolymerized with gelatin, and a constant amount of protein was seperated by electrophoresis in each lane. Following electrophoresis, gels were washed with 2.5% Triton X-100 and incubated for 24 to 48 h at 37°C in incubation buffer. Gels were then stained with 0.05% Coomassie Brilliant Blue in a mixture of methanol/acetic acid/water and destained in aqueous 4% methanol/8% acetic acid. Zymograms were digitally scanned, and band intensities were quantified using Quantity One software (Bio-Rad, Hercules, CA) and expressed as a ratio to the internal standard.

5.3.6 Western Blot

Heart tissue homogenized by adding homogenization buffer and PMSF to the sample. For phosphoprotein determination a NaF, Na_3VO_4 solution was added to the sample after which samples were sonicated for 3 times 10 seconds and then centrifuged at 5000rpm for 10 minutes. Protein concentration was determined through the biconcic acid test kit method.

Samples were diluted with Laemmli sample buffer and boiled, after which 40 μg of protein was separated by SDS-PAGE electrophoresis. After electrophoresis the proteins were transferred to PVDF membranes. Non specific binding was blocked by overnight incubation in 5% fat free milk in TBST. Membranes were then incubated with primary antibodies that recognize Erk p42/44 (Thr^{202}/Thr^{204}) and PKB (Thr^{308}). Membranes were subsequently washed and incubated with secondary antibody. After thorough washing with TBST, membranes were covered with ECL and exposed to autoradiography films which were densitometrically analyzed.

5.3.7 Nitrotyrosine Elisa

Myocardial nitrotyrosine levels were measured by ELISA test kit (supplied by Cayman chemical). This assay is based on competition between nitrotyrosine in the sample and an actylcholinesterase-linked tracer for limited binding sites on antibodies. After incubation with sample and tracer, washing and addition of the enzyme substrate, a distinct yellow color can be measured spectrophotometricaly

at a wavelength of 412nm. These results are inversely proportional to the levels of nitrotyrosine present in the sample.

5.3.8 Serum Cholesterol measurement

Whole blood of rats was centrifuged to obtain serum. Serum cholesterol levels were measured using a test kit supplied by Diagnosticum Zrt. (Budapest, Hungary). This test kit makes use of cholesterol esterase and cholesterol oxidase in order to produce hydrogen peroxide from cholesterol present in the sample. Hydrogen peroxide together with phenol and 4-aminoantipyrine then forms a red quonone in the presence of peroxidase. This red quonone can be measured spectrophotometrically at a wavelength of 505nm.

5.3.9 Tryglyceryde measurement

Whole blood of rats was centrifuged to obtain serum. Serum triglyceride levels were measured using a test kit supplied by Diagnosticum Zrt. (Budapest, Hungary). Triglycerydes present in the sample are degraded to dihydroxiacetone-phosphate and hydrogen peroxide. Hydrogen peroxide together with 4-aminoantipyrine and P-chlorophenol forms a red quonone in the presence of peroxidase. This red quonone can be measured spectrophotometrically at a wavelength of 505nm.

5.3.10 Statistical Methods

All values are presented as mean plus or minus standard error of the mean. Significance between groups was determined with one way ANOVA with Tukey Kramer post hoc test. P was considered significant if it was less than 0.05.

5.4 Results

5.4.1 Animal mass

The weight of rats from the RPO supplemented group was significantly less than that of the control groups after the feeding period (381.25 ± 7.59g *versus* 422.25 ± 8.85g for the SRC and 403.63 ± 7.69g for the SFO supplemented group). As animals were randomly divided into groups before the feeding period this is an indication that rats supplemented with RPO gained less weight in this time period (Table 5.1).

5.4.2 Heart mass

The weight of hearts isolated from the SRC group was significantly increased when compared to those of the RPO-supplemented group (1.57 ± 0.06g *versus* 1.40 ± 0.03g). However, hearts from the SFO-supplemented group showed no difference when compared to the other two groups (1.44 ± 0.04g) (Table 5.1).

Table 5.1. Weight of rats, and rat hearts after a five week supplementation period. *P<0.05 vs SRC, #P<0.05 vs SFO.

	SRC	Sunflower oil	Red palm oil
Animal mass (gram)	422.35±8.85	403.62±7.69	381.25±7.59 *
Heart mass (gram)	1.57±0.06	1.44±0.04	1.40±0.03 *#

5.4.3 Perfusion data

Table 5.2: Functional parameters of hearts before and after ischaemia. *P<0.05 *versus* the same group before ischaemia

	Coronary effluent before ischaemia (ml/10min)	Coronary effluent after ischaemia (ml/10min)	Heart rate before ischaemia (BPM)	Heart rate after ischaemia (BPM)
SRC	151.88±11.18	91.38±4.87*	347.13±16.13	158.2±32.59
SFO	195.0±20.35	127.25±16.65*	349.13±12.22	251.5±40.36
RPO	152.5±12.92	100.0±6.88*	344.13±11.99	328.0±40.66

There were no significant differences between the groups with respect to heart rate and coronary effluent before or after ischaemia. Coronary effluent was significantly decreased after ischaemia in all groups when compared to pre-ischaemic values (Table 5.2).

5.4.4 Infarct Size

Infarct size was significantly reduced in the RPO-supplemented group when compared to the SRC and the SFO-supplemented groups (9.17 ± 1.03% *versus* 30.20 ± 3.97% for the SRC group and 27.19 ± 2.43% for the SFO group, respectively). However, dietary SFO-supplementation did not reduce infarct size associated with ischaemia/reperfusion injury when compared to the SRC fed control rats (Figure 5.2). For an example of a TTC stained heart see Figure 5.1.
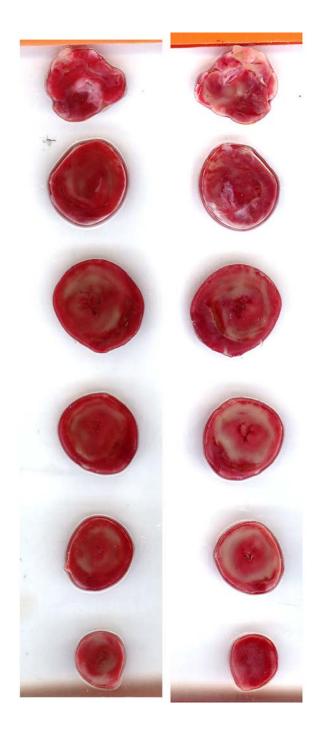
Figure 5.1 An example of a TTC stained heart cut in to 6 slices and scanned from the top and the bottom.
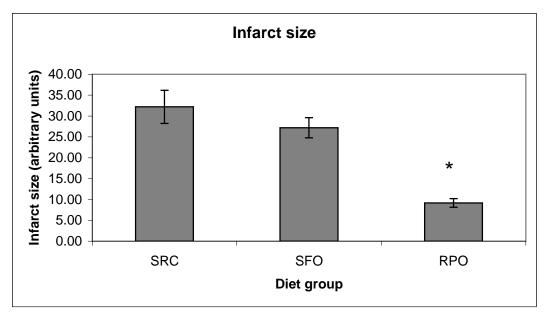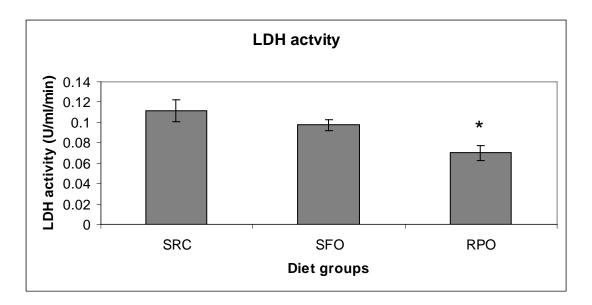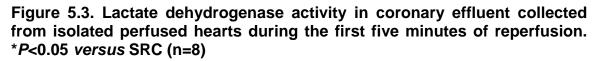
Figure 5.2. Infarct size expressed as percentage of the area at risk of hearts that was subjected to 30 minutes global ischaemia and 120 minutes reperfusion. *P<0.05 vs SRC and SFO (n=8)

Figure 5.3. Lactate dehydrogenase activity in coronary effluent collected from isolated perfused hearts during the first five minutes of reperfusion. *P<0.05 versus SRC (n=8)

5.4.5 Lactate dehydrogenase (LDH)

LDH levels in the reperfusion coronary effluent of the SRC group was significantly increased when compared to the RPO-supplemented group (0.11 ± 0.01U/ml/min *versus* 0.07 ± 0.01U/ml/min) (Figure 5.3).

5.4.6 Matirx Metalloproteinase 2 (MMP2)

MMP2 activity in both the 75kDa and 72kDa isoforms were significantly decreased in the RPO and SFO-supplemented groups when compared to the SRC group (75kDa: 1389,27 ± 124,34 arbitrary units and 1433.61 ± 103.65 arbitrary units *versus* 1724,42 ± 69,77 arbitrary units; 72kDa: 2635,03 ± 163,02 arbitrary units and 2597,92 ± 158,5 arbitrary units *versus* 3201,63 ± 104,97 arbitrary units). There were no differences between the RPO-supplemented and SFO-supplemented groups for either the 75kDa or the 72kDa isoforms (Figure 5.4).
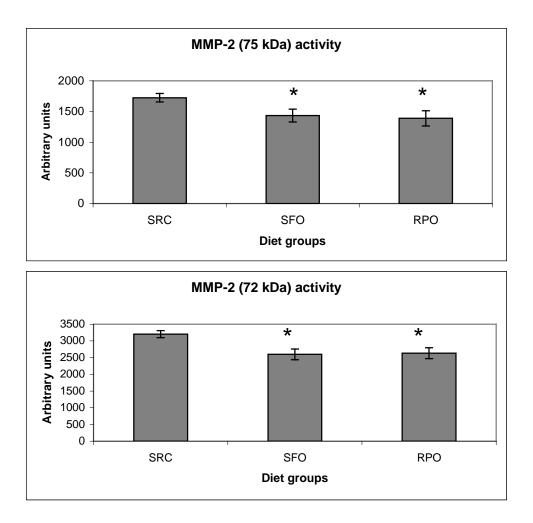
Figure 5.4. MMP2 75 and 72 kDa isoform activities in coronary effluent of isolated perfused rat hearts, collected during the first ten minutes of reperfusion. *$P<0.05$ *versus* SRC (n=8)

5.4.7 3-Nitrotyrosine

The RPO-supplemented group had significantly lower 3-nitrotyrosine levels when compared with the SFO-supplemented group (0.53 ± 0.07ng/mg *versus* 1.17 ± 0.17ng/mg for the SFO group). Neither of these groups differed significantly from the SRC-control group. Nitrotyrosine is considered to be a good marker of peroxynitrite levels (Onody *et al.,* 2003) (Figure 5.5).
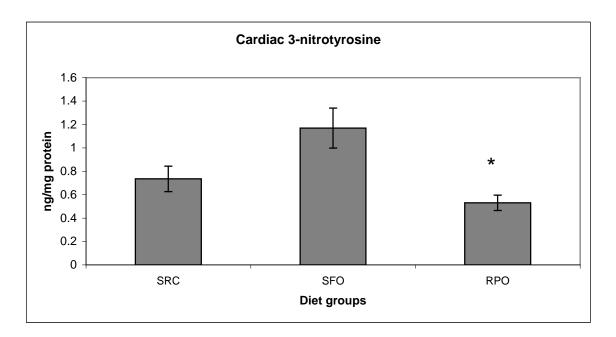
Figure 5.5. 3-Nitrotyrosine levels in myocardial tissue of rats at ten minutes into reperfusion. **P<0.05 versus SFO (n=8) (P=0.2606 for SRC versus SFO)*

5.4.8 Western blot analysis

The RPO supplemented group showed significantly increased Akt (thr) phosphorylation when compared to the SFO supplemented group (475.28 ± 83.51 arbitrary units *versus* 207.66 ± 49.19 arbitrary units). The total Akt for all groups were similar. There were no significant differences between any of the groups for total Erk 44 and 42 or phosphorylated Erk 44 and 42 (Figure 5.6).
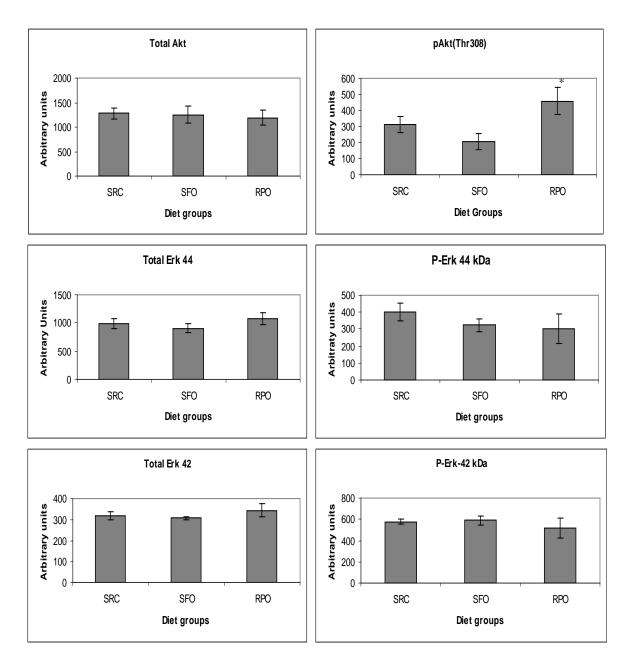
Figure 5.6. Pro-survival kinase Western blots at 10 minutes into reperfusion. *P<0.05 vs SFO (n=8)

5.4.9 Serum lipid profile

The total serum cholesterol levels were similar in all the groups. There was,

however, a significant decrease in serum triglyceride levels of both the SFO and

RPO groups when compared to the SRC group (0.75 ± 0.12 mmol/L and 0.71 ± 0.06 mmol/L *versus* 1.16 ± 0.12 mmol/L) (Figure 5.7).
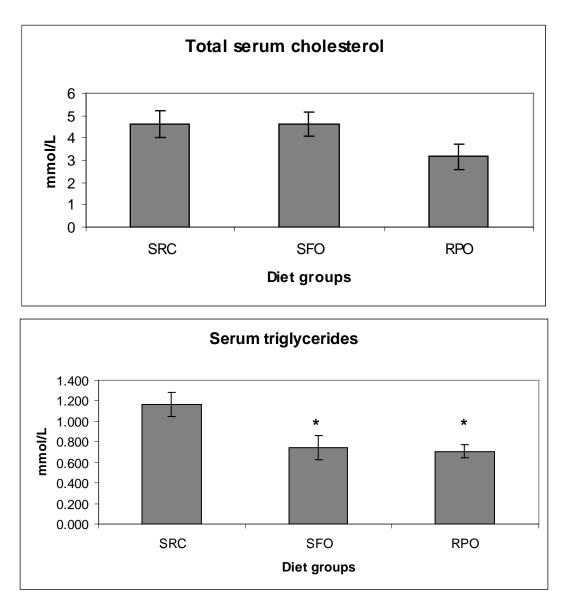
Total serum cholesterol

Serum triglycerides

Figure 5.7. Total serum cholesterol and triglyceride measurements performed after diet period. *$P<0.05$ vs SRC (n=8)

5.5 Discussion

Our results demonstrate that dietary RPO-supplementation was able to reduce myocardial infarct size. Sunflower oil supplementation however, was not able to reduce myocardial infarct size when supplemented to the diet in equal proportions as RPO, suggesting that the protection offered by RPO is not a result of additional energy added to the diet. This confirms results by Bester and co-workers (2006) which showed that RPO supplementation could offer protection against ischaemia/reperfusion injury in diets of different fat content when compared to isocaloric control groups. The reduction in infarct size in the RPO supplemented group also correlates well with functional results from previous studies where aortic output recovery was measured during reperfusion (Esterhuyse *et al.,* 2005; Esterhuyse *et al.,* 2005b; Esterhuyse *et al.,* 2006; Bester *et al.,* 2006). Infarct size however, may be used as a longer term predictor of myocardial recovery (Chareonthaitawee *et al.,* 1995; Wu *et al.,* 2009). The results of this study together with the results of previous studies show for the first time that dietary RPO is able to offer protection against ischaemia/reperfusion injury by improving reperfusion function and reducing myocardial infarct size. The difference in heart weight between the RPO and SRC groups could be explained by the difference in body weight. These results indicate that dietary RPO-supplementation does not lead to increased weight gain or cardiac hypertrophy.

Reduction of MMP2 activity is normally associated with protection against ischaemia/reperfusion injury (Hayashidani *et al.,* 2003; Menon *et al.,* 2004; Bergman *et al.,* 2006; Fert-Bober *et al.,* 2008). Under normal physiological conditions the 75kDa and 72kDa isoforms of MMP2 are inactive. The autoinhibitory propeptide domain has not been cleaved from these MMP2 isoforms. These isoforms may however, display activity when partial cleavage of the autoinhibitory propeptide domain takes place in the presence of peroxynitrite and glutathione (Okamoto *et al.,* 2001; Viappiani *et al.,* 2006). The fact that both RPO-supplementation and SFO-supplementation reduced the MMP2 activity suggests that both of these oils may offer some protection against ischaemia/reperfusion injury when supplemented to the diet. Alternatively, the reduction of MMP2 activity may not be the only pathway involved in RPO-mediated protection. The reduction of MMP2 (75kDa and 72kDa) activity suggests that oxidative stress has been reduced by the supplemented oils. However, dietary SFO-supplementation failed to decrease infarct size in this study. The reduction in MMP2 activity in the SFO-supplemented group was therefore not enough to offer protection against ischaemia/reperfusion injury. This suggests that MMP2 activity is not the only role player in the protection offered by RPO supplementation. Van Rooyen and co-workers (2008) proposed that several mechanisms synergistically offered RPO-mediated protection. Our results suggest that reduction in MMP2 activity may together with other pathways such as 1) the NO-cGMP pathway, 2) the PKB/Akt pathway and 3) the inhibition

of caspases, play a role in offering protection in dietary RPO supplemented hearts.

The reduced coronary effluent LDH levels in RPO supplemented rats confirmed that RPO supplementation was able to offer protection against ischaemia/reperfusion injury. This supports the infarct size result, as LDH release from the heart is a marker of myocardial tissue damage.

Our results also demonstrate that RPO is more effective than SFO in offering protection against myocardial ischaemia/reperfusion injury. This may be attributed to several possible mechanisms. One such mechanism may be the higher antioxidant content in RPO as compared to SFO (Nagendran *et al.,* 2000; Sundram *et al.,* 2003). The antioxidant content of RPO may also offer an explanation for the significantly lower levels of 3-nitrotyrosine in the hearts of RPO-supplemented rats. 3-Nitrotyrosine is a good indicator of protein nitration, and is thus a good indicator of peroxynitrite levels (Onody *et al.,* 2003). Previous results have shown that dietary RPO-supplementation led to increased levels of cGMP production, accompanied by increased intracellular nitric oxide levels in myocytes (Esterhuyse *et al.,* 2006). These authors suggest that antioxidants present in RPO scavenged superoxide and thus lead to the conservation of nitric oxide. This would in turn lead to increased cGMP- and decreased peroxynitrite production (Du Toit *et al.,* 2001; Ferdinandy *et al.,* 2003). This increase in cGMP and nitric oxide, accompanied by the decreased peroxynitrite is suggested to be

one of the pathways of protection of dietary RPO-supplementation (Esterhuyse *et al.,* 2005; Esterhuyse *et al.,* 2006; Bester *et al.,* 2006; Van Rooyen *et al.,* 2008). Nitrotyrosine results of our studies suggest that the antioxidants present in RPO were able to scavenge more ROS than the antioxidants present in SFO could. Alternatively, RPO may have prevented the formation of peroxynitrite more effectively than SFO.

Yang and co-workers (2009) found that simvastatin decreased myocardial infarct size associated with ischaemia/reperfusion. They suggested that this is due to increased NO signaling which was responsible for RPO mediated protection against ischaemia/reperfusion injury (Esterhuyse *et al.,* 2006). Based on our results we propose that increased NO signaling may have played a role in reducing myocardial infarct size.

Serbinova and co-workers (1992) and Das and co-workers (2008) used the vitamin E fraction of palm oil, named tocotrienol rich fraction (TRF) as dietary supplement or introduced it into the perfusate of isolated perfused hearts. These studies showed that TRF could offer protection against ischaemia/reperfusion injury. In the study by Das and co-workers (2008) the protective effects of TRF is ascribed to its ability to increase Akt phosphorylation. This places the Akt phosphorylation results of this study into perspective, as we were able to confirm that increased Akt phosphorylation takes place when RPO is supplemented to the diet. This is also in agreement with results by Engelbrecht and co-workers

(2006) who showed that Akt is phosphorylated by dietary RPO-supplementation. In this study, Akt_{ser473} was shown to be phosphorylated, while the current study demonstrates that the Akt_{thr308} residue is also phosphorylated. Das *et al.* (2008) suggested that it is the tocotrienol content of palm oil that is responsible for Akt phosphorylation. This data supports our finding that RPO, which contains high levels of tocotrienols, could induce phosphorylation of Akt. SFO also contains vitamin E, but predominantly tocopherols (Slover, 1971). We can therefore conclude from our studies that tocotrienols were responsible for Akt phosphorylation.

Furthermore, our results confirm previous studies which showed that RPO does not have hypercholesterolaemic effects (Theriault *et al.,* 1999; Kritchevsky *et al.,* 2000). RPO has also been shown to decrease triglycerides to a similar level as SFO. These results demonstrate that dietary RPO-supplementation does not have negative effects on the serum lipid profile normally associated with saturated fats. Wistar rats are, however, resistant to changes in the serum lipid profile, and show only minor increases in cholesterol levels when supplemented with cholesterol (Ferdinandy *et al.,* 1997; Onody *et al.,* 2003).

5.6 Conclusion

Our results demonstrate that dietary RPO-supplementation reduces myocardial infarct size when compared to SRC fed controls. We were also able to demonstrate that RPO supplementation is more effective in reducing myocardial infarct size than SFO fed group. MMP2 seems to have played at most, a minor role in the reduction of myocardial infarct size in this study, as its activity was reduced in both the RPO- and SFO-supplemented groups. The effects of reduced MMP2 activity may however contribute to RPO mediated protection against ischaemia/reperfusion by working synergistically with other mechanisms, as suggested by Van Rooyen and co-workers (2008). Our results confirm that Akt phosphorylation and possibly antioxidant activity of RPO may have been more effective in the protection offered by RPO against ischaemia/reperfusion injury.

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CHAPTER 6

Dietary red palm oil supplementation is able to attenuate increased infarct size associated with dietary cholesterol supplementation.

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6.1 Abstract

Background and Aims: Previous studies in our laboratory showed that dietary RPO supplementation could reduce myocardial infarct size in the isolated perfused rat heart model. Dietary RPO supplementation has also been shown to increase functional recovery after ischaemia in cholesterol supplemented rats. However, little is known about its effect on myocardial infarct size in this model. Only a few mechanisms of RPO associated protection have been described in cholesterol fed rat hearts. Mechanisms that have been suggested include the inhibition of apoptosis through MAPK signaling pathways. The aims of this study were to investigate the effects of dietary RPO supplementation in a cholesterol supplemented rat model and to investigate MMP2 activity as a possible mechanism of protection.

Materials and Methods: Male Wistar rats were divided into three groups. The first group (Ctrl) received a standard rat chow (SRC) diet for the entire nine week feeding period. The second group, named the hypercholesterol (HC) group, received SRC plus 2% cholesterol for nine weeks. The third group named hypercholesterol and RPO (HCRPO), received SRC supplemented with 2% cholesterol for the first four weeks of the feeding period. For the last five weeks of the feeding period the third group received SRC plus 2% cholesterol plus 200µl RPO per day. After the feeding period hearts were excised and perfused on a Langendorff perfusion apparatus. Hearts were subjected to thirty minutes of normothermic global ischaemia and two hours of reperfusion. Infarct size was measured by 2,3,5- triphenyltetrazolium chloride (TTC) staining and analyzed

using infarct size analysis software. Coronary effluent was collected during the first ten minutes of reperfusion and used to measure lactate dehydrogenase (LDH) activity by spectrophotometry and MMP2 activity by gelatin zymography.

Results: Cholesterol supplementation showed increased myocardial infarct size when compared to SRC fed control rats (37,16 ± 3.58% *versus* 23,45 ± 2,96%). RPO supplementation reduced infarct size in cholesterol fed rats to levels comparable to the SRC fed control rats (26,87 ± 2,96%). There was, however, no significant differences in LDH activity in any of the groups. MMP2 activity before ischaemia was significantly reduced in the HCRPO group when compared to the HC group (228.43 ± 28.06 arbitrary units *versus* 450.83 ± 33.62 arbitrary units). However, the MMP2 activity of the HCRPO group was significantly higher when compared to the HC group after ischaemia (2107.06 ± 50.99 arbitrary units *versus* 1821.90 ± 56.92 arbitrary units).

Conclusions: Dietary RPO attenuated the increased susceptibility of cholesterol fed hearts to ischaemia/reperfusion injury, as shown by reduced infarct size. Myocardial MMP2 activity is most probably not a major role player in RPO mediated protection against ischaemia/reperfusion injury in cholesterol supplemented hearts.

Key words: Red Palm Oil; Ischaemia; Reperfusion; Infarct size; Matrix metalloproteinase; Heart; Cholesterol supplementation

6.2 Introduction

Many cardiovascular ischaemia/reperfusion injury studies use healthy rats for their research protocols. In clinical conditions, unhealthy diets and lifestyle are normally associated with increased risk of myocardial infarction (Ferdinandy *et al.,* 1998). Previous studies using healthy animals contributed to elucidating certain mechanisms of cardiovascular protection. However it has recently been shown that cholesterol feeding may reverse the beneficial effects of preconditioning (Girics *et al.,* 2006). One such a model is a mildly dislipidaemic rat model, where the rat is supplemented with low doses of cholesterol for a short time period. In this model, peroxynitrite was increased with a subsequent reduction of myocardial function (Onody *et al.,* 2003). This ultimately led to contractile failure of the myocardium, or myocardial stunning injury. Additionally, this model was shown to inhibit the cardioprotective effects of myocardial preconditioning (Girics *et al.,* 2006). Osipov and co-workers (2009) demonstrated in an *in vivo* hypercholesterolaemic pig model increased infarct size when compared with normal control animals. Increased infarct size was accompanied by an increase in oxidative stress and apoptotic markers in hypercholesterolaemic pig hearts, as well as decreased phosphorylation of survival kinases such as Akt. The authors concluded that increased infarct size in hypercholesterolaemic hearts are associated with increased oxidative stress and inflammation, together with downregulation of cell survival pathways and induction of apoptosis.

RPO is an antioxidant rich oil which contains approximately 50% saturated and 50% unsaturated fatty acids (Nagendran *et al.,* 2000; Sundram *et al.,* 2003). Carotenoids and vitamin E (75% of which is tocotrienol) are the most abundant antioxidants in this oil. Both of these antioxidants are contained at a level of at least 500ppm in RPO (Nagendran *et al.,* 2000; Sundram *et al.,* 2003). The cocktail of antioxidants within RPO is believed to have synergistic effects (Wilson *et al.,* 2005; Schroeder *et al.,* 2006). The oil offers cardioprotection, by activation of several different protective pathways which work synergistically together (Van Rooyen *et al.,* 2008).

Dietary RPO supplementation has previously been shown to offer protection against ischaemia/reperfusion injury in the isolated perfused heart (Esterhuyse *et al.,* 2005b; Bester *et al.,* 2006; Kruger *et al.,* 2007). Esterhuyse and co-workers (2005b) showed that dietary RPO supplementation could improve post ischaemic functional recovery in rats fed a standard rat chow diet (SRC), and rats fed a SRC plus two percent cholesterol for six weeks. They suggested that RPO mediated protection against ischaemia/reperfusion injury may be induced through different pathways in hearts of SRC fed rats and cholesterol fed rats. Kruger and co-workers (2007) showed that inhibition of apoptosis may play a role in cholesterol fed rats, through the downregulation of p38 and JNK phosphrylation, and upregulation of ERK phosphorylation.

Matrix metalloproteinases (MMPs) are calcium and zinc dependant endopeptidases. Under normal physiological conditions they facilitate cell migration and tissue remodelling (Nagase *et al.,* 1999). Recently it was found that MMP2 plays a role in ischaemia/reperfusion damage in the heart (Cheung *et al.,* 2000). Increased MMP2 activity has also been associated with hypercholesterolaemic diets (Girics *et al.,* 2006). This may be due to the increased peroxynitrite production within the myocardium (Onody *et al.,* 2003). ROS, and peroxynitrite have been shown to activate MMP2 through redox modification of the regulatory site of this enzyme (Rajagopalan *et al.,* 1996; Okamoto *et al.,* 2001; Siwik *et al.,* 2001; Viappiani *et al.,* 2006). Activated MMP2 damages cardiomyocytes during reperfusion. This is achieved by cleaving the contractile protein regulatory element, troponin I and possibly other structural and cytoskeletal proteins (Hein *et al.,* 1995; Matsumura *et al.,* 1996; Gao *et* al., 1997; Bolli *et al.,* 1999; McDonough *et al.,* 1999; Wang *et al.,* 2002). Activation of MMP2 during an ischaemic insult is therefore normally associated with decreased functional recovery and larger infarct size of the heart (Cheung *et al.,* 2000; Hayashidani *et al.,* 2003; Menon *et al.,* 2004; Bergman *et al.,* 2006; Fert-Bober *et al.,* 2008). This has been confirmed through inhibition of MMP2 by antibodies or chemical agents (Clark *et al.,* 1997; Cursio *et al.,* 2002; Roach *et al.,* 2002; Bendeck *et al.,* 2003; Krishnamurthy *et al.,* 2009). Upon activation MMP2 is released from myocytes, and is therefore rapidly depleted within the cell (Cheung *et al.,* 2000). MMP2 activity is therefore best measured in coronary effluent early into reperfusion.

The aims of this study were: 1) to investigate the effects of dietary RPO supplementation on myocardial infarct size in a hypercholesterolaemic rat heart model and 2) to determine whether MMP2 activity was involved as a mechanism of protection in this protection.

6.3 Materials and Methods

For full description of the materials and methods, see chapter 4.

All rats received humane animal care in accordance with the Guide for the Care and Use of Laboratory Animals, published by the United States National Institutes of Health (NIH publication 8523, revised 1985). Ethical aproval was obtained from the Ethics comitee of the Faculty of Health and Wellness Sciences at the Cape Peninsula University of Technology on the 26th of January 2007.

6.3.1 Experimental design

Male Wistar rats were divided into three groups. Rats in these groups were placed on the following diets, respectively:

Control group 1: Standard rat chow diet (SRC) for nine weeks

Control group 2: SRC diet plus 2% cholesterol for nine weeks

Experimental group: SRC diet plus 2% cholesterol for nine weeks, plus 200µl red palm oil (RPO) for the last five weeks of the nine week period

Rats were individually housed to ensure that each animal received equal amounts of supplements. RPO and supplements were prepared on a daily basis in order to prevent spoiling. Rats were allowed *ad libitum* access to food and water.

6.3.2 Whole heart perfusion

After the feeding period, rats were anaesthetized using diethyl ether. Hearts were rapidly excised, then mounted on a Langendorff perfusion apparatus and were perfused at 37°C using a Krebs Henseleit buffer solution which was constantly gassed with 5% carbon dioxide, balance oxygen and a perfusion pressure of 100mmHg was maintained. After mounting, hearts were subjected to 10 minutes of stabilization, followed by 30 minutes of normothermic global ischaemia and 120 minutes of reperfusion. At the end of the protocol ventricular tissue was frozen at -20°C overnight. Hearts for biochemical analysis were snap frozen after the 10 minute stabilization period and also after 10 minutes reperfusion.

6.3.3 Infarct size determination

Frozen hearts were cut into 3mm thick cross-sectional slices. These slices were stained in 2,3,5-triphenyltetrazolium chloride (TTC) for 10 minutes at 37°C. After TTC staining, the slices were transferred to a formalin solution for ten minutes and then placed in phosphate buffer (pH 7.4).

Heart slices were then placed between two sheets of glass and scanned into a computer and analyzed using infarct size planimetry software (Infarct size). Infarct size was represented as percentage of the area at risk.

6.3.4 LDH measurement

Lactate dehydrogenase (LDH) release from hearts was measured in coronary effluent samples that were collected for the first 5 min of reperfusion, using a LDH-P kit (Diagnosticum, Budapest, Hungary). The kit makes use of spectrophotometric measurement to determine the absorbance of substrate catalyzed by the enzyme.

6.3.5 MMP2 Zymography

Coronary effluent collected for the first 10 minutes of reperfusion was concentrated by ultra filtration using Amicon ultra filtration tubes. The concentrated coronary flow was then subjected to gelatin zymography.

Gelatinolytic activities of MMPs were examined as previously described (Cheung et al., 2000). Briefly, polyacrylamide gels were copolymerized with gelatin, and a constant amount of protein was seperated by electrophoresis in each lane. Following electrophoresis, gels were washed with 2.5% Triton X-100 and incubated for 24 to 48 h at 37°C in incubation buffer. Gels were then stained with 0.05% Coomassie Brilliant Blue in a mixture of methanol/acetic acid/water and destained in aqueous 4% methanol/8% acetic acid. Zymograms were digitally scanned, and band intensities were quantified using Quantity One software (Bio-Rad, Hercules, CA) and expressed as a ratio to the internal standard.

6.3.6 Serum cholesterol measurement

Whole blood of rats was centrifuged to obtain serum. Serum cholesterol levels were measured using a test kit supplied by Diagnosticum Zrt. (Budapest, Hungary). This test kit makes use of cholesterol esterase and cholesterol oxidase in order to produce hydrogen peroxide from cholesterol present in the sample. Hydrogen peroxide together with phenol and 4-aminoantipyrine then forms a red quonone in the presence of peroxidase. This red quonone can be measured spectrophotometrically at a wavelength of 505nm.

6.3.7 Triglyceryde measurement

Whole blood of rats was centrifuged to obtain serum. Serum triglyceride levels were measured using a test kit supplied by Diagnosticum Zrt. (Budapest, Hungary). Triglycerydes present in the sample are degraded to dihydroxiacetone-phosphate and hydrogen peroxide. Hydrogen peroxide together with 4-aminoantipyrine and P-chlorophenol forms a red quonone in the presence of peroxidase. This red quonone can be measured spectrophotometrically at a wavelength of 505nm.

6.3.8 Statistical Methods

All values are presented as mean plus or minus standard error of the mean. Significance between groups was determined with one way ANOVA with Tukey Kramer post hoc test. P was considered significant if it was less than 0.05.

6.4 Results

6.4.1 Animal mass

Table 6.1. Weight of rats, and rat hearts after a five week supplementation period. *P<0.05 vs SRC, #P<0.05 vs SFO.

	SRC	HC	HCRPO
Animal mass (gram)	494.0±18.14	472.63±13.52	511.5±14.44
Heart mass (gram)	1.64±0.05	1.74±0.09	1.73±0.09

There were no significant differences in body weight or heart weight between any

of the groups after the feeding period (Table 6.1).

6.4.2 Perfusion data

Table 6.2: Functional parameters of hearts before and after ischaemia. #P<0.05 *versus* SRC, *P<0.05 *versus* the same group before ischaemia (n=8)

	Coronary effluent before ischaemia (ml/10min)	Coronary effluent after ischaemia (ml/10min)	Heart rate before ischaemia (BPM)	Heart rate after ischaemia (BPM)
SRC	205.0±5.67	59.17±3.96*	387.38±56.45	338.0±128.56
HC	185.88±21.65	67.13±6.53*	390.75±37.25	346.0±115.31
HCRPO	141.87±12.53#	61.88±3.81*	327.25±17.56	339.0±29.23

Coronary effluent of the HCRPO group was significantly decreased when

compared to the SRC group, after the supplementation period (185.88±21.65

ml/min for HC *versus* 205.0±5.67). Coronary effluent of all groups were

significantly decreased after ischaemia, when compared to their baseline values. There were no significant differences in the heart rates before or after ischaemia (Table 6.2).
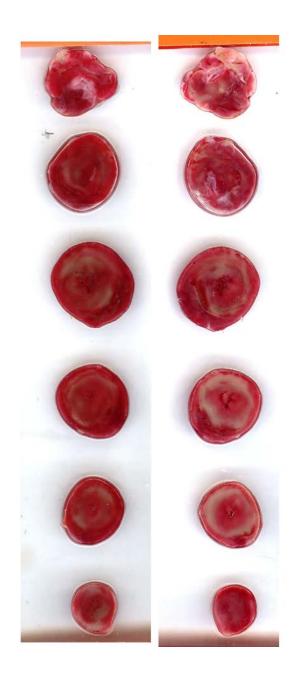

Figure 6.1 An example of a TTC stained heart cut in to 6 slices and scanned from the top and the bottom.
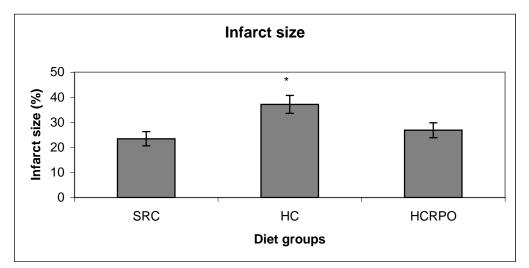
6.4.3 Infarct size

Figure 6.2. Myocardial infarct size of rats fed different diets for a nine week period. Infarct size was expressed as a percentage of the area at risk. *_P_<0.05 _versus_ SRC (n=8)

Cholesterol supplementation significantly increased infarct size when compared to SRC fed controls (37,16 ± 3.58% _versus_ 23,45 ± 2,96%). RPO supplementation of the hypercholesterol group decreased infarct size to levels comparable with the SRC group (26,87 ± 2,96%) (Figure 6.2). For an example of an TTC stained heart see Figure 6.1.

6.4.4 LDH

There were no significant differences in the LDH levels in the coronary flow of any of the groups (Figure 6.3).
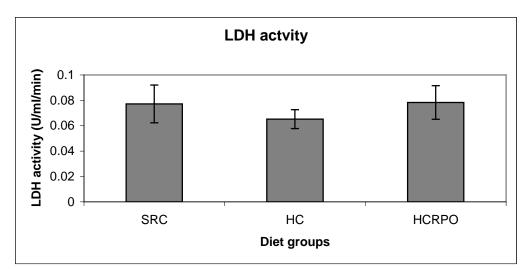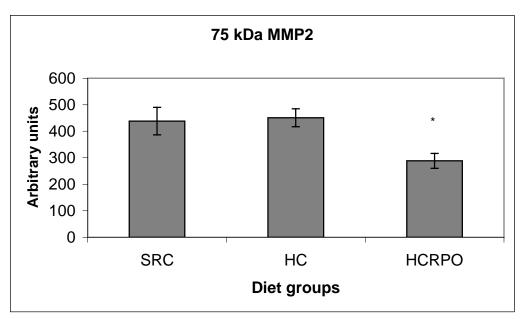
Figure 6.3. Lactate dehydrogenase activity in the coronary effluent collected during the first five minutes of reperfusion of isolated perfused rat hearts. (n=8)

6.4.5 MMP2

Activity of the 75kDa isoform of MMP2 was significantly lower in the HCRPO group when compared to the HC group before ischaemia (228.43 ± 28.06 arbitrary units *versus* 450.83 ± 33.62 arbitrary units). MMP2 activity showed no other significant differences before ischaemia (Figure 6.4).
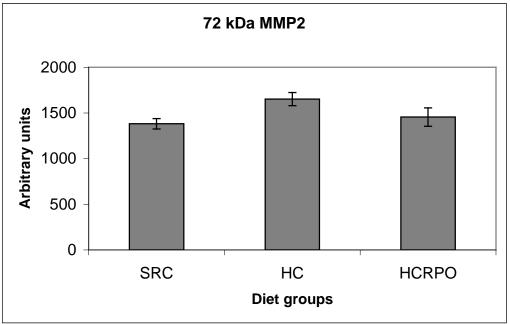
Figure 6.4. MMP2 activity in coronary flow collected in coronary effluent of isolated perfused rat hearts for ten minutes before ischaemia. *P<0.05 *versus* HC (n=8)

The HCRPO group had significantly higher activity of the 72kDA isoform of

MMP2 than the HC group (2107.06 ± 50.99 arbitrary units *versus* 1821.90 ±

56.92 arbitrary units). There were no other significant differences in MMP2

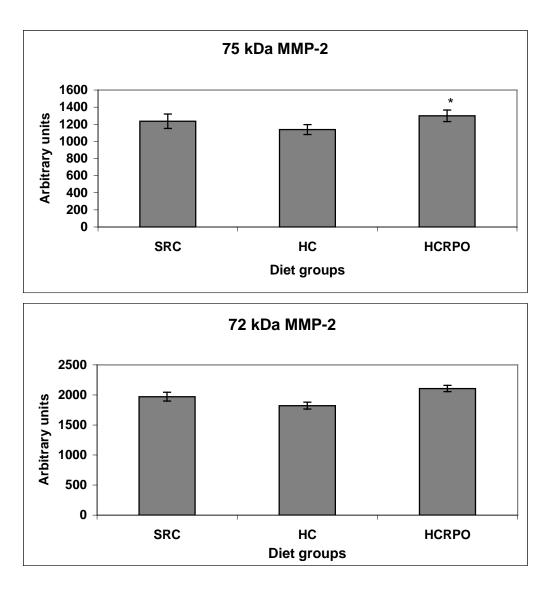activity between the groups after ischaemia. (Figure 6.4)

Figure 6.5. MMP2 activity in coronary effluent of isolated perfused rat hearts, collected during the first ten minutes of reperfusion. *$P<0.05$ *versus* HC (n=8)

6.4.6 Serum lipid profile

There were no significant differences in the total serum cholesterol levels and

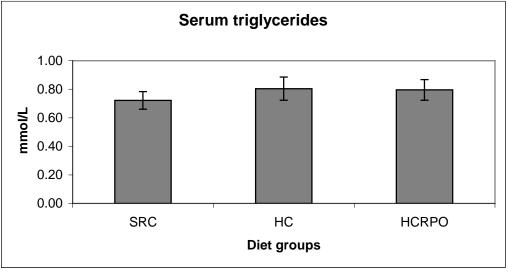triglycerides values between the groups (Figure 6.6).

Figure 6.6. Serum cholesterol and triglycerides of rats after nine weeks of dietary supplementation. (n=8)

6.5 Discussion

Our results show that dietary RPO supplementation is able to reverse the increased susceptibility of cholesterol fed rats to ischaemia/reperfusion injury. We demonstrated, for the first time, that RPO can reduce myocardial infarct size in cholesterol supplemented rats. Previous studies have shown that dietary RPO supplementation improved functional recovery of cholesterol fed rats after ischaemia (Esterhuyse *et al.,* 2005b; Kruger *et al.,* 2007). In the present study cholesterol supplementation was carried out for a longer period (nine weeks *versus* six weeks in previous studies). This indicates that RPO could effectively protect hearts against ischaemia/reperfusion injury, despite a more hypercholesterolaemic diet. Osipov and co-workers (2009) found that hypercholesterolaemic pigs had increased left ventricular function throughout the ischaemia/reperfusion period when compared to normal pigs. This was, however, associated with an increased infarct size and increased apoptotic markers. Our results together with previous studies (Esterhuyse *et al.,* 2005b; Kruger *et al.,* 2007) showed that dietary RPO can attenuate the harmful effects of cholesterol supplementation in the ischaemia/reperfusion model. Decreased coronary flow in the HC and HCRPO groups before ischaemia, when compared to the SRC group suggests that cholesterol feeding may reduce vascular function.

Despite the lack of increased total serum cholesterol levels in cholesterol fed rats, it is clear from the infarct size results that the cholesterol feeding did have a negative impact on the cardiovascular health of the rats. A similar model of

cholesterol supplementation has been previously employed in rats without increased total serum cholesterol levels being achieved (Giricz *et al.,* 2003; Esterhuyse *et al.,* 2006).

Our results demonstrate for the first time that dietary RPO supplementation may alter myocardial oxidative stress before ischaemia, as MMP2 activity was reduced before ischaemia. In previous studies performed with RPO significant differences were only found after ischaemia. The reduction in MMP2 activity before ischaemia in rats supplemented with both cholesterol and RPO suggests that RPO was able to reduce oxidative stress in these rats. This would most probably be achieved through quenching of ROS, which is generated in greater proportions in cholesterol supplemented rats (Giricz *et al.,* 2003; Onody *et al.,* 2003). Increased generation of ROS and oxidative stress would normally be associated with activation of MMP2 (Rajagopalan *et al.,* 1996; Siwik *et al.,* 2001; Viappiani *et al.,* 2006). As increased activity of MMP2 may lead to either cardiac remodelling, or tissue damage (Gao *et* al., 1997; Bolli *et al.,* 1999; McDonough *et al.,* 1999; Wang *et al.,* 2002), this reduction in MMP2 activity may play a role in RPO mediated protection against ischaemia/reperfusion injury. However, MMP2 activity of the rats supplemented with both cholesterol and RPO was increased during reperfusion, when compared to cholesterol supplemented rats. This would normally be associated with increased myocardial susceptibility to ischaemia/reperfusion injury (Cheung *et al.,* 2000; Hayashidani *et al.,* 2003; Menon *et al.,* 2004; Bergman *et al.,* 2006; Fert-Bober *et al.,* 2008). RPO was able

to reduce myocardial infarct size in cholesterol fed rats, despite increased activity of MMP2 in reperfusion. This suggests that other protective pathways are responsible for this protection. MMP2 activity was only measured in the coronary effluent in this study, as it is known to be rapidly washed out of the heart tissue upon activation (Cheung *et al.,* 2000). The levels of active MMP2 in the tissue would therefore be low irrespective of the amount that has been activated within the tissue.

The aim of this study was to investigate whether MMP2 activity was involved in RPO mediated protection of cholesterol fed rat hearts against ischaemia/reperfusion. As this does not seem to be the case, we can only suggest that pathways proposed by Kruger and co-workers (2007) are involved in this protection. These pathways include: 1) decreased phosphorylation of pro-apoptotic molecules, p38 and JNK together with 2) increased phosphorylation of the pro-survival kinase ERK early in reperfusion which leads to reduced apoptosis. Apoptosis has been shown to play a role in the detrimental effects of hypercholesterolaemia in the heart. Inhibition of apoptosis may therefore explain the protective effects of RPO in this model.

6.6 Conclusion

Dietary RPO supplementation attenuated the increased susceptibility of cholesterol fed rat hearts to ischaemia/reperfusion injury as evidenced by reduced infarct size. Myocardial MMP2 activity can not be considered as a major role player in RPO mediated protection against ischaemia/reperfusion injury in hearts of dietary cholesterol supplemented rats.

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CHAPTER 7

Dietary red palm oil oleïen attenuates myocardial ischaemia/ reperfusion injury: Effects on glutathione peroxidase transcription and extracellular signal-regulated kinases 1/2.

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7.1 Abstract

Background and Aims: It has been shown that dietary red palm oil (RPO) supplementation offers protection against ischaemia/reperfusion injury. Different pathways have been suggested for this protection. It has recently been shown that RPO supplementation increases glutathione peroxidase (GPX) activity in the myocardium. The mechanism for this increase in GPX activity remains unknown. Little is known about the effect of RPO supplementation on the genetic control of antioxidant enzyme systems in the heart. The involvement of extracellular signal regulated kinases (ERK) early in reperfusion gave different results in previous studies, depending upon the diet to which RPO was supplemented.

The aims of this study were to investigate the effects of dietary RPO supplementation on ERK 1/2 phosphorylation and GPX1, GPX3 and GPX4 transcription.

Materials and Methods: Male Wistar rats were randomly divided into three groups. The first control group received a standard rat chow diet (SRC) for 6 weeks. The experimental group received a standard rat chow diet supplemented with 2ml of RPO oleïen per day. An additional control group received a standard rat chow diet supplemented with 2ml of sunflower oil per day. After the feeding period, rats were sacrificed and hearts perfused on a working heart perfusion apparatus. Cardiac function was measured before and after ischaemia in order to determine aortic output recovery. Hearts were also freeze clamped at 20 minutes perfusion, 10 minutes reperfusion and 25 minutes reperfusion, in order to

perform biochemical analysis. Freeze clamped hearts were used for the determination of ERK and phosphorylated ERK by Western blotting. Regulation of glutathione peroxidase genes was determined before ischaemia using polymerase chain reaction (PCR).

Results: RPO supplementation did not produce significant changes in GPX1, GPX3 or GPX4 expression when compared to the SRC control group. The SFO control group increased the expression of GPX1 versus the SRC control group (2.08 ± 0.32 fold increase *versus* 1.25 ± 0.12 fold increase). ERK44 phosphorylation was significantly higher in the RPO supplemented group when compared to the SFO supplemented group at 20 minutes perfusion (79,21 ± 1.28% *versus* 76,19 ± 1,70%). ERK42 phosphorylation was significantly decreased in RPO supplemented hearts when compared to the SFO supplemented hearts at 25 minutes reperfusion (82.45 ± 2.49% *versus* 91.20 ± 1.23%).

Conclusions: Our results confirmed improved aortic output recovery as reported in previous studies after ischaemia/reperfusion injury. The minor changes found in ERK phosphorylation in this study may suggest that RPO has little effect on this pathway. Our findings also suggest that if RPO influences glutathione peroxidase activity, it would not be through genetic regulation of this enzyme.

7.2 Introduction

Recent studies have shown that dietary RPO supplementation is able to offer protection against ischaemia/reperfusion injury (Esterhuyse *et al.,* 2005b; Esterhuyse *et al.,* 2006; Bester *et al.,* 2006). Several pathways of protection have been suggested for this protection. RPO is an oil, containing several antioxidants (Van Rooyen *et al.,* 2008). The most abundant antioxidants in RPO are carotenoids and vitamin E. RPO contains at least 500ppm carotenoids of which the majority is in the form of α- and β-carotene (Nagendran *et al.,* 2000; Sundram *et al.,* 2003). RPO also contains approximately 500ppm vitamin E of which the majority is in the form of tocotrienols.

Since RPO contains high levels of antioxidants, it would be of value to know whether it influences the antioxidant enzymes *in vivo*. Little is known about the effects of dietary supplementation of RPO on the regulation of antioxidant enzymes. A study using rats fed a high fat diet, showed that antioxidant supplementation may reduce transcription of GPX1 (Sreekumar *et al.,* 2002). Research has shown that RPO may increase glutathione peroxidase (GPX) activity in rat hearts (Narang *et al.,* 2004), but the mechanism for this increase in GPX activity is still unknown. Increased GPX activity would lead to decreased oxidative stress, as all isoenzymes of GPX reduce hydrogen peroxide and alkyl hydroperoxides (Brigelius-Flohe, 1999). GPX1 reduces only soluble hydroperoxides and is expressed intracellularly. GPX 3 may reduce hydroperoxides from complex lipids. GPX 4 can reduce hydroperoxides which

are integrated in cellular membranes, as well as hydroperoxide groups of complex lipids, lipoproteins, cholesterol esters and thymine (Brigelius-Flohe, 1999).

The effect of RPO on the reperfusion induced salvage kinase (RISK) pathway has been a key feature in recent studies (Engelbrecht *et al.,* 2006; Kruger *et al.,* 2007; Engelbrecht *et al.,* 2009). The PKB/Akt branch of this pathway was shown to be upregulated during reperfusion in normal rats receiving RPO supplementation (Engelbrecht *et al.,* 2006; Engelbrecht *et al.,* 2009). It is well known that ERK induces anti-apoptotic effects through phosphorylation of p90 ribosomal S6 kinase (P90RSK) and Bcl-2-associated death promotor (BAD) (Robinson *et al.,* 1997; Bonni *et al.,* 1999; Abe *et al.,* 2000; Bueno *et al.,* 2002). It is also well known that ERK is involved in cardiac hypertrophy and heart failure (Haq *et al.,* 2001; Bueno *et al.,* 2002). However, it has not yet been established whether ERK plays a protective or causative role in heart failure. The pro-survival effects of ERK may also be dependant upon more variables than the Akt pathway. Badrian and co-workers (2006) suggested that pro-survival effects of ERK phosphorylation are dependant on the insult and also the length of time of ERK activation. It may therefore be necessary to investigate the effects of dietary RPO supplementation on ERK activation in greater depth.

Our aims were therefore to investigate: 1) the effect of dietary RPO supplementation on GPX1, GPX3 and GPX4 gene transcription, and 2) to

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determine the involvement of ERK in RPO mediated protection against ischaemia/reperfusion injury.

7.3 Materials and Methods

For full description of the materials and methods, see chapter 4.

All rats received humane animal care in accordance with the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH publication 8523, revised 1985). Ethical aproval was obtained from the Ethics comitee of the Faculty of Health and Wellness Sciences at the Cape Peninsula University of Technology on the 26th of January 2007.

7.3.1 Experimental design

Male Wistar rats were divided into three diet groups at random. These groups were placed on the folowing diets for a 5 week period:

Control group 1: Standard rat chow diet (SRC)

Control group 2: SRC plus 2ml sunflower oil (SFO) per day

Experimental group: SRC plus 2ml red palm oil (RPO) per day

Rats were individually caged in order to ensure that they consume similar amounts of supplements. Rats were allowed *ad libitum* access to rat chow and water, after supplements were consumed.

7.3.2 Working heart perfusion

At the end of the feeding period rats were sacrificed by injection with Euthanase (sodium pentabarbitol). Hearts were rapidly excised and mounted on the working heart perfusion apparatus. Once the aorta was canulated retrograde perfusion

was initiated and sustained for the 10 minute stabilization period. After the stabilization period, the heart was switched to the working heart mode for 20 minutes. Hearts were then subjected to 20 minutes of normothermic global ischaemia and 25 minutes reperfusion, of which the first 10 minutes was retrograde perfusion and the last 15 minutes working heart perfusion.

During the working heart perfusion period, aortic output and coronary flow was measured every five minutes. Hearts were freeze clamped for biochemical analysis at the following time points: 20 minutes perfusion, 10 minutes ischaemia, 10 minutes reperfusion and 25 minutes reperfusion.

7.3.3 Western Blot

Heart tissue homogenized by adding homogenization buffer and PMSF to the sample. For phosphoprotein determination a NaF, Na_3VO_4 solution was added to the sample after which samples were sonicated for 3 times 10 seconds and then centrifuged at 5000rpm for 10 minutes. Protein concentration was determined through the biconcic acid test kit method.

Samples were diluted with Laemmli sample buffer and boiled, after which 40 μg of protein was separated by SDS-PAGE electrophoresis. After electrophoresis the proteins were transferred to PVDF membranes. Non specific binding was blocked by overnight incubation in 5% fat free milk in TBST. Membranes were then incubated with primary antibodies that recognize Erk p42/44 (Thr^{202}/Thr^{204})

and PKB (Thr[308]). Membranes were subsequently washed and incubated with secondary antibody. After thorough washing with TBST, membranes were covered with ECL and exposed to autoradiography films which were densitometrically analyzed.

7.3.4 Quantitative real-time polymerase chain reaction

Total RNA was isolated from snap frozen left ventricular tissue by homogenisation in Trizol (1ml/100 mg tissue) using a bead mill homogenizer (Precellys 24, Bertin Technologies). Insoluble material was removed from the homogenate by centrifugation, and the supernatant with 200 µl chloroform was transferred to a phase lock gel (Eppendorf, Hamburg, Germany) for separation of total RNA from genomic DNA. After DNase treatment and ammoniumacetat precipitation, total RNA was purified using RNeasy Mini Kit (Qiagen), and the concentration measured on a NanoDrop Spectrophotometer (ND 1000). First strand cDNA was synthesised from 1µg total RNA primed with 4 ul of oligo(dT) using qScript cDNA SuperMix (Quanta Biosences) in a total volume of 20 µl and subjected to PCR. 2,5µl cDNA synthesis template were mixed with 5µl PerfeCta SYBR Green 2x FastMix (Quanta Biosences), 0.5uM forward and reverse primers (Gpx1, Gpx3 and Gpx4; qPCR primers assays from SA Biosences) and RNase free water to a total volume of 10µl. The qPCR was performed on a Roche Light Cycler 480. The level of each Gpx mRNA was analysed and expressed relative to the housekeeping gene GAPDH using the Light Cycler$_R$ 480 SW software

(f´CCAAGGTCATCCATGACAACTT, ´AGGGGCCATCCACAGTCTT; Invitrogen).

7.3.5 Statistical Methods

All values are presented as mean plus or minus standard error of the mean. Significance between groups was determined with one way ANOVA with Tukey Kramer post hoc test. P was considered significant if it was less than 0.05.
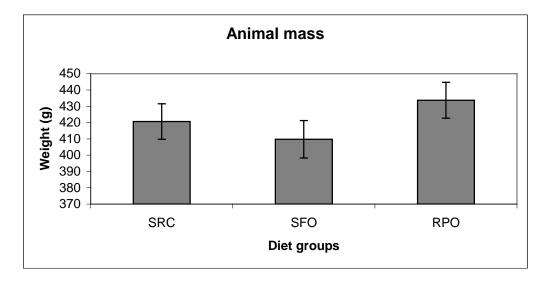
7.4 Results

7.4.1 Animal mass

Figure 7.1. Body weight of rats fed different diets for a 5 week period. (n=8)

There was no significant difference between the body weights of any of the diet groups after the feeding period (Figure 7.1).

7.4.2 Coronary effluent

Coronary effluent of the SFO group was significantly increased when compared to the SRC group, before ischaemia (20.57 ± 1.49 ml/min *versus* 16.29 ± 1.46 ml/min). Coronary effluent of both the SFO and RPO groups were siginificantly increased when compared to the SRC group after ischaemia (18.85 ± 0.93 ml/min for SFO and 17.62 ± 0.73 ml/min for RPO *versus* 14.14 ± 1.13 ml/min) (Figure 7.2).

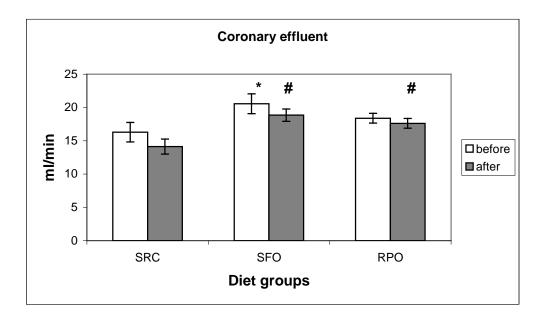**Figure 7.2. Coronary effluent of different diet groups before and after ischaemia.** *P<0.05 *versus* SRC before ischaemia; #P<0.05 *versus* SRC after ischaemia (n=8)

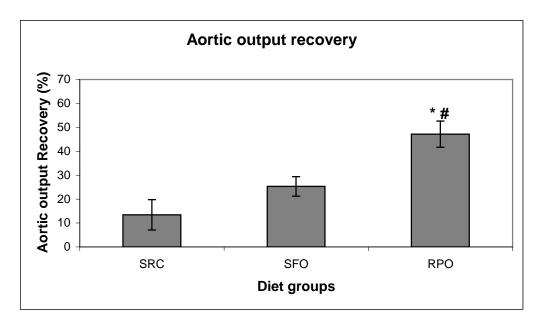## 7.4.3 Aortic output recovery:

Figure 7.3. Aortic output recovery of rat hearts after 25 minutes of global ischaemia and 25 minutes of reperfusion. *P<0.05 *versus* control; #P<0.05 *versus* sunflower oil (n=8)

Aortic output recovery of the RPO supplemented group was significantly higher than that of the SRC and SFO groups (47.16 ± 5.46 % *versus* 13.44 ± 6.34 % and 25.33 ± 4.06 %, respectively) (Figure 7.3).

7.4.4 ERK 42/44

ERK 44 phosphorylation was significantly increased in RPO supplemented hearts compared to the SFO group, at the 20 minute perfusion time point (79.21 ± 1.28 % *versus* 76.18 ± 1.70 %). This may suggest that dietary RPO supplementation may have a more significant cardio-protective effect than SFO, as ERK is associated with cardiac protection. At the 10 minutes reperfusion time point however, the RPO supplemented group showed significantly lower phosphorylation of ERK 42 when compared with the SFO group (82.45 ± 2.49 % *versus* 91.20 ± 1.23 %). ERK 44 phosphorylation was significantly decreased in the RPO supplemented group between the 20 minute perfusion time point and the 10 minute reperfusion time point, and again at the 25 minute reperfusion time point (79,21 ± 1.28 % *versus* 72.05 ± 1.55 % *versus* 63.92 ± 3.18 %) (Figures 7.4 and 7.5).

Figure 7.4. ERK 44 phosphorylation in rat hearts after 20 minutes perfusion, 10 minutes reperfusion and 25 minutes reperfusion. *P<0.05 *versus* SFO at the same time point (n=7)
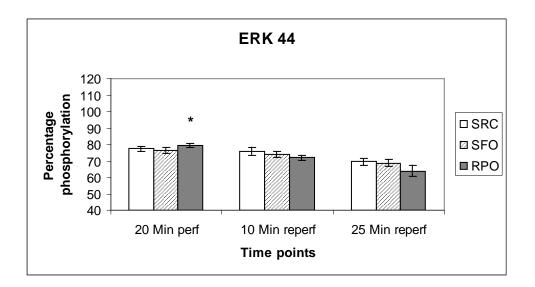

Figure 7.5. ERK 42 phosphorylation in rat hearts after 20 minutes perfusion, 10 minutes reperfusion and 25 minutes reperfusion. *P<0.05 *versus* SFO at the same time point; #P<0.05 *versus* the same group at 20 minutes perfusion and 10 minutes reperfusion (n=7)

7.4.5 Glutathione peroxidase transcription

GPX1

Figure 7.6. Glutathione peroxidase 1 expression in rat hearts after 20 minutes perfusion. ***P*<0.05 *versus* SRC (n=5)

GPX3

Figure 7.7. Glutathione peroxidase 3 expression in rat hearts after 20 minutes perfusion. (n=5)

Figure 7.8. Glutathione peroxidase 4 expression in rat hearts after 20 minutes perfusion. **P<0.05 versus* **SRC (n=5)**

GPX 1 expression was significantly higher in the SFO group when compared to the SRC (2.08 ± 0.32 arbitrary units *versus* 1.25 ± 0.12 arbitrary units). GPX 4 expression was also significantly increased in the SFO group when compared to the SRC group (1.15 ± 0.20 arbitrary units *versus* 0.53 ± 0.11 arbitrary units). No statistical significant differences were achieved in the RPO supplemented group (Figures 7.6, 7.7 and 7.8).

7.5 Discussion

Our results show that dietary RPO supplementation was able to increase functional recovery of hearts when compared to hearts of dietary SFO supplemented rats and SRC fed controls. This confirms results of previous studies which also found improved functional recovery in RPO supplemented hearts (Esterhuyse *et al.,* 2005b; Esterhuyse *et al.,* 2006; Bester *et al.,* 2006). The significant increase in coronary flow after ischaemia in both the SFO and RPO groups, when compared to the SRC group, may indicate that both sunflower oil and red palm oil was able to reduce the vascular effects of ischaemia.

It was of interest to note that coronary flow was significantly increased in the SFO group when compared to the SRC group after supplementation. This difference was not observed in our previous studies, but may be explained by the higher supplementation dose of sunflower oil employed in this study design.

Narang and co-workers (2004) found that dietary RPO supplementation was able to increase GPX and other antioxidant enzyme activity in myocardial tissue. They suggested that this increase was followed by reduced oxidative stress in an ischaemia/reperfusion model. We were unable to detect any effects of dietary RPO supplementation on GPX transcription. Our results therefore suggest that increased GPX activity found by Narang and co-workers (2004) is upregulated by RPO supplementation by another mechanism. This may be due to the increased

amount of antioxidants provided in the diet by RPO supplementation. Increased antioxidants in the tissue may aid in the recycling of glutathione peroxidase through improvement of intracellular oxidative stress status (Ferrari *et al.,* 1991). It may therefore be advisable to measure the ratio of glutathione to reduced glutathione in the myocardium in future studies, in order to obtain a better understanding of the intracellular oxidative stress. In our study we did not perform transcription analysis in samples after ischaemia. In most other studies we showed that RPO's mechanism of protection was maximized after ischaemia. It would therefore be advisable to analyse GPX transcription after ischaemia. However, Borchi and co-workers (2009) reported increased GPX activity in the failing human heart with no significant change in mRNA or protein expression. They suggested that this may take place through post-translational alterations, such as tyrosine phosphorylation of the protein. This also suggests that increased transcription of GPX1 and GPX4 in the SFO group may have been due to chronic, rather than acute increase in oxidative stress. Increased polyunsaturated fatty acids in the diet may be associated with increased oxidative stress (Diniz *et al.,* 2004), which may acount for chronic increased oxidative stress in the SFO group.

Our results regarding ERK phosphorylation are in agreement with those of Engelbrecht and co-workers (2006). There seems to be no significant effects of RPO supplementation on ERK phosphorylation early in reperfusion. The fact that ERK 42 phosphorylation showed decreased levels of phosphorylation at 25

minutes reperfusion, when compared to the SFO supplemented hearts, supports the suggestion that ERK phosphorylation does not play a role in RPO mediated protection. The increased ERK 44 phosphorylation in hearts of dietary RPO supplemented rats before ischaemia is lost during ischaemia, and is therefore probably not involved in protection against ischaemia/reperfusion injury. Similar studies in cholesterol fed rats may, however, have yielded different results (Kruger *et al.,* 2007). Our results together with those of Engelbrecht and co-workers (2006 and 2009) show that RPO supplementation to SRC fed rats induced upregulation of mainly the protein kinase B/Akt (PKB/Akt) branch of the reperfusion induced salvage kinase (RISK) pathway. However, further investigation of ERK phosphorylation before ischaemia may be needed in order to draw a conclusion.

7.6 Conclusion

Our results are in agreement with previous studies, showing that dietary RPO supplementation improves reperfusion functional recovery in the isolated perfused rat heart. Furthermore, RPO supplementation did not have any significant effect on GPX transcription. Finally, it is inconclusive whether phosphorylation of ERK 1/2 plays any significant role in RPO mediated protection against ischaemia/reperfusion injury.

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CHAPTER 8

DISCUSSION

Our results showed that dietary RPO supplementation offered protection against ischaemia/reperfusion injury in all the experimental models employed. To our knowledge we showed for the first time that dietary RPO supplementation can reduce myocardial infarct size in a rat model which was on standard rat chow and hypercholesterolaemic diets. This protection may be induced *via* different pathways, as suggested by Esterhuyse and co-workers (2005b). Ferdinandy and co-workers (1998) concluded in a review article that certain models, like a hypercholesterolaemic diet, inhibit the beneficial effects of ischaemic preconditioning. This was confirmed by Girics *et al.,* (2006) who demonstrated that MMP2 activation plays a major role in ischaemic preconditioning, and that pharmacological inhibition of MMP2 activation may lead to reduced infarct size in hypercholesterolaemic and normal rats. Based on our results, we propose that dietary RPO supplementation offers protection against ischaemia/reperfusion injury by various mechanisms, of which some are not employed by ischaemic preconditioning.

From our work it was evident that RPO supplementation is able to reduce myocardial infarct size and increase post ischaemic functional recovery. This suggests that dietary RPO supplementation is able to reduce myocardial cell death (infarction) (Pfeffer *et al.,* 1979; Chareonthaitawee *et al.,* 1995; Wu *et al.,*

2009) and also contractile failure (Yang *et al.,* 2004). These effects may be achieved *via* several mechanisms, which may or may not function synergistically.

We have also shown that a reduction in myocardial infarct size in dietary RPO supplemented rats can be associated with a decrease in MMP2 activity early in reperfusion when compared to SRC fed rats. These findings are supported by research that has shown that activated MMP2 can be associated with myocardial cell death by degradation of intracellular targets within the myocyte (Hein *et al.,* 1995; Matsumura *et al.,* 1996; Gao *et* al., 1997; Bolli *et al.,* 1999; McDonough *et al.,* 1999; Wang *et al.,* 2002). Although, SFO supplemented rats also showed a decrease in MMP2 activity early in reperfusion, there was no reduction in infarct size in this group of animals. Our results indicate that RPO and SFO supplementation may reduce MMP2 *via* different mechanisms. Alternatively, RPO supplementation may simply exert additional mechanisms of protection which was not the case with SFO supplementation. Nitrotyrosine results in this study indicate that there is a definite difference in the oxidative state of the myocardium between the RPO- and SFO supplemented groups. The SFO supplemented group showed significantly higher levels of nitrotyrosine, indicating that there was more peroxynitrite present in the hearts of SFO supplemented rats than in the hearts of RPO supplemented rats (Onody *et al.,* 2003). Peroxynitrite is known to be one of the major free radicals involved in redox modification of the MMP2 regulatory sub-unit and thereby leading to the activation of this enzyme (Rajagopalan *et al.,* 1996; Okamoto *et al.,* 2001; Siwik *et al.,* 2001; Viappiani *et*

al., 2006). Girics and co-workers (2006) showed that the pharmacological inhibition of MMP2 activity is associated with cardioprotection. They showed that cholesterol supplementation was able to block the effects of ischaemic preconditioning by increasing MMP2 activity. Cholesterol supplementation could however not achieve this in the presence of pharmacological inhibition of MMP2. Furthermore, the cholesterol supplemented group also showed increased levels of nitrotyrosine when compared to control hearts. This may suggest that peroxynitrite plays a role in the cardiotoxic effects of MMP2. In our study decreased peroxynitrite was also associated with a decrease in MMP2 activity in the RPO supplemented animal group. The SFO group was able to reduce MMP2 activity but not peroxynitrite levels. This indicates that RPO supplementation was able to induce different mechanisms of protection than in the case of SFO. In the hearts of cholesterol plus RPO supplemented rats however, RPO was able to reduce MMP2 activity before ischaemia, when compared to hearts of cholesterol supplemented controls. However, in hearts of rats supplemented with both RPO and cholesterol there was significantly higher MMP2 activity than in cholesterol fed controls after ischaemia. This argues that pre-ischaemic MMP2 activity may play a major role in protection against ischaemia/reperfusion injury. Alternatively this may suggest that inhibition of MMP2 activity is not the only pathway of protection associated with dietary RPO supplementation.

Although Narang and co-workers (2004), showed that dietary RPO supplementation was able to increase GPX activity in the isolated perfused rat

heart, our results demonstrated that myocardial GPX transcription was not affected by dietary RPO supplementation in the same model. The reason for this may be the provision of increased levels of antioxidants in the diet, which could improve the overall oxidative stress status of the heart. Improved oxidative regulation would lead to more glutathione molecules being in the reduced state, and therefore able to reduce glutathione peroxidase. This can improve glutathione peroxidase activity by leading to improved recycling time from the oxidized to the reduced state (Dolphin *et al.,* 1985; Ferrari *et al.,* 1991; Bjornstedt *et al.,* 1994; Griffith, 1999; Grant, 2001; Moran *et al.,* 2001). Interestingly, SFO supplementation was able to increase transcription of GPX1 and GPX4 significantly when compared to the SRC controls. Diniz and co-workers (2004) showed that diets rich in PUFAs may be associated with increased oxidative stress due to lipid peroxidation. Therefore, increased GPX transcription in hearts of SFO supplemented rats may be due to higher oxidative stress levels in hearts of SFO supplemented rats than in hearts of RPO supplemented rats, as GPX transcription has been shown to be associated with hydroperoxide levels (Flohe, 1989; Brigelius-Flohe, 1999).

Research has shown that RPO-mediated protection against ischaemia/ reperfusion injury in the isolated perfused rat heart is associated with increased PKB/Akt phosphorylation (Engelbrecht *et al.,* 2006; Van Rooyen *et al.,* 2008; Engelbrecht *et al.,* 2009). Our results confirm that Akt phosphorylation is upregulated in hearts of RPO supplemented rats early in reperfusion, which is

associated with a reduction in apoptosis by phosphorylation of BAD (Datta *et al.,* 1997).

ERK phosphorylation was not significantly affected at the 10 minutes reperfusion time point in any of the models employed. There was, however, an increase in ERK 44 phosphorylation before ischaemia, and a reduction in ERK 42 phosphorylation after ischaemia. This suggests that ERK does not play a major role in RPO-mediated protection against ischaemia/reperfusion injury, as ERK is dephosphorylated after ischaemia. The increased ERK phosphorylation before ischaemia may be associated with protection, or hypertrophy (Haq *et al.,* 2001; Bueno *et al.,* 2002). It is, however, unlikely that ERK in this case is involved in hypertrophic remodeling of the heart, as heart weights of RPO supplemented rats were significantly lower than that of the SRC fed controls. Research has shown that ERK phosphorylation is involved in RPO mediated protection against ischaemia/reperfusion injury in cholesterol fed rats (Kruger *et al.*, 2007). This emphasizes the fact that RPO offers protection against ischaemia/reperfusion injury *via* several mechanisms of protection. These mechanisms may differ partly, or in full, because the models employed were different; one used cholesterol and the other not.

Lactate dehydrogenase release in our study suggests that dietary RPO supplementation was able to reduce myocardial necrosis in SRC fed rats, but not cholesterol fed rats. Intramyocardial enzyme release is normally associated with

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myocardial necrosis (Ravkilde *et al.,* 1995; Ishikawa *et al.,* 1997). This may indicate that mechanisms involved in RPO-mediated protection in cholesterol supplemented rats could be anti-apoptotic pathways.

Serum lipid levels observed in the current study are in agreement with other studies, which showed that dietary RPO supplementation does not increase cholesterol levels, despite its relatively high content of saturated fatty acids (Quereshi *et al.,* 1991; Khor *et al.,* 1995; Quereshi *et al.,* 1995; Theriault *et al.,* 1999).

RPO supplementation was associated with a decreased body weight and heart weight when compared to the SRC group in our first experimental model, where RPO baking fat was used and cholesterol was excluded. In the other two models there were no significant differences. This may suggest that dietary RPO supplementation can in fact reduce weight gain. This should however be further investigated.
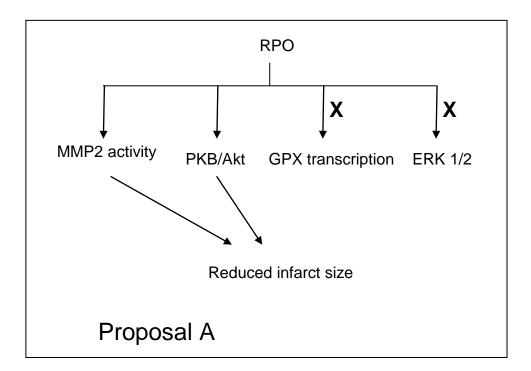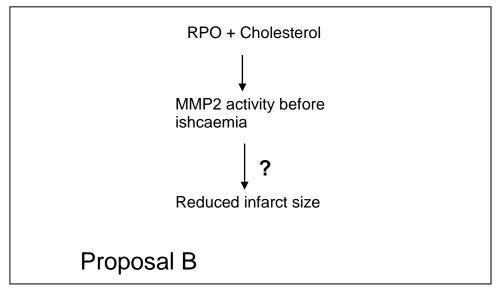
CHAPTER 9

CONCLUSION

Dietary RPO supplementation was shown to be able to reduce myocardial infarct size in standard rat chow fed rats and cholesterol supplemented rats. This reduction in infarct size was associated with a decrease in MMP2 activity after ischaemia in SRC fed rats. Although MMP2 activity was also decreased by SFO supplementation, it could not reduce infarct size in SFO fed rats. This argues that reduction of MMP2 activity is not a major pathway of RPO mediated protection in the SRC fed rats. In hypercholesterolaemic rats supplemented with RPO, MMP2 activity was decreased before ischaemia and increased after ischaemia. This suggests that involvement of MMP2 activity may play a role in RPO mediated protection against ischaemia/reperfusion injury in cholesterol fed rats.

GPX transcription is not affected by dietary RPO supplementation.

Our results show that PKB/Akt phosphorylation early in ischaemia play a role in RPO mediated protection against ischaemia/reperfusion injury in the SRC fed rats. ERK phosphorylation is however, not involved in protection against ischaemia/reperfusion injury in this model.

X   The pathway was not affected by RPO supplementation

?   The influence of MMP2 activity before ischaemia on infarct size is unknown.

Figure 9.1. Proposed pathways of cardiac protection offered by dietary RPO supplementation against myocardial ischaemia/reperfusion injury. A) RPO alone and B) RPO and Cholesterol

CHAPTER 10
RECOMMENDATIONS

Our studies create opportunities for further investigation;

1) Both RPO and SFO supplementation can reduce MMP2 activity. SFO did not show reduced 3-nitrotyrosine levels. Therefore, the involvement of peroxynitrite (nitrotyrosine) with MMP2 activity and associated tissue damage should be further investigated in a SFO supplemented group.

2) It is unclear whether MMP2 activity before ischaemia would influence myocardial infarct size after ischaemia/reperfusion injury. The importance of MMP2 activity before ischaemia should therefore be confirmed in SRC and cholesterol supplemented models.

3) Literature shows that dietary RPO supplementation increases GPX activity in the isolated perfused rat heart. We demonstrated that RPO supplementation does not have any effect on GPX transcription. Mechanisms by which RPO increases GPX activity should therefore be further investigated and study models standardized.

4) It is well known that ERK phosphorylation after ischaemia may lead to reduced cell death and improved myocardial recovery. It is however, unclear whether increased ERK phosphorylation before ischaemia would have similar effects on myocardial recovery after ischaemia/reperfusion injury. Effects of ERK phosphorylation before ischaemia should therefore be investigated in greater depth.

5) It was demonstrated that dietary RPO baking fat supplementation was able to reduce weight gain by rats in the absence of cholesterol feeding. The effect of dietary RPO supplementation on weight gain should be further investigated in SRC fed rats.

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