THE EFFECT OF DIETARY RED PALM OIL ON THE FUNCTIONAL RECOVERY AND THE PKB/Akt PATHWAY IN THE ISCHAEMIC/REPERFUSED ISOLATED RAT HEART.

By

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Declaration

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

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ABSTRACT

Introduction

Cardiovascular disease is one of the leading causes of death in the world. Formation of harmful reactive oxygen species (ROS) is associated with several pathological conditions, and contributes to ischaemia/reperfusion injury. Antioxidants can be added to the diet in an attempt to decrease the prevalence of cardiovascular disease by decreasing the harmful effects of ischaemia/reperfusion injury.

Red Palm Oil (RPO) consists of saturated, monounsaturated and polyunsaturated fatty acids and is rich in antioxidants such as β-carotene, tocopherols and tocotrienols.

It has previously been shown that RPO-supplementation improved reperfusion mechanical function. In these studies it was found that RPO might exert its beneficial effects during reperfusion through increased PKB/Akt pathway activity, which may lead to inhibition of apoptosis and improved mechanical function.

Aims

The aims of this study were: 1) to determine whether RPO-supplementation protected against ischaemia/reperfusion injury in the isolated perfused rat heart, 2) to confirm RPO-supplementation's effect on the PKB/Akt pathway activity and, 3) to elucidate the regulators in the PKB/Akt pathway that RPO-supplementation influenced.

Methods

Male Wistar rats were divided into 4 groups, 2 control groups and 2 experimental groups. The 2 control groups were fed a standard rat chow (SRC) for 4 weeks. The two experimental groups received SRC and RPO-supplementation for 4 weeks. Hearts were excised and transferred to a Langendorff perfusion apparatus and perfused with Krebs-Henseleit buffer.

Mechanical functional recovery was measured after 25 min of total global no-flow ischaemia. The following parameters were also measured during various time points in the protocol: left ventricular develop pressure, heart rate, coronary flow, rate pressure product. Hearts were also freeze-clamped for biochemical analysis at 10 min during reperfusion. The biochemical analysis was aimed at determining PKB/Akt involvement.

In a second protocol, hearts were subjected to the same perfusion protocol, but wortmannin was also added to the perfusion fluid, in order to inhibit PI3-kinase.

Results

Hearts from the RPO-supplemented rats showed an improved RPP recovery (92.26 ± 5.89 % *vs* 63.86 ± 7.74 %) after 10 min of reperfusion. This finding corroborated the findings of previous studies. Hearts of the RPO-supplemented rats perfused with wortmannin, showed increased RPP recoveries at several time points.

Biochemical results showed that wortmannin did indeed inhibit PI3-K phosphorylation in the RPO-supplemented group, as was expected. The RPO-supplemented group that was perfused with wortmannin had an increased PKB/Akt (Ser^{473}) phosphoyrylation, when compared to the wortmannin control group. It was also found that the combination of RPO and wortmannin had prosurvival effects.

Discussion

This study showed that RPO-supplementation offered protection against ischaemia/reperfusion injury in the Langendorff-perfusion apparatus at 10 min into reperfusion. Thereafter the significance of the protection was lost. This protection has been confirmed in several previous studies and several mechanisms have been proposed for this protection.

Since no conclusive evidence exists on the precise mechanism of protection, our investigation focused on the regulators of the pro-survival PKB/Akt pathway.

An improved functional recovery was also seen in the RPO-supplemented group that was perfused with wortmannin. This was an unexpected finding, because Wortmannin is a known PI3-kinase inhibitor (as was confirmed by our biochemical data). PI3-kinase phosphorylation leads to PKB/Akt phosphorylation and therefore, activation of a pro-survival pathway. It would be expected that wortmannin would inhibit PKB/Akt and thus decrease the survival of the cells. The RPO-supplementation thus reversed wortmannin's detrimental effect to such an extent that the functional recovery was far better than RPO-supplementation alone.

In the RPO + wortmannin group, PKB/Akt (Ser^{473}) phosphorylation was increased, contrary to previous findings. This is an indication that RPO may have the ability to override wortmannin's inhibitory effect on PI3-kinase, or that PKB/Akt (Ser^{473}) may be phosphorylated independently of PI3-kinase.

OPSOMMING

Inleiding

Kardiovaskulêre siektes is een van die hoof oorsake van sterftes in die wêreld. Die vorming van skadelike reaktiewe suurstof spesies word geassosieer met verskeie patologiese kondisies en dra ook by tot isgemie/reperfusie skade. ‘n Moontlike manier om die voorkoms van isgemie/herperfusie skade asook kardiovaskulêre siektes te voorkom, is om antioksidante by die dieet te voeg.

Rooi Palm Olie (RPO) bevat versadigde, mono-onversadigde en poli-onversadigde vetsure. RPO bevat ook ‘n oorvloed van antioksidante soos β-karoteen en tokoferole en tokotriënole.

Dit is bewys in vorige studies dat RPO-aanvulling verbeter funksionele herstel. Hierdie voordelige effekte mag dalk wees agv verhoogde PKB/Akt pad aktiwiteit. Die PKB/Akt pad word geassosieer met die inhibisie van apoptose en verhoogde meganiese funksie.

Doelwitte

Die doelwitte van hierdie studie was om te bepaal of 1) RPO-aanvulling beskermende effekte teen isgemie/herperfusie skade in die geisoleerde rotharte het, 2) Bevestig of RPO-aanvulling wel die PKB/Akt pad beïnvloed 3). om die effekte wat RPO-aanvulling het op die reguleerders van die PKB/Akt pad te onthul.

Metodes

Manlike Wistar rotte is in 4 groepe verdeel. 2 Groepe kontrole rotte is ‘n standaard rotkosmengsel gevoer vir 4 weke. Die 2 eksperimentele groepe het ook ‘n standaard rotkosmengsel gekry plus ‘n RPO-aanvulling vir 4 weke. Harte is uitgesny en op ‘n Langendorff perfusie sisteem gemonteer en met Krebs-Henseleit buffer geperfuseer. Meganiese funksie herstel is gemeet na 25 min totale globale geen-vloei isgemie. Linker ventrikulêre ontwikkelde druk, harttempo, koronêre vloei en tempo druk produk is gemeet by

verskillende tydpunte. Sommige harte is na 10 min herperfusie vir biochemiese analiese gevriesklamp. Die biochemiese analisiese was beoog om die PKB/Akt pad betrokkenheid te bepaal.
'n Tweede stel harte is aan dieselfde perfusie protokol blootgestel, maar wortmannin (PI3-kinase inhibitor) is ook bygevoeg by die perfusie vloeistof.

Resultate

Die groep wat met RPO aangevul is, het na 10 min herperfusie, 'n verbeterde tempo druk produk herstel getoon (92.26 ± 5.89 % *vs* 63.86 ± 7.74. Hierdie bevinding is ook met ander studies bevestig. 'n Interessante bevinding was dat die groep wat met RPO aangevul is en met wortmannin geperfuseer is, 'n verbeterde meganiese funksionele herstel getoon het.

Biochemiese resultate het getoon dat wortmannin wel PI3-K fosforilering geinhibeer het. Die harte van die rotte in die groep wat aangevul is met RPO en daarna met wortmannin geperfuseer is, het 'n toename in PKB/Akt (Ser^{473}) fosforilering getoon, relatief tot die wortmannin geperfuseerde harte van die rotte in die kontrole groep. Hierdie groep (RPO-aanvulling en wortmannin perfusie) het beskermende effekte getoon.

Bespreking

Hierdie studie het getoon dat RPO-aanvulling beskerming gebied het teen isgemie/herperfusie skade in die Langendorff geperfuseerde rothart na 10 min herperfusie. Daarna is die beduidenheid van die beskerming verloor. Hierdie bevindings ondersteun die resultate van vorige studies. Verskeie moontlike meganismes is voorgestel vir die beskerming, maar die presiese meganisme is nog nie duidelik nie.

In hierdie studie is daar gekyk na die reguleerders van die PKB/Akt pad. Geen vorige studies het al gefokus op RPO-aanvulling en sy effek op die reguleerders van die PKB/Akt pad nie.

'n Onverwagte bevinding is dat harte van die rotte in die RPO + wortmannin groep 'n verbeterde funksionele herstel getoon het. Wortmannin is 'n PI3-

kinase inhibitor. PI3-K fosforilering lei tot PKB/Akt fosforilering, wat tot sel beskerming lei. Dus, aangesien wortmannin PI3-K inhibeer, sou dit verwag word dat wortmannin sel beskerming sal verminder. Die RPO het egter die wortmannin se nadelige effekte tot so ‘n mate oorskrei dat die funksionele herstel baie beter was as die RPO-aanvulling alleen.

Die verhoogde PKB/Akt (Ser^{473}) fosforilering, wat gesien is in die RPO + wortmannin groep kan toegeskryf word aan RPO se vermoë om wortmannin se nadelige effekte te oorskrei. ‘n Moontlike verduideliking vir hierdie bevinding mag wees dat rooi palm olie PKB/Akt (Ser^{473}) op ‘n PI3-K onafhanklike manier fosforileer.

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α	Alpha
AFX	Forkhead transcription factors
ASK1	Apoptosis signal-regulating kinase 1
ATP	Adenosine 5'-triphosphate
BAD	Pro-apoptotic protein BAD
Bcl-2	Integral membrane protein
β	Beta
C	Control
Ca^{2+}	Calsium ion
cAMP	Cyclic adenosine monophosphate
CF	Coronary flow
cFLIP	Cellular FLICE-inhibitory protein
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
cm	Centimetre
CoQ_{10}	Coenzyme Q_{10}
CO_2	Carbon dioxide
CREB	cAMP response element-binding proteins
CVD	Cardiovascular disease

DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
FADD	Fas Associated protein with Death Domain
FKHR	Forkhead transcription factor
FKHRL1	Forkhead transcription factor
γ	Gamma
GSK	Glycogen synthase kinase-3
HDL	High density lipoproteins
HDL-c	High density lipoproteins cholesterol
H_2O_2	Hydrogen peroxide
H_2O	Water
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A
HR	Heart rate
IAP	Inhibitor of apoptosis
JNK	c-JunN-terminal kinase
kg	Kilogram
LDL	Low density lipoprotein
LDL-c	Low density lipoprotein cholesterol
LVDevP	Left ventricular developed pressure
LVDP	Left ventricular diastolic pressure

LVSP	Left ventricular systolic pressure
MAPK	Mitogen-acitvated protein kinase
mg	Milligram
min	Minutes
mM	Millimolar
mmHG	Millimetres of mercury
mRNA	Messenger Ribonucleic Acid
MUFA	Monounsaturated fatty acids
NF-$_{\kappa}$B	Nuclear factor-kappa B
nM	Nanomolar
NO	Nitric oxide
O_2	Oxygen
O_2^-	Superoxide
OH	Hydroxyl radical
p38	p38 Mitogen-activated protein kinase
P/S	Polyunsaturated/saturated fatty acid ration
PARP	Poly(ADP-ribose) polymerase
PDK-1	Phosphoinositide-dependent kinase-1
PIAK	Phospholipids independent Akt/PKB kinase
PI3-K	Phosphatidylinositol 3-kinase

PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
PKB/Akt	Serine/threonine protein kinase, protein kinase B or AKT
PKC	Protein kinase C
PMSF	Phenylmethyl sulfonyl fluoride
PPM	Parts per million
PTEN	Phoshoinositide-lipid-3-phosphotase
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
RPO	Red palm oil
RPP	Rate pressure product
SEM	Standard error of the mean
SFA	Saturated fatty acids
SHIP	Inositol-specific lipid phosphatase
SRC	Standard rat chow
TC	Total cholesterol
U/S	Monounsaturated + polyunsaturated/saturated fatty acid ratio
UFA	Unsaturated fatty acids
Wn	Wortmannin

ω-6	Omega-6 fatty acids
ω-3	Omega-3 fatty acids
%	Percentage

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Chapter 1 INTRODUCTION

1.1 Motivation for study

Cardiovascular disease and heart failure is a leading cause of morbidity and mortality in industrialized countries (Ho *et al*., 1993), and is related to risk factors such as elevated blood pressure, cholesterol, or glucose levels and smoking (Yusuf *et al*., 2001.). Recent studies have also suggested that the generation of reactive oxygen species (ROS) increased the incidence of heart failure (Belch *et al*., 1991; Hill and Singal, 1996; Mallat *et al*., 1998).

The excessive formation of ROS has a harmful effect on the functional and structural integrity of biological tissue (McCord, 1985). ROS have been implicated in a wide range of pathological conditions including ischaemia/reperfusion injury, neurodegenerative diseases and aging. ROS cause contractile failure and structural damage in the myocardium. However, their toxic effects can be prevented by scavenging enzymes known as antioxidants.

It is thus clear that the prevention of ROS generation during ischaemia/reperfusion is increasingly important in ischamiea/reperfusin injury and heart failure prevention. Antioxidants play an important role in minimizing the damaging effects of ROS generation and oxidative stress on cells (Rao *et al*., 2006). Dietary supplementation with a substance rich in antioxidants can be a means of preventing ischaemia/reperfusion injury, especially in heart failure.

The carotenoids and vitamin E in RPO have strong antioxidant properties (Bagchi & Puri, 1998; Theriault *et al*., 1999) and can act as scavengers of damaging oxygen free radicals. RPO also has a high content of SFAs, and this has created the perception that the oil may be detrimental to health. However, the characteristic composition of the oil and the position of SFAs and MUFAs on the triaglyceride backbone make it unique (Kritchevsky, 2000; Ong and Goh,

2002). Furthermore, a few studies have shown that RPO protected against ischaemia/reperfusion injury (Abeywardena *et al*., 1991; Charnock *et al*., 1991; Abeywardena & Charnock, 1995; Esterhuyse *et al*,. 2005). Other evidence suggests that RPO protected against cardiovascular disease (Kritchevsky *et al.,* 1999) and may protect against cancer (Yu *et al*., 1999).

Additional evidence is provided by studies in Africa and India where RPO-supplementation improved vitamin A status in vitamin A deficient children that were either breastfed by mothers (Canfield *et al*., 2001) or received RPO in a spread applied to bread (Van Stuijvenberg, 2005).

The precise mechanism of protection of RPO against ischaemia/reperfusion has not been resolved. Esterhuyse *et al.* (2006) suggested that the NO-cGMP pathway is involved by offering protection during ischaemia. Engelbrecht *et al.* (2006) showed that the PKB/Akt and MAPK pathways played a protective role during reperfusion. However, no conclusive protective mechanism was described by any of these studies.

When investigating the MAPK pathway, it was found that RPO might exert its beneficial effects through increased p38 phosphorylation and dephosphorylation of JNK (Engelbrecht *et al.,* 2006). Phosphorylation of p38 protects the heart from ischaemia/reperfusion injury (Mackay and Mochly-Rosen, 2000; Marais *et al.,* 2001) and dephosphorylation of JNK appears to be anti-apoptotic (Obata T *et al.,* 2000; Park *et al.,* 2000). It was also found that RPO significantly increased phosphorylation of PKB/Akt (Engelbrecht *et al.,* 2006). PKB/Akt activation promotes the survival of myocytes and protected against ischaemia/reperfusion injury (Fujio *et al.,* 2000).

Research has shown that RPO activated PKB/Akt (Engelbrecht *et al.,* 2006), but uncertainty prevails about the regulatory proteins that are involved. Therefore we were interested in studying the protective mechanism of RPO and how it relates to PKB/Akt pathway activity.

We used the Langendorff perfused heart model where hearts were subjected to ischaemia/reperfusion to investigate the effects of RPO on the function of the heart and regulators of PKB/Akt pathway at a cellular level.

1.2 Aim

The aims of this study were:

1. to determine whether dietary Carotino Premium RPO-supplementation protected against ischaemia/reperfusion in the isolated perfused rat heart
2. to confirm that RPO-supplementation influenced the PKB/Akt pathway activity
3. to determine upstream or downstream involvement of the PKB/Akt pathway with RPO-supplementation.

Chapter 2 LITERATURE REVIEW

2.1 Introduction

Cardiovascular disease (CVD) is one of the major causes of death in the Western world (Ho *et al.,* 1993). Risk factors which are associated with CVD include elevated blood pressure and glucose levels, as well as smoking (Yusuf *et al.,* 2001). Several studies also demonstrated that the generation of reactive oxygen species (ROS) are increased during heart failure (Belch *et al*., 1991; Hill and Singal, 1996; Mallat *et al*., 1998).

The excessive formation of ROS and has a harmful effect on the functional and structural integrity of biological tissue (McCord, 1985). These harmful effects of ROS can be prevented by scavenging enzymes that are known as antioxidants. Antioxidants play an important role in attenuating the damaging effects of ROS on cells (Rao *et al*., 2006).

The production of ROS is associated with ventricular function impairment, arrhythmias and coronary dysfunction during ischaemia/reperfusion injury (Bolli, 1991; Cai & Harrison, 2000; Toufektsian *et al*., 2001). In trying to prevent the increasing prevalence of heart failure, it is thus important to decrease the extent of ischaemia/reperfusion injury.

2.2 Ischaemia/reperfusion injury in the rat heart

Ischaemia/reperfusion injury occurs when blood flow to the heart is disrupted and then subsequently reintroduced. In this situation much of the damage occurs during the reperfusion period, when blood flow is restored after coronary occlusion. A primary factor in the initiation of the pathological response to reperfusion injury is the generation of ROS, which can covalently modify protein and lipid macromolecules, leading to cell damage, DNA mutation and initiation of the necrotic and apoptotic cascades (Li & Jackson, 2002).

Furthermore, there is also a restriction of oxygen to the heart during an ischaemic episode. This restriction of oxygen leads to an accumulation of lactate and protons. This build-up is due to the uncoupling of glycolysis from glucose oxidation and continued H^+ production during ischaemia (Liu *et al.,* 1996), which results in a decrease in intracellular pH. The accumulation of protons and lactate is detrimental to heart function and the magnitude of the pH decrease determines the severity of the ischaemic episode (Kloner & Jennings, 2001; Liu *et al.,* 2002).

If the ischaemic episode is not too severe, injury to the myocardium can be reversed during reperfusion. Fatty acid oxidation rapidly recovers during reperfusion. Even though fatty acid oxidation is restored, the uncoupling of glycolysis form glucose oxidation still occurs during reperfusion. The proton accumulation that is associated with ischaemia and reperfusion is detrimental to the normal heart function as it potentially leads to an accumulation of intracellular Ca^{2+} during reperfusion (Liu *et al.,* 1996; Du Toit *et al.,* 2001), which is detrimental to the heart. Furthermore, it was shown by Liu *et al.,* (1996) that a sudden rise in intracellular Ca^{2+} could potentially cause cell death.

Proton accumulation also results in impaired functional recovery during reperfusion and a reduction in cardiac efficiency (cardiac work/myocardial O_2 consumption). H^+ production can be decreased by improving glycose to glycose oxidation coupling. This decrease in H^+ production and/or inhibiting Na^+/H^+ exchange, can improve functional recovery as well as cardiac efficiency during reperfusion (Liu *et al.,* 1996*)*. It can thus be seen that the altered metabolism of the heart during ischaemia/reperfusion contributes to cell death which is responsible for the impaired functional recovery of the post-ischaemic heart.

2.3 Apoptosis during ischaemia/reperfusion-induced injury

The significance of cell death by apoptosis during ischaemia/reperfusion injury has gained great interest and unlike necrosis, which is thought to be an

essentially irreversible process, the step-by-step nature of apoptosis suggests that it may be amenable to therapeutic intervention.

Apoptosis (programmed cell death) is essential to many biological processes including embryonic development, immune responses, tissue homeostasis and normal cell turnover (Lin, 2003). It has been shown that apoptosis plays a significant role in myocardial ischaemia/reperfusion injury (Kajstuara *et al.,* 1996). The increase in the amount of oxygen free radicals during reperfusion can account for the increased apoptosis in cell culture and the isolated perfused heart (Feuerstein and Young, 1999).

Apoptosis is an active mode of cell death where the cell itself designs and executes the program of its own death. There are several regulatory systems, which include the Bcl-2/bax family of proteins (Hockenberry, 1995; Reed, 1994), the cysteine-proteases (caspases) (Fernandes-Alnemri *et al*., 1995; Lazebnik *et al*., 1994; Nicholson *et al.*, 1995) and possibly also serine- proteases (Bruno *et al*., 1992; Hara *et al*., 1996; Weaver *et al*., 1993).

The release of cytochrome c from the mitochondria is an important event in apoptosis. Cytochrome c release triggers the activity of caspases and other downstream apoptotic effectors (Chinnaiyan *et al*., 1996; Wu *et al*., 1997; Yang *et al*., 1997, Kluck *et al*., 1997).

2.3.1 The molecular pathways through which apoptosis is induced

The activation of cysteine proteases (caspases) is one of the biochemical features of apoptosis (Lazebnik, 1998). Caspases are present in cells as inactive procaspases that can be cleaved and activated in response to an apoptotic stimulus. The activation of caspases can follow two routes. The first one being the transduction of a signal from the membrane death receptors (Ashkenazi & Dixit, 1998). Stimulation of the death receptors lead to the activation of caspase-8, which further activates caspase-3, a key protein in apoptosis. The second pathway for activation is mediated through a mitochondrial pathway (Green &

Kroemer, 1998). Cytochrome c is released from the mitochondria, which leads to activation of caspase-9 and subsequent activation of caspase-3.

Apoptosis is energy dependent and highly regulated and is controlled by the complex interaction of numerous pro-survival and pro-death signals. These regulatory proteins include the Bcl-2 family of proteins which exert their effects mainly through the mitochondria. This family of proteins can be anti-apoptotic (Bcl-2, Bcl-X_L) or pro-apoptotic (Bad, Bid) (Adams & Cory, 1998). Other important regulators of apoptosis act at the level of caspases. These include cellular FADD-like inhibitory protein (cFLIP) and the inhibitor of apoptosis (IAP) family (Schmitz *et al.,* 2000).

There are also several other factors that are involved in the regulation of apoptosis. These include, growth factors, mitogen activated protein kinases (MAPKs), PKB/Akt, calcium, and oxidants. The outcome of the interaction of all these molecules determines the fate of the cell: life or death.

2.3.2 Caspases and the apoptotic pathway

An essential phenomenon of apoptotic cell death is the activation of caspases. Caspases are a unique class of aspartate-specific proteases. As many as 14 members have been identified (Nicholson & Thornberry, 1997). All of the caspases are composed of a prodomain and an enzymatic region. Differences among the proteases can be seen, regarding the structure of the prodomain. This region defines functional differences between caspases. Caspase-1, -2, -4, -5, -8, -9, and -10 contain a long prodomain versus caspases-3, -6, and -7, which contains a much shorter prodomain. For caspases to be activated, the proform has to be cleaved within the enzymatic domain. Activation can only occur through auto activation or cleavage by other caspases.

Caspases may work in a cascade fashion. It was found that deletion of caspase-3 resulted in failure of neural apoptosis. These mice were born with overlarge brains and they die soon after birth (Kuida *et al*., 1996).

There are many different proteins that act as substrates for caspases. These include nuclear proteins, proteins involved in signal transduction, and cytoskeletal targets (Cardone *et al*., 1997; Kothakota *et al*., 1997; Sakahira *et al*., 1998). Most of these proteins appear to be cleaved by caspases-3 and -7. Before apoptosis can take place, there must be a cleavage of a cytoplasmic inhibitor of the apoptosis-specific endonucleases (Enari *et al*., 1998; Sakahira *et al*., 1998; Liu *et al*., 1997). Only after this cleavage, the endonuclease translocate to the nucleus and degrade the genomic DNA (Enari *et al*., 1998; Sakahira *et al*., 1998). Thus, the activity of caspases-3 and -7 leads to the breakdown of cellular target proteins.

2.3.3 PARP

One of the targets of caspases is the enzyme poly-(ADP-ribose) polymerase (PARP). The cleavage of PARP by caspase-3 during apoptosis facilities nuclear disassembly and may help to ensure the completion of apoptosis (Szabo, 2005). It has been postulated that PARP cleavage occurs in order to prevent depletion of energy pools required for later stages of apoptosis (Earnshaw, 1995). Oliver and co-workers (1999) also claim that PARP cleavage facilitates cellular disassembly, ensuring cell death completion.

In contrast to PARP cleavage, PARP activation induces necrotic cell death. The obligatory trigger for its activation is nicks and breaks in the DNA strand. The generation of free radicals and oxidants in cardiac myocytes during ischaemia/reperfusion leads to such DNA strand breakage. This initiates an energy consuming and inefficient metabolic cycle with transfer of the ADP-ribosyl moiety of NAD^+ to protein acceptors. Resynthesis of NAD^+ requires ATP and poses a heavy demand on the cellular energy capacities. Failure to overcome this crisis leads to cell death, apoptotic or necrotic, depending on ATP availability. In this way, ATP is rapidly diminishing and its usage for other processes, including apoptosis is prevented. Therefore, the energy depletion caused by PARP activation induces rapid necrotic rather than delayed apoptotic

cell death. In other words, PARP activation shifts cellular death towards necrosis, away from apoptosis. In this way PARP may prevent several damaged cells from attempting to repair themselves and surviving with a high mutation frequency (Martin *et al.*, 2005).

2.4 Signalling pathways and apoptosis in the heart

2.4.1 Introduction

Several efforts have been made to disentangle the intricate relationships between signal transduction and apoptosis. Analysis is complicated by the fact that receptor agonsits may activate several signal transduction mechanisms with opposing effects on apoptosis regulation. However, it has become clear that the PKB/Akt signalling pathway plays a major role in the regulation of apoptosis in the heart.

2.4.2 Protein kinase B (PKB)/Akt pathway (Fig 2-1)

The serine/threonine protein kinase, protein kinase B or Akt (PKB/Akt), is an important regulator of many cellular processes. These processes include apoptosis, proliferation and differentiation. Three members of the PKB/Akt family have been isolated. They are PKBα (Akt1), PKBβ (Akt2), and PKBγ (Akt3). These members show an 80% homology in their amino acid composition.

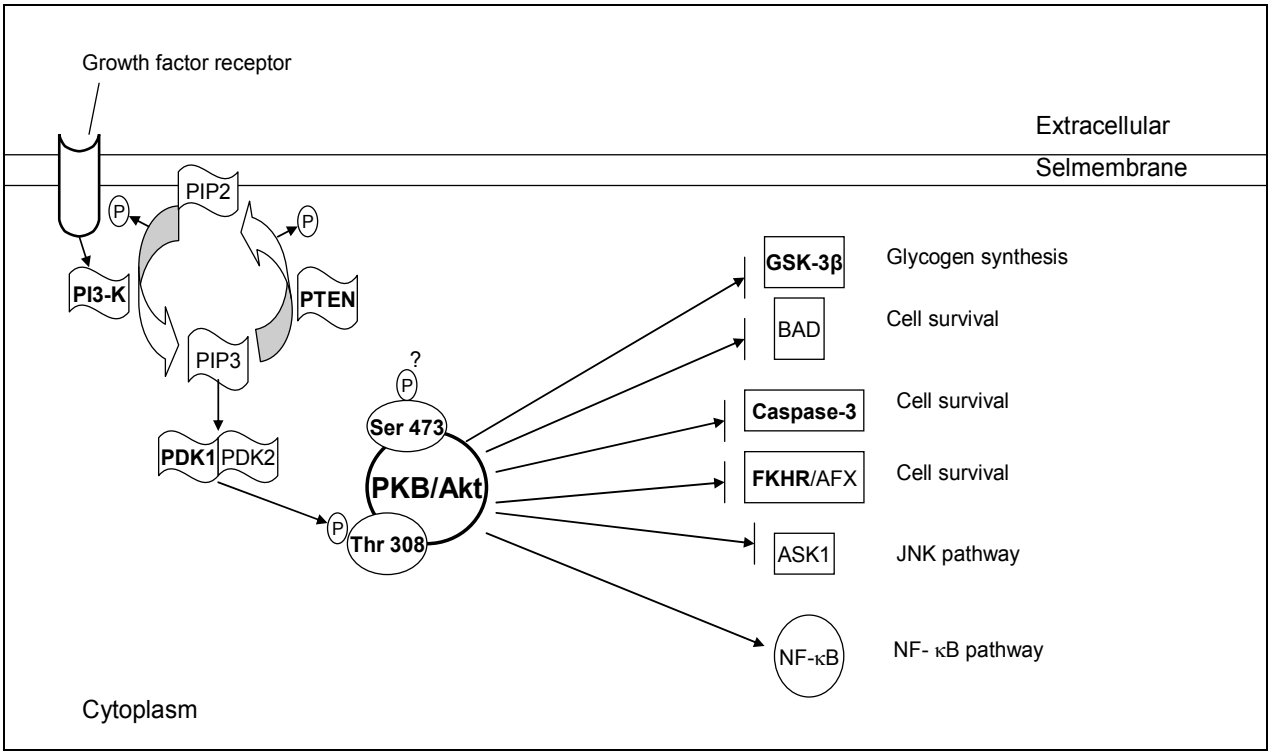

Figure 2-1: The PKB/Akt pathway

PKB/Akt is a downstream target for (phosphatidylinositol 3-kinase) PI3-K (Burgering & Coffer, 1995; Franke *et al*., 1995). For the activation of PKB/Akt to take place, inositol-containing membrane lipids must be phosphorylated by PI3-K. Factors that stimulate PI3-K to phosphorylate these lipids include thrombin, platelet-derived growth factor, and insulin (Downward, 1998). There has been speculation about other pathways leading to activation of PKB/Akt (Vanhaesebroeck & Alessi, 2000), but the identity of these pathways remain unclear. One mechanism considered is the activation of PKB/Akt by heat shock and oxidative stress (Konishi *et al.,* 1996; Shaw *et al.,* 1998).

PI3-K is activated by tyrosine kinases and G-protein coupled receptors. Activated PI3-K then phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2). This generates the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PIP_3 levels are regulated by phosphatases such as PTEN and SHIP. PTEN removes phosphate from the 3-OH position and SHIP

dephophorylates at the 5-OH position. These phosphatases thus inhibits the generation of the second messenger.

PIP_3 does not activate PKB/Akt directly. PIP_3 recruits PKB/Akt to the plasma membrane and alters PKB/Akt's conformation so that phosphorylation can take place by phosphoinositide-dependent kinase-1 (PDK-1).

PDK-1 is a 63-kDa serine/threonine kinase that contains a C-terminal PH domain that binds with high affinity to 3-phosphoinositides. PDK-1 phosphorylates PKB/Akt. This regulates access to the catalytic site of PKB/Akt, where phosphorylation *in vitro* is enhanced by 3-phosphoinositides. This phosphorylation takes place at the Thr^{308} site of PBK/Akt. It has been suggested that lipids induce both a favourable conformation of PKB/Akt as well as PDK-1. This allows access to the acceptor phosphorylation site (Alessi *et al.,* 1997; Stokoe *et al.,* 1997).

The phosphorylation at Thr^{308} partially activates PKB/Akt (Alessi *et al.,* 1996). For full activation, phosphorylation must also take place on Ser^{473}. Phosphorylation of Thr^{308} alone is able to increase PKB/Akt activity, while the phosphorylation of Ser^{473} alone does not stimulate the kinase (Alessi *et al.,* 1996; Bellacosa *et al.,* 1991)

In most situations, phosphorylation of PKB/Akt at Ser^{473} occurs together with Thr^{308} (Alessi *et al.,* 1996), but some studies have shown that phosphorylation at the two sites occur independently (Kroner *et al.,* 2000). PDK-1 null embryonic stem cells maintain the ability to undergo Ser^{473} phosphorylation (Williams *et al.,* 2000). However, the regulation of PKB/Akt is a complex process, but it is clear that the phosphorylation of Ser^{473} alone does not increase PKB/Akt activity.

There is increasing evidence that the PKB/Akt pathway participates in ischaemia/reperfusion-induced injury (Brar *et al.,* 2002; Andreucci *et al.,* 2003). Expression of active PI3-kinase itself is cardioprotective (Brar *et al.,* 2002). This

finding is consistent with a number of studies where the activation of PI3-kinase prevented ischaemic- (Matsui *et al.,* 1999; Fujio *et al.,* 2000) and ischaemic/reperfusion induced cell death of cardiac myocytes (Fujio *et al.,* 2000).

One of the substrates of PKB/Akt, glycogen synthase kinase (GSK), also play a pivotal role in ischaemia/reperfusion injury. GSK is an unusual protein because its kinase activity is high under basal conditions, and stimuli result in its inactivation. Also, many of GSK's substrates are functionally inhibited by phosphorylation. This means that signals that inhibit or inactivate GSK-3 often cause activation of its downstream target proteins (Kockeritz *et al.,* 2006; Cross *et al.,* 1995).

GSK-3 has two isoforms, α (51kDa) and β (47kDa), and they are characterized as serine/threonine kinases. These two isoforms have a 98% identity in their central 30-kDa catalytic domain (Woodgett, 1990; Juhaszova *et al.,* 2004). These isoforms exhibit different catalytic activities toward different intracellular substrates. The β isoform has a higher activity than the α isoform (Plyte *et al.,* 1992). GSK is a pivotal kinase as it receives inputs from different pathways that regulate its enzymatic activity. GSK-3 is involved in the control of a variety of cellular processes through several intracellular signalling pathways. The most important role of GSK-3 is that it phosphorylates and inactivates glycogen synthase (Rylatt *et al.,* 1980; Plyte *et al.,* 1992).

Cardioprotection may be mediated by the phosphorylation of GSK-3β (Juhaszova *et al.,* 2004). Gross *et al* (2004) showed that the addition of GSK-3β inhibitors before the start of reperfusion resulted in a significant reduction in infarct size. Tong and co-workers (2002) also demonstrated that inhibition of GSK-3 reduced infarct size and improved post-ischaemic function.

GSK-3β is a substrate of PKB/Akt and also participates in regulating the cell cycle in various cell types (Liang and Slingerland, 2003). PKB/Akt phosphorylates GSK-3β at Ser^9. The phosphorylation decreases the activity of

the enzyme. This leads to reduced glycogen synthase phosphorylation at the sites phosphorylated by GSK-3 and also mediates insulin-stimulated upregulation of glycogen synthase activity (Cross *et al.,* 1995; McManus *et al.,* 2005; Kerr *et al.,* 2006). PTEN increases GSK-3 activity by exerting an inhibiting effect on PKB/Akt (Persad *et al.,* 2001; Sharma *et al.,* 2002).

2.4.3 Involvement of PKB/Akt in anti-apoptotic mechanisms

All cells have the intrinsic capacity to undergo apoptosis. This capacity is suppressed by survival signals. Several studies have shown that PKB/Akt is critical for cell survival. These studies showed that there is a reduction in the ability of growth factors to maintain cell survival in the absence of activated PKB/Akt. Furthermore, cells can be rescued from stress-induced apoptosis when there is an overexpression of activated PKB/Akt (Kauffmann-Zeh *et al*., 1997; Kennedy *et al*., 1997; Khwaja *et al*., 1997; Kulik *et al*., 1997).

It is clear that PKB/Akt promotes cell survival, but the mechanisms involved are not yet clear. There are several PKB/Akt substrates. Some of these either participate directly in the apoptotic cascade or regulate the transcription of pro- and anti-apoptotic genes. Prosurvival substrates of PKB/Akt signaling include ASK1, BAD, CREB, Forkhead family (FKHR, FKHRL1, AFX), NFκ-B kinase and procaspase-9.

A way in which PKB/Akt might promote cell survival is through the direct phosphorylation of transcription factors controlling the expression of pro-and anti-apoptotic genes. PKB/Akt inhibits the pro-apoptotic genes and stimulates the survival genes. Although not directly, PKB/Akt exerts these effects on factors that influence these genes. An example where PKB/Akt negatively regulates the apoptotic genes is on the forkhead family of transcription factors. There are three identified mammalian members of the forkhead family, FKHR, FKHRL1, and AFX. All of these members contain a PKB/Akt phosphorylation sequence that can be phosphorylated by PKB/Akt *in vitro* (Biggs *et al*., 1999; Brunet *et al*., 1999; Rena *et al*., 1999). The phosphorylation of forkhead proteins by PKB/Akt

alters their location in the cell. When PKB/Akt activity is increased, it leads to the export of FKHRL1 from the nucleus (Biggs *et al*., 1999). The forkhead proteins are not able to influence the forkhead genes, located in the nucleus, to transcribe pro-apoptotic proteins. Thus, PKB/Akt negatively regulates forkhead activity.

PKB/Akt also regulates other transcription factors: NF-κB has survival-promoting activity. PKB/Akt is a critical regulator of NF-κB-dependent gene transcription and may play a critical role in promoting cell survival (Romashkova & Makarov, 1999; Ozes *et al*., 1999). PKB/Akt is also involved in the expression of the anti-apoptotic gene bcl-2 (Skorski *et al*., 1997; Pugazhenthi *et al*., 2000). PKB/Akt is believed to increase its expression. It has been found that PKB/Akt also plays a role in the regulation of the expression of c-FLIP (Panka *et al.,* 2001). C-FLIP is a caspase-8 homologue that acts as a negative inhibitor of TNF receptor family-induced apoptosis.

PKB/Akt also phosphorylates and deactivates pro-caspase-9, thus inhibiting the apoptotic pathway (Cardone *et al.,* 1998).

PKB/Akt not only influences the expression of proteins at the gene level, but can also promote survival by directly phosphorylating key regulators of the apoptotic cascade. BAD is a member of the Bcl-2 family that promotes apoptosis by binding to prosurvival members of the same family and antagonizing their actions. PKB/Akt phosphorylates BAD which leads to the sequestration of BAD in the cytosol, thus preventing BAD from interacting with the prosurvival genes at the mitochondrial membrane (Del Peso *et al*., 1997; Datta *et al*., 1997). PKB/Akt-induced phosphorylation of BAD may also occur indirectly through the enhanced expression of active Raf-1 on the mitochondrial membrane. (Majewski *et al*., 1999; Shurmann *et al*., 2000; Tang *et al*., 2000).

All these findings indicate that PKB/Akt regulates apoptosis prior to the release of cytochorme c from the mitochondria. However, it was also found that

PKB/Akt can influence postmitochondrial events of apoptosis (Cardone *et al.,* 1998; Zhou *et al.,* 2000).

2.4.4 Negative regulation of PKB/Akt activity

The activity of PKB/Akt depends on the balance between the signals that activates PKB/Akt (like PIP_3) and the signals that negatively regulate PKB/Akt (dephosphorylation of PKB/Akt) (Andjelkovic *et al*., 1996; Meier *et al*., 1997, 1998).

A decrease in PI3-K activation leads to a rapid dephosphorylation of Ser^{473} and a slower dephosphorylation of Thr^{308}. This dephosphorylation causes a loss of PKB/Akt activity. PKB/Akt can also be inactivated by ceramide (Schubert *et al*., 2000) and osmotic stress (Meier *et al*., 1998; Chen *et al*., 1999) through the dephosphorylation of Ser^{473}. The dephosphorylation of Thr^{308} can occur independently of Ser^{473} (Schubert *et al*., 2000) and a recent study also indicates that PDK-1 might participate in this dephosphorylation, but the mechanism is still unknown (Yamada *et al*., 2001). Thus, PDK-1 might be involved in phosphorylating and dephosphorylating of PKB/Akt.

PTEN can dephosphorylate the inositol ring of the second messenger phosphatidylinositol (3,4,5)-triphosphate (PIP_3), thus inhibiting cell survival due to responses from PI3-kinase and PKB/Akt (Maehama and Dixon, 1998; Stambolic *et al.,* 1998; Wu *et al.,*1998). The overexpression of PTEN significantly reduced the $PI(3,4,5)P_3$ production which was induced by insulin. It was also found that PTEN-null cells have higher levels of $PI(3,4,5)P_3$ (Haas-Kogan *et al*., 1998; Maehama & Dixon, 1998; Stambolic *et al*., 1998). Thus, it is clear that PTEN inhibits PI3-kinase activity, thereby influencing PKB/Akt activity. Experiments conducted with inactive PTEN and PTEN-null fibroblasts showed that these cells exhibit high basal activity of PKB/Akt (Meyers *et al*., 1998; Li & Sun, 1998; Wu *et al*., 1998). Genetic studies done in *C elegans* indicated that PTEN lies in the same pathway as PI3-kinase/PKB and inhibits PKB/Akt (Ogg & Ruvkun, 1998).

All of the above mentioned studies confirmed PTEN's ability to negatively regulate PKB/Akt.

SHIP is another lipid phosphatase that can negatively regulate PKB/Akt. The over-expression of SHIP has been shown to inhibit PKB/Akt activity and SHIP-null cells induced prolonged activation of PKB/Akt upon stimulation (Liu *et al*., 1995; Aman *et al*., 1998).

It is clear from the previously discussed pathways that apoptosis is a highly regulated and complex process, controlled by numerous checkpoints and signalling networks. This is of particular importance in fully differentiated cardiomyocytes where it prevents unnecessary death of salvageable cells. Although the extent to which apoptosis is involved in cardiac disease remains to be established, the evidence that has emerged clearly supports a role for this mode of cell death. A better understanding of the underlying pathways may lead to the development of therapies to treat coronary heart disease (CHD) which are anti-apoptotic with minimum or no side effects.

2.5 Lipids in cardiovascular health

The concept that dietary lipids affect the incidence of CHD is widely accepted, based on both epidemiological and experimental evidence. Lipids, which refer to both fats and oils (fats refer to lipids that are solids at room temperature while oils are usually liquids at room temperature), form an important part of our diets and are also essential for cardiovascular health. They provide calories for metabolic activities, supply essential fatty acids and assist in the absorption of fat-soluble vitamins. Lipids are also required for cell structure and membrane function. Lipid components like cholesterol and phospholipids regulate membrane-associated functions such as activities of membrane bound enzymes, receptors and ion channels (Clandinin *et al.,* 1991). Lipids are thus important and essential in our every day life.

Dietary lipids consist of triglycerides (esters of glycerol and the three fatty acids) and minor amounts of phospholipids and sterols. The most common sources of lipids derived from plants are: soybean oil, palm oil, sunflower seed oil and rapeseed oil (Ong & Goh, 2002). The fatty acid composition of the most important oils and fats are summarized in Table 1. These are classified according to degree of saturation, into saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs).

SFAs are straight chain structures with no double bonds and an even number of carbon atoms. Human MUFAs have an even number of carbon atoms, a chain length of 12-22C and a double bond with the *cis* configuration. PUFAs are mostly confined to the C18 and C20 acids, and have more than one double bond. PUFAs are further divided into omega-6 (ω-6) or omega-3(ω-3) fatty acids based on the position of the first double bond nearest to the methyl end of the carbon chain.

2.6 Red Palm Oil as a therapeutic agent for cardiovascular disease

2.6.1 Introduction

Palm oil is the second most commonly consumed vegetable oil in the world (Edem, 2002) and is mainly used as an edible oil. Crude palm oil is obtained from the fruit of a tropical plant*, Elais guineensis* (Manorama *et al.,* 1993; Nagendran *et al.,* 2000), and is grown in India, Malaysia and in some African countries (Hariharan *et al.,* 1996). The use of palm oil dates back as far as 5 000 years. The palm tree bears 10-12 fruit bunches annually, each weighing between 20-30 kg.

Table 2-1: Saturated, monounsaturated and polyunsaturated fatty acid composition of dietary oils and fats (Ong & Goh, 2002).

Oil	*SFA (%)*	*MUFA (%)*	*PUFA(%)*	*P/S ratio*	*U/S ratio*
Rape seed	5	71	24	4,8	19
Canola	7	61	32	4,67	13,3
Sunflower	11,7	18	68,6	5,9	7,4
Olive	13	79,1	7,9	0,6	6,7
Corn	13,3	28,4	58,3	4,4	6,5
Soybean	16	23,5	60,5	3,8	5,3
Groundnut	20	38,7	41,3	2,1	4
Cotton seed	27,7	19,8	52,5	1,9	2,6
Lard	43	47	10	0,2	1,3
Palm olein	46,8	41,5	12	0,3	1,1
Palm oil	49,5	40,3	9,6	0,2	1
Red palm oil	**50,8**	**38,3**	**10,9**	**0,2**	**1**
Cocoa butter	60	36,5	3,4	0,2	0,7
Butter	63,4	32,5	4,5	0,1	0,6
Hydrogenated soybean	64 + trans	26	4	0,1	0,5
Palm kernel	84	14	2	0,02	0,2
Coconut	92,2	6,2	1,6	0,02	0,1

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

(reproduced from Ong and Goh, 2002)

2.6.2 Composition of Red Palm Oil

Crude palm oil consists mainly of glycerides and small quantities of non-glyceride components. These non-glyceride components include free fatty acids, trace metals, moisture and impurities, as well as minor components. The minor components include some of the most important components of crude palm oil. They are carotenoids, tocopherols and tocotrienols (Goh *et al.,* 1985).

Together, these components contribute to the stability and nutritional properties of crude palm oil (Ooi *et al.,* 1996). RPO consist of 51 % SFAs, 38 % MUFAs, 11 % PUFAs (Table 1) and contains 500 parts per million (ppm) carotenoids, 60 % as β-carotene and 25 % as α-carotene. The vitamin E content of RPO is between 500 - 800 ppm. Of the total vitamin E, 70 % is tocotrienols and 30 % tocopherols (Nagendran *et al.,* 2000; Sundram *et al.,* 2003). RPO also contains small amounts of CoQ_{10}.

The carotenoids are precursors of vitamin A. Drummon & Coward observed in 1920 that the red palm oil pigment was largely carotene and that it possessed vitamin A activity in rats (Aykroyd & Wright, 1937). The tocopherols and tocotrienols are vitamin E isomers and are potent antioxidants (Nagendran *et al.,* 2000).

Crude palm oil is not very stable. Due to some of the non-glyceride components, the oil needs to undergo a refining process during which the oil is rendered stable. Unfortunately the refining process also results in both the removal of some of the tocopherols and tocotrienols and the destruction of all the carotenoids present. This is how carotenoid-free palm oil is produced. A modified refining process has thus been developed to decrease the loss of carotenoids. The product is a stable, red palm oil that retains at least 80 % of the carotenoids and the vitamin E that is originally found in crude palm oil. The oil contains just over 500 ppm carotene, 85 % of which is present as α- and β-carotene. This makes it the world's richest food source of carotenoids. It is also a very good source of vitamin E (30 % tocopherols & 70 % tocotrienol) (Sundram

et al., 2003). Ubiquinones and sterols are also other important minor components present in the oil (Nagendran *et al.,* 2000). Red palm oil is a non-genetically modified, cholesterol-free and trans fatty acid free oil (Goh *et al,* 1985; Sundram *et al*, 2003). The RPO that was used in this study is commercially available Carotino Premium, and its components are provided in Table 2-2.

The saturated fatty acids of red palm oil consist of palmitic acid (44 %) and stearic acid (5 %), while the UFAs are oleic acid (39 %) and linoleic acid (10 %) (Ong and Goh, 2002). The SFA and UFA are evenly distributed in RPO.

Table 2-2: The components of Carotino Premium red palm oil (per 100 ml).

Total fats	92 g
Monounsaturates	43 g
Polyunsaturates	12 g
Saturates	37 g
Trans fat	0 g
Cholesterol, Sodium	0 g
Protein, Carbohydrate, Dietary fibre	0 g
Natural Carotenes	46 mg
Natural Vitamin E	74 mg
Co-Enzyme Q10	4 mg

(reproduced from Premium Carotino Palm fruit oil label)

2.7 Protection provided by the individual components of Red Palm Oil

2.7.1 Fatty acids

The type of dietary fat influences the serum lipid concentrations (Idris & Sundram, 2002) as well as the incidence of coronary heart disease (CHD).

Dietary saturated fatty acids cause an elevation in plasma cholesterol levels. Saturated fatty acids have been positively implicated in raising plasma total cholesterol (TC) and low density lipoprotein (LDL) cholesterol, and enhances the risk for coronary heart disease (CHD) (Idris & Sundram, 2002). Saturated animal fats also increase the susceptibility to develop cardiac arrhythmias under ischaemic stress (Charnock *et al.,* 1991). Therefore, it is not recommended to consume saturated fats, as they are linked to an unhealthy cholesterol and cardiovascular status (Kritchevsky, 1995).

It is generally acknowledged that increasing PUFA intake and reducing the intake of saturated fats exert a beneficial effect on plasma cholesterol levels (Hegsted *et al,* 1965; Keys *et al,* 1965). It has been shown that when saturated fatty acids, e.g. palmitic acid, is substituted with a monounsaturated fatty acid, e.g. oleic acid, and a polyunsaturated fatty acid, e.g. linoleic acid, the plasma TC as well as plasma LDL-C was significantly reduced (Mattson & Grundy, 1985). It is clear from the literature that different dietary saturated fatty acids do not exert the same detrimental cholesterolaemic impact, e.g. lauric acid (12:0), myristic acid (14:0) and palmitic acid (16:0) were found to be equally detrimental (Keys *et al,* 1965). Furthermore, Hegsted *et al.* (1965) have shown that lauric acid and myristic acids are more hypercholesterolaemic than palmitic acid. Of these, lauric acid was found to be the most hypercholesterolaemic (Denke & Grundy, 1992). Stearic acid (18:0) was found to be relatively neutral (Hegsted *et al,* 1965; Keys *et al,* 1965) or even hypocholesterolaemic in some studies (Horlick & Graig, 1957; Denke & Grundy, 1992; Bonanome & Grundy, 1988). This implies that lauric, myristic and palmitic acids are the saturated fatty acids that can raise

cholesterol levels, with palmitic acid being less adverse than the other two. Hayes *et al.* (1991) also showed that palmitic acid as a component of palm oil failed to raise cholesterol levels.

The question can thus be raised as to why the other long-chained saturated fatty acids showed a cholesterol-raising effect and not stearic acid. The answer might lie in a process that occurs shortly after absorption. Stearic acid is desaturated to oleic acid, which does not raise cholesterol concentrations (Elovson, 1965). Lauric acid, on the other hand, presumably retains its status as a saturated fatty acid and exerts its cholesterol-raising properties (Brett *et al,* 1971). There is thus an overall misconception that all saturated fatty acids are detrimental to health.

Polyunsaturated fatty acids reduce the plasma cholesterol levels when they are exchanged for saturated fatty acids. It is recommended that PUFAs should be increased and SFA are decreased in the diet. This would lower serum cholesterol and indirectly prevent atherosclerosis (Heyden, 1994). It is not clear whether this increase in PUFA consumption is really as good for health as is expected (Sturdevant *et al,* 1973; Shepherd *et al,* 1978; Vessby *et al,* 1980; Vega *et al,* 1982), despite the decrease in serum cholesterol that is associated with an increase in PUFA.

Fats that have a high PUFAs content are more susceptible to oxidation than SFAs. In the absence of adequate antioxidants, PUFAs will increase the oxidative stress in the heart and contribute to dysfunction and myocardial damage by increasing cardiac susceptibility to lipid peroxidation (Mehta *et al.,* 1994; Esposito *et al.,* 1999; Hart *et al.,* 1999; Droge, 2002; Faine *et al.,* 2002; Novelli *et al.,* 2002; Diniz *et al.* 2004). The number of double bonds of a fatty acid determines the inclination with which the fatty acid is peroxidized. As PUFAs have more than one double bound, it has a higher rate of peroxidation, and without adequate antioxidants, this can be deleterious. From the above

mentioned studies, it is thus clear that increased concentrations of PUFAs in the diet is not necessarily as healthy as is generally accepted.

On the other hand, monounsaturated fatty acids appeared to be neutral. It was found by Sundram and co-workers (1995) that the substitution of dietary palmitic acid with monounsaturated oleic acid (C18:1n-9) did not result in significant differences in plasma TC. These results are in agreement with previous studies (Vergroesen & Gottenbos, 1975; Ng *et al.,* 1992; Ghafoorunissa & Reddy, 1993). When comparing a diet high in PUFA with a diet high in MUFA, no significant differences were found in the high density lipoprotein (HDL) cholesterol levels (Fernandez & McNamara, 1989; Berry *et al.,* 1991).

The effects of the different fatty acids in diets are well researched, but it can be concluded from the above mentioned studies that not all saturated fats are equally detrimental to health; increased PUFA consumption is not necessarily healthy; and MUFAs are mostly neutral. Fats that have a high MUFA content are seen as a healthier choice than a fat with a high SFA or PUFA content. This is also why oils like rape seed-, canola- and olive oil are considered as such healthy oils (Ong and Goh, 2002). The high MUFA:SFA ratio implies that lipid levels will stay unchanged, and this is favourable when consuming a fat.

Questions can be raised about the high content of saturated fatty acids in RPO and the implication of this on cardiovascular health. However, RPO has certain attributes that potentially place it in a category of a healthy oil. One of these attributes is that the fatty acids in the sn-2 position of RPO are predominantly unsaturated (87%), whilst the sn-1 and sn-3 positional fatty acids are more highly saturated.

All oils and fats have one of three triacylglicerol or triglyceride structures. The differences between fats lie in the types of fatty acids (ROOH) that are attached to the glycerol backbone in position sn-1, sn-2, and sn-3. The position of attachment (sn-1, 2, or 3) to the glycerol backbone plays a role in determining the preferential absorption and subsequently the levels of blood triacylglycerols

and cholesterol (Ong & Goh, 2002). Studies have shown that the sn-2 positional fatty acids are mostly absorbed. This is due to specific actions of pancreatic lipase enzymes (Mattson & Volpenhein, 1959; Small, 1991; Kritchevsky *et al.,* 1996; Goh, 1999; Willis *et al.,* 1998; Kritchevsky *et al.,* 1998; Kritchevsky *et al.,* 1999). The sn-1 and sn-3 positional fatty acids are less readily absorbed, especially if they are long-chain saturated fatty acids (Kritchevsky *et al,* 1995; Willis *et al,* 1998; Kritchevsky *et al,* 1998; Goh, 1999).

RPO's SFAs (palmitic- and stearic acid) are mostly situated in postions sn-1 and sn-3, whilst the UFAs (oleic and linoleic acid) is mostly situated in position sn-2 (USADA, 1979; Small, 1991; Gunstone *et al.,* 1994; Gunstone, 1996; Willis *et al.,* 1998; Siew, 2000). This implies that the unsaturated fatty acids of RPO will be more readily absorbed than the saturated fatty acids. Thus, the relatively high content of saturated free fatty acids in the digested RPO, when compared to other fats and oils, will be less easily absorbed. Most of the sn-1 and sn-3 position unsaturated fatty acids are absorbed while the saturated fatty acids from these positions are excreted as salts. Only a minimal amount of saturated fatty acids from the sn-2 position will be absorbed.

As a result, none of the fatty acids in RPO, whether saturated (palmitic & stearic) or unsaturated (oleic & linoleic) has cholesterol elevating effects. RPO behaves like a monounsaturated oil, because most of the fatty acids that are absorbed are UFAs, even though it is classified as a saturated oil (Mattson & Volpenhein, 1959; Kritchevsky, 1995; Kritchevsky *et al,* 1999; Ong & Goh, 2002). Coconut oil and many animal fats have large amounts of saturation on the sn-2 positional fatty acids and this account for the hypercholesterolaemic properties in these fats (Ong & Goh, 2002).

2.7.2 Carotenoids

Carotenoids are a group of red, orange and yellow pigments found in plants, especially in fruit and vegetables, but are not synthesized by animals (Stahl and Sies, 2003). Red Palm oil is a well known source of carotenoids.

Of the carotenes present in RPO, only α-, β-, and γ-carotenes have provitamin A activity. The main component of these carotenoids is β-carotene, which is a precursor of vitamin A (table 2) (Choo *et al.,* 1992; Choo, 1995; Scrimshaw, 2000).

Table 3 shows a comparison of the vitamin E and carotene content between Carotino palm oil and other plant oils. In comparison to other oils, red palm oil is naturally much richer in carotenoids. It is 15 times richer in carotenes compared to carrots and contains 50 times more carotenes than tomatoes (Kamen, 2000).

Table 2-3: Comparison between Carotino palm oil and other plant oils

	Carotino Premium (Red Palm oil)	Carotino classic (Red Palm oil)	Sunflower seed oil	Safflower seed oil	Corn oil	Olive oil
Vitamin E mg/kg	80	50	39	17,4	20.7	7.6
Carotene mg/kg	50	12.5	0	0	0	0

(Reproduced from Kamen, 2000)

β-Carotene is an effective antioxidant because it is one of the most powerful singlet oxygen quenchers. It can disperse the energy of a singlet oxygen, thus preventing this active molecule from generating free radicals (Bagchi & Puri,

1998). It thus protects cells and tissue from oxidative damage. This protection is only seen at low partial oxygen pressure, as found in the body. When the partial pressure of oxygen is increased, β-carotene looses its antioxidant activity and exhibited a pro-oxidant effect (Palozza *et al.,* 1995). This illustrates the importance of oxygen tension on the antioxidant/pro-oxidant effects of β-carotene.

In certain animal models, carotenoid compounds can act as antioxidants, cancer-preventative agents and anti-atherosclerotic agents. However, animal models cannot be directly correlated to humans, because most laboratory animals absorb carotenoids differently to humans (Pavia and Russell, 1999). Observational studies showed an inverse relationship between various cancers and carotenoid intake, especially with cardiovascular disease. However, other studies showed no protective effects against cancer or cardiovascular disease when the diet was supplemented with high doses of β-carotene (Pavia and Russell, 1999). However, this was an extracted form of β-carotene, and not its natural form. It is also possible that the combination of carotenes and other antioxidants exert a better effect when they are supplemented individually.

There is also conflicting evidence about the effect of β-carotene on LDL oxidation. High doses of β-carotene supplementation resulted in an increased susceptibility of LDL to oxidation (Gaziano *et al.,* 1995). However, Lin *et al.,* (1998) showed that a depletion of β-carotene also lead to an increased susceptibility of LDL to oxidation. The only protection can be seen when a normal intake of β-carotene was provided.

Scientific evidence indicates that carotenes are healthy when taken at physiological levels, but when they are taken in high dosages or in the presence of highly oxidative conditions, their effects maybe adverse. It is also possible that mixtures of carotenoids with other antioxidants, as in RPO, can increase the ability of the antioxidant to offer protection against lipid peroxidation. Implying that when carotene is extracted from its natural environment, it has different

effects than when it is in its natural environment. Even though carotenoids are not essential for human health, they have biological actions that may be important in maintaining health and preventing the appearance of serious diseases.

Lycopene is also a carotenoid antioxidant. Among the many natural carotenoids, lycopene is recognized to be the most potent singlet oxygen quencher and free radical scavenger (Di Mascio *et al.,* 1989). The radical quenching activity of lycopene is twice that of β-carotene and ten times that of β-tocopherol (Böhm *et al*., 2001; Woodall *et al*., 1995; Di Mascio *et al*., 1989).

In contrast to β-carotene, lycopene is not a precursor of vitamin A in humans. Besides its antioxidant effect, it influences the expression of various proteins (Banhegyi, 2005). Epidemiological and animal studies have provided convincing evidence that supports the role of lycopene in the prevention of chronic deseases (Rao *et al.,* 2006; Shao & Hathcock, 2006).

Dietary consumption and serum levels of lycopene have been linked to a reduced risk of cardiovascular disease (Arab & Steck, 2000; Willcox *et al*., 2003) as well as prostate cancer (Chan *et al*., 2005; Giovannucci, 2005). Placebo-controlled intervention trials showed that the consumption of lycopene can reduce DNA damage (Astley *et al*., 2004; Zhao *et al*., 2006) and lung cancer (Liu *et al*., 2003, 2006; Wang, 2005). Matulka *et al*., (2004) found no indication of adverse effects with natural sources of lycopene.

Lycopene has been reported to decrease the infarct size in ischaemia/reperfusion brain injury (Hsiao *et al*., 2004). Some studies have reported that diets rich in lycopene lower cholesterol and lipid peroxidation (De Lorgeril *et al*., 1999; Rissanen *et al*., 2001). However, it was more recently found that lycopene does not affect plasma lipids or antioxidant status of healthy subjects (Collins *et al*., 2004).

It has also been found that lycopene is able to reduce the risk of atherosclerosis and cardiovascular disease by decreasing the susceptibility of LDL to oxidative modification (Hadley *et al*., 2003; Kohlmeier *et al*., 1997).

2.7.3 Vitamin E (tocopherols and tocotrienols)

Palm oil is a rich source of vitamin E and vitamin E associated compounds. Vitamin E is the collective name for eight compounds, four of which are tocopherols and the other four are tocotrienols (Bagchi & Puri, 1998). All vegetable oils have tocopherols, but palm oil also has an abundance of tocotrienols. An important attribute of palm oil is the fact that among vegetable oils, it is the only rich source of tocotrienols (Kamen, 2000).

Vitamin E has antioxidant properties, especially against lipid peroxidation in biological membranes (Theriault *et al.,* 1999). Tocopherols and tocotrienols are known natural antioxidants (Goh *et al.,* 1990; Ong & Packer, 1992; Baskin & Salem, 1997; Ong & Packer, 1998; Goh *et al.,* 1998) that protect the oil from oxidation.

Vitamin E has a high lipophilic activity and is the major lipid-soluble chain-breaking antioxidant found in blood plasma. It also protects polyunsaturated fatty acids in cell membranes from peroxidation by retarding oxidation (Bagchi & Puri, 1998). The oxidation of PUFAs leads to disturbances in membrane structure and function. Vitamin E protects against these damaging effects of oxidation by preventing the auto-oxidation of these lipids (Burton & Ingold, 1981). Vitamin E is a singlet oxygen quencher and neutralises these highly reactive and unstable molecules (Kamal-Eldin & Appelqvist, 1996).

Serbinova and co-workers (1992) were the first to show that palm oil vitamin E (containing both α-tocopherol and α-tocotrienol) improved reperfusion functional recovery in a Langendorff-perfused rat heart. The protection was attributed to the ability of both tocopherols and tocotrienols to scavenge free

radicals during reperfusion. Thereafter, Bagchi & Puri (1998) also showed that vitamin E protected against ischaemia and reperfusion injury. Venditti and co-workers (1999) also reported protection against ischaemia/ reperfusion-induced oxidative stress with vitamin E treatment.

It can be speculated that tocopherols and tocotrienols capture and destroy damaging free oxygen radicals that have been implicated in cellular aging, atherosclerosis and cancer (Kok *et al.,* 1987; Salonen *et al.,* 1988; Teoh *et al.,* 1992; Tomeo *et al.,* 1995). Laboratory experiments performed on isolated rat hearts have shown that a tocopherol/tocotrienol concentrate from palm oil is more efficient than α-tocopherol alone in protecting the heart against the oxidative injury usually associated with reperfusion (Esterbauer *et al.,* 1989; Steinberg *et al.,* 1989; Serbinova *et al.,* 1991; Serbinova *et al.,* 1992). This abovementioned concentrate, was also used in an experiment to treat patients with vascular disorders that cause a limitation in blood flow. The subjects that received the concentrate showed a significant increase in walking distance before onset of pain, in comparison with the groups given aspirin or a placebo. The oxidation in their blood lipids was also significantly reduced. These natural antioxidants protect LDLs (which are involved in the formation of atheriosclerotic lesions) from oxidation.

In a cross-cultural epidemiological study done by Gey *et al*, (1991) the amount of vitamin E in the plasma showed a strong inverse correlation with age-specific mortality from coronary heart disease. It was also found that there was protection against protein oxidation induced during resting as well as exercise, when supplemented with tocopherol and tocotrienols (Reznick *et al.,* 1992).

Tocopherol is considered the most active form of vitamin E, but tocotrienol the most potent antioxidant (Goh *et al.,* 1990; Serbinova *et al.,* 1991; Suzuki *et al.,* 1993; Goh *et al.,* 1998). Tocotrienols have been shown to be an effective inhibitor of breast and liver cancer cell growth (Sundram *et al.,* 1989; Goh *et al.,* 1990; Goh *et al.,* 1994; Nesaretnam *et al.,* 1995). It can also prevent oxidation of

protein and lipids after strenuous bouts of exercise. Differing from α-tocopherol, tocotrienols are not accumulated in the liver and seem to benefit the epidermis of the skin (Hew *et al.,* 1994; Traver *et al.,* 1998).

The more potent antioxidant activity of tocotrienols has been attributed to a number of mechanisms which include efficient interaction with free radical species, higher recycling efficiency of the chromanoxyl radical and uniform distribution of tocotrienols in membrane bilayers (Serbinova *et al.,* 1991; Theriault *et al.,* 1999).

It was concluded from studies done by Yoshidu (2003) and Sen (2000) who compared the antioxidants activities of tocopherols and tocotrienols, that tocotrienols were more effective due to the fact that they are absorbed/incorporated more effectively.

Non-antioxidant functions of vitamin E have been proposed, especially that of a “gene regulator”. These effects of vitamin E have been observed at the level of mRNA or protein and can be due to regulation of gene transcription, mRNA stability, protein translation, protein stability and post-translational events (Ricciarelli *et al.,* 2001; Azzi *et al.,* 2002).

Tocotrienols also possess cholesterol lowering effects together with the ability to reduce the atherogenic apolipoprotein B and lipoprotein (Aa serum levels (Hood, 1995). It has also been suggested that tocotrienols have anti-thrombotic and anti-tumourigenic effects. This indicates that tocotrienol may serve as an effective agent in the prevention and/or treatment of cardiovascular disease and cancer (Guthrie *et al.,* 1995; Qureshi *et al.,* 1997). Animal studies have shown that tocotrienols inhibit the enzyme HMG-CoA reductase and thus the synthesis of cholesterol (Parker *et al.,* 1993; Khor *et al.,* 1995). Tocotrienols can reduce the aggregation of blood platelets, and thus reducing the tendency for blood clot formation (Ong and Goh, 2002).

Hornstra (1988) showed that palm oil (containing tocopherols and tocotrienols) has anti-clotting effects, and the same antithrombotic effect as the highly unsaturated sunflower seed oil. Scientific evidence suggests that a palm oil containing diet either increases the production of prostacyclin (inhibits blood-clotting) or decreases formation of thromboxane (induces blood-clotting). These findings indicate that palm oil potentially has the same antithrombotic effect as polyunsaturated oils (Rand *et al.,* 1988; Abeywardena *et al.,* 1987; Abeywardena *et al.,* 1997).

Epidemiological studies also showed that high vitamin E intake is associated with a reduced risk of coronary heart disease. Vitamin E treatment significantly reduced the risk of cardiovascular deaths as well as non-fatal myocardial infarctions (Stephens *et al.,* 1996). Tocopherol and tocotrienol inhibits human platelets from coagulation, this in itself prevents thrombosis. In conjunction with this, tocotrienol supplementation, derived from palm oil, can reduce restenosis of patients with carotid atherosclerosis (Tomeo *et al.,* 1995; Kooyenga *et al.,* 1996).

2.7.4 Ubiquinones

Crude palm oil and RPO contains small amounts of ubiquinones. Of these, coenzyme Q_{10} (CoQ_{10}) is the most common. CoQ_{10} has been reported to be beneficially involved in several mechanisms: It can boost the immune system, relieve angina, afford protection against heart disease and reduce blood pressure at relatively low concentrations of CoQ_{10} intake (Nagendran *et al.,* 2000).

It is also known for its role in the mitochondrial electron-transport system and it also has antioxidant properties in the ischaemic/reperfused myocardium in rats (Hano *et al.,* 1994). CoQ_{10} might be a free radical scavenger and may preserve coronary vessel mechanical function during ischaemia or reperfusion through a direct antioxidant mechanism (Yokoyama *et al.,* 1996).

CoQ_{10} may also have a protective effect against breast cancer (Portakal *et al.,* 2000), and may even be used as a treatment for breast cancer (Lockwood *et al.,* 1994; Lockwood *et al.,* 1995). It is also protective against heart damage related to chemotherapy (Folkers, 1996).

2.8 The beneficial effects of RPO: a review of the evidence

2.8.1 Cancer and Vitamin A deficiency

It is well documented that a high dietary fat intake increases the risk of colon cancer (Slattery *et al.,* 1997; Dwivedi *et al.,* 2003; Nkondjock *et al.,* 2003). However, it has been proposed that dietary fat, depending on the source, quantity and fatty acid composition (saturated, monounsaturated or polyunsaturated) can reduce the incidence of colon cancer (Dwivedi *et al.,* 2003; Nkondjock *et al.,* 2003).

Nakayama *et al.* (1993) observed an increase in tumour size in rats that were fed a corn oil diet, but no increase was seen in those fed palm oil. Red palm oil and its bioactive components are, based on recent scientific evidence, displaying increased protection against many cancers (Manorama *et al*., 1993; Yu *et al.,* 1999; Boateng *et al*., 2006).

It was also found that RPO in the maternal diet improved the vitamin A status of lactating mothers and their infants (Lietz *et al.,* 2000). In another study, children were supplemented with RPO and/or retinol. The increase in serum retinol levels was similar in both groups after 2 months of supplementation (Manorama, *et al.,* 1996). Aykroyd & Wright (1937) gave vitamin A-deficient rabbits crude RPO and found that the oil cured the characteristic eye lesions of vitamin A deficiency in animals. This was followed with clinical trials that showed that RPO was an effective substitute for cod liver oil in the treatment of keratomalacia (Vitamin A deficiency).

Investigations performed in India have established that 5 to 8g of RPO given daily to children as a supplementary food can improve their vitamin A status within about six months (Manorama *et al.* 1996; Mahapatra *et al.,* 1997).

2.8.2 RPO and its effect on serum cholesterol

Palm oil is also beneficial as it does not promote the formation of plaques in the arteries. When high-cholesterol diets along with certain saturated fats (milk fat, tallow, and coconut oil) are fed to rabbits, quails, pigs and monkeys, atherolsclerosis can be induced. Honstra *et al*, (1987) tested the effects of palm oil on atherosclerosis. It was found that after 18 months of feeding, the rabbits that received palm oil and sunflower oil diets, had the lowest degree of atherosclerosis in comparison with fish oil, linseed oil, and olive oil. In a subsequent study by Honstra (1988), it was found that palm oil showed no difference in a number of atherosclerosis lesions when compared to cottonseed oil, hydrogenated cottonseed oil and an experimental fat blend. These oils were all found to be anti-atherosclerotic. Kritchevsky *et al.,* (1999) also showed that RPO reduces atherosclerosis in rabbits.

A desirable serum lipid profile is one that has low plasma total cholesterol (TC), triglycerides (TG) and low-density lipoprotein cholesterol (LDL-C) levels and high high-density lipoprotein cholesterol (HDL-C) levels (Matton & Grundy, 1985).

Human feeding studies performed before 1990, showed that palm oil diets resulted in a reduction in blood cholesterol values (Ahrens *et al.,* 1957; Keys *et al.,* 1965; Baudet *et al.,* 1984; Mattson & Grundy, 1985; Bonanome & Grundy, 1988).

Recent nutritional studies confirmed that the effects of palm oil on blood cholesterol and lipoprotein profiles are beneficial. These studies showed that palm olein (the liquid fraction of palm oil) had similar effects as olive oil on

lowering cholesterol levels (Ng *et al*., 1992; Choudhury *et al*., 1995). This was also shown by another study that indicated that a palm oil supplemented diet had the same effects as an oleic acid supplemented diet (oleic acid is the main fatty acid in olive oil) (Sundram, 1997). Palm oil induced an increase in the "good" HDL-cholesterol and a decrease in the "bad" LDL- cholesterol as well as the total blood cholesterol (Ng *et al*., 1991; Truswell *et al*., 1992; Farooq *et al*., 1996; Zhang *et al*., 1997; Chandrasekharan, 1999; Theriault *et al.,* 1999; Kritchevsky, 2000). Palm olein also did not raise cholesterol (Ghafoorunissa *et al.,* 1995).

The above evidence indicates that the effect of palm oil on blood lipid levels more closely resembles that of a monounsaturated oil rather than a saturated oil. These studies suggest that palm oil does not raise serum TC or LDL cholesterol levels to the extent expected from a fat that has such a high saturated fatty acid content.

2.8.3 RPO and ischaemia/reperfusion injury

During myocardial reperfusion, there is an increase in free oxygen radicals due to the sudden oxygen burst induced by reoxygenation of the heart. These radicals include: superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH), which are implicated in the pathogenesis of ischaemia/reperfusion injury. They interact with nucleic acids, proteins and lipids and thus cause damage to cell membranes or intracellular organelles (Serbinova *et al.,* 1992; Wall, 2000; Illarion *et al.,* 2002).

In a recent study by Esterhuyse *et al.* (2005), it was found that a RPO-supplemented diet caused an improved functional recovery during reperfusion in the working heart model. This recovery was associated with increased levels of cGMP and decreased levels of cAMP in the heart. The increased cGMP levels possibly protected the myocardium by decreasing reperfusion induced cytosolic Ca^{2+} overload. An increase in cAMP activity would increase Ca^{2+} and aggrevates ischaemia/reperfusion injury (Du Toit *et al.,* 2001). Thus, it seems that cGMP may be an endogenous intracellular cardioprotectant (Pabla *et al.,*

1995). An increase in functional recovery was also seen in a RPO-supplemented high fat diet group. This improvement could not be linked to changes in NO-cGMP pathway activity (Esterhuyse *et al.,* 2005). There must be another pathway that activates the beneficial effects of RPO in a high fat diet. It is proposed that both the antioxidants as well as the fatty acids play a role in the protection induced by RPO under these conditions.

RPO also protected against ischaemai/reperfusion injury in diets that are rich in SFAs and PUFAs. Together these results indicate that RPO was effective to protect against ischaemia/reperfusion in a normal rat chow diet (Esterhuyse *et al.,* 2005; Engelbrecht *et al.,* 2006), a high cholesterol diet (Esterhuyse *et al.,* 2006; Kruger *et al.,* 2007), an oxidative risk induced diet and a high fat diet (Bester *et al.,* 2006).

In other studies performed on RPO it was found that RPO-supplementation showed a significant increase in PKB/Akt phosphorylation (Engelbrecht *et al.,* 2005) and a significant inhibition of pro-apoptotic proteins (Kruger *et al.,*2007) during reperfusion. These changes were also associated with increased mechanical functional recovery of the heart. This indicated that the improved physiological function associated with RPO-supplementation (Esterhuyse *et al.,* 2005) was due to cellular signalling effects of RPO.

Although lipids have been an essential component of human diets, it seems that an important basic level of control of cardiac function by a simple dietary choice of RPO has been largely overlooked. Much work, especially carefully planned basic research to further explore the mechanisms as well as clinical trials are needed to test whether this natural oil will fulfil the need of a safe and effective therapeutic agent.

Chapter 3 MATERIAL AND METHODS

3.1 Animal Care

The animals used in this study were male, Wistar rats. All animals used in the study received humane care in accordance with the Principle of Laboratory Animal Care of the National Society of Medical Research and the Guide for the Care and use of Laboratory Animals of the National Academy of Sciences. (National Institutes of Health Publications no. 80-23, revised 1978). Rats had ad lib access to food and water. They were housed in an animal house at a constant temperature of 27^{o}C and were exposed to a twelve-hour artificial day-night cycle.

3.2 Experimental Model

3.2.1 Experimental Groups

Male Wistar rats were divided into four groups: two control groups receiving standard rat chow and two experimental groups receiving standard rat chow plus 2ml RPO for 4 weeks. Carotino Premium, supplied by Carotino, Malaysia, was used for the experimental group. The reason for using 2 ml RPO was that in previous studies (Esterhuyse *et al.,* 2005), 0.2 ml of a baking fat form of RPO was used. The Carotino Premium that we used was much less concentrated than the baking fat. Therefore, an increased dosage was used. The energy and macronutrient content of the two diets are shown in Table 3-1.

3.2.2 Heart perfusion

At the end of the feeding programme, rats weighing 300-400g were anaesthetized with an intraperitoneal injection of 2mg/kg intraval sodium (sodium pentobarbital), before the hearts were rapidly excised and placed in ice-cold Krebs-Henseleit buffer. Hearts were transferred to the Langendorff perfusion apparatus and perfused with a Krebs-Henseleit buffer equilibrated with 95% O_2

and 5% CO_2 at 37^{o}C (118,5 mM NaCl; 4,75 mM KCl; 1,2 mM MgCl 6 H_2O ; 1,36 mM $CaCl_2$; 25,0 mM $NaHCO_3$; 1,2 mM KH_2PO_4; 11,0 mM glucose). Pressure was kept constant at 100 cm H_2O with a flow pump.

Table 3-1: The energy and macronutrient content of the two diets used in this study

Nutrient	Standard rat chow *	RPO 2 ml	Standard rat chow+RPO**
Energy (kJ)	272,5	68	**340.5**
Total non structural carbohydrates (g)	8,375	0	**8,375**
Total Protein (g)	4,5	0	**4,5**
Total Fat (g)	0,625	1.84	**2.465**
Total SFA (g)	0,139	0.74	**0.879**
Total MUFA (g)	0,168	0.86	**1.028**
Total PUFA (g)	0,297	0.24	**0.537**

***25 g of standard rat chow per day**

****25 g of standard rat chow plus 2ml red palm oil per day**

SFA = saturated fatty acids

MUFA = monounsaturated fatty acids

PUFA = polyunsaturated fatty acids

The aorta was cannulated and retrograde perfusion with Krebs-Henseleit buffer was initiated. Immediately after cannulation, excess tissue was removed from the heart and the left atrium was removed. A thermomix waterbath was used to keep the Langendorff perfusion system's temperature constant

throughout the experiment by circulating warm water through the water-jacketed glass reservoir. The temperature was monitered at regular intervals to ensure that the temperature was maintained at 37^{o}C irrespective of coronary flow. A balloon, made from transparent sandwich wrap film and filled with water, was inserted into the left ventricle through the opening of the left atrium. The balloon is connected to a Powerlab system (ADInstruments Pty Ltd. Castle Hill, Australia) on a computer. After insertion, the balloon is inflated to 2 mmHg, and the contraction force of the heart against the balloon causes water displacement that is converted to pressure. Through this the systolic and diastolic pressures as well as the heart rate were read on the computer. The first 10 min of perfusion was used to stabilize the heart.

3.2.3 Exclusion criteria:

Rats with body mass outside of the range of 300-400 g were not used for experiments. Hearts with a heart rate above 350 or below 210 beats per min and LVDevP above 150 or below 70 mmHg during the stabilization phase were also excluded from the experiment.

3.3 Perfusion protocol

The study was divided into two perfusion protocols (Figure 3-1). In the first protocol, hearts were perfused with Krebs-Henseleit buffer for 10 min stabilization, followed by 20 min, during which mechanical function was documented. Hearts were then subjected to 25 min of total global ischaemia. After the ischaemic period, hearts were reperfused for 30 min, and mechanical function was again documented. In order to reduce the incidence of reperfusion arrhythmias, 2% lignocaine solution was used for the last min of pre-ischaemia perfusion as well as the initial 3 min of reperfusion of all hearts.

Functional and biochemical measurements were taken. Mechanical functional parameters measured during pre-ischaemia and during reperfusion at

timepoints 5 min, 10 min, 15 min and 25 min. Biochemical measurements were only done at 10 min reperfusion.

In the second protocol, hearts were stabilized for 10 min and perfused for 15 min with normal Krebs-Henseleit buffer, before being subjected to a wortmannin solution (100 nM) for 5 min pre-ischaemia. After the 25 min total global ischaemia period, hearts were reperfused for 3 min with the wortmannin solution, before reverting to the drug-free Krebs-Henseleit buffer for the rest of the 27 min reperfusion period. A set of functional and biochemical measurement were again taken.

3.4 Mechanical function parameters measured:

Functional measurements were taken during pre-ischaemia (20 min perfusion) and at 5 min, 10 min, 15 min and 25 min into reperfusion. All the results are given as reperfusion mechanical function percentage recovery. This was determined by dividing the value obtained during reperfusion by the pre-ischaemic value and multiplying it by 100.

3.4.1 Coronary flow (ml/min)

The coronary flow was measured by collecting the coronary effluent from each sampling point for 1min, in a measuring cylinder.

3.4.2 Heart rate (bpm)

The heart rate was determined by the Powerlab system that was connected to the balloon that was inserted into the left ventricle of the heart.

3.4.3 Left Ventricular developed pressure (LVDevP) (mmHg)

Left ventricular systolic (LVSP) and diastolic (LVDP) pressure were measured by the Powerlab system by means of the generation of pressure on the balloon inserted into the left verntricle of the heart. LVDevP (the difference

between systolic and diastolic pressure) was calculated and used as a measure of mechanical function of the heart.

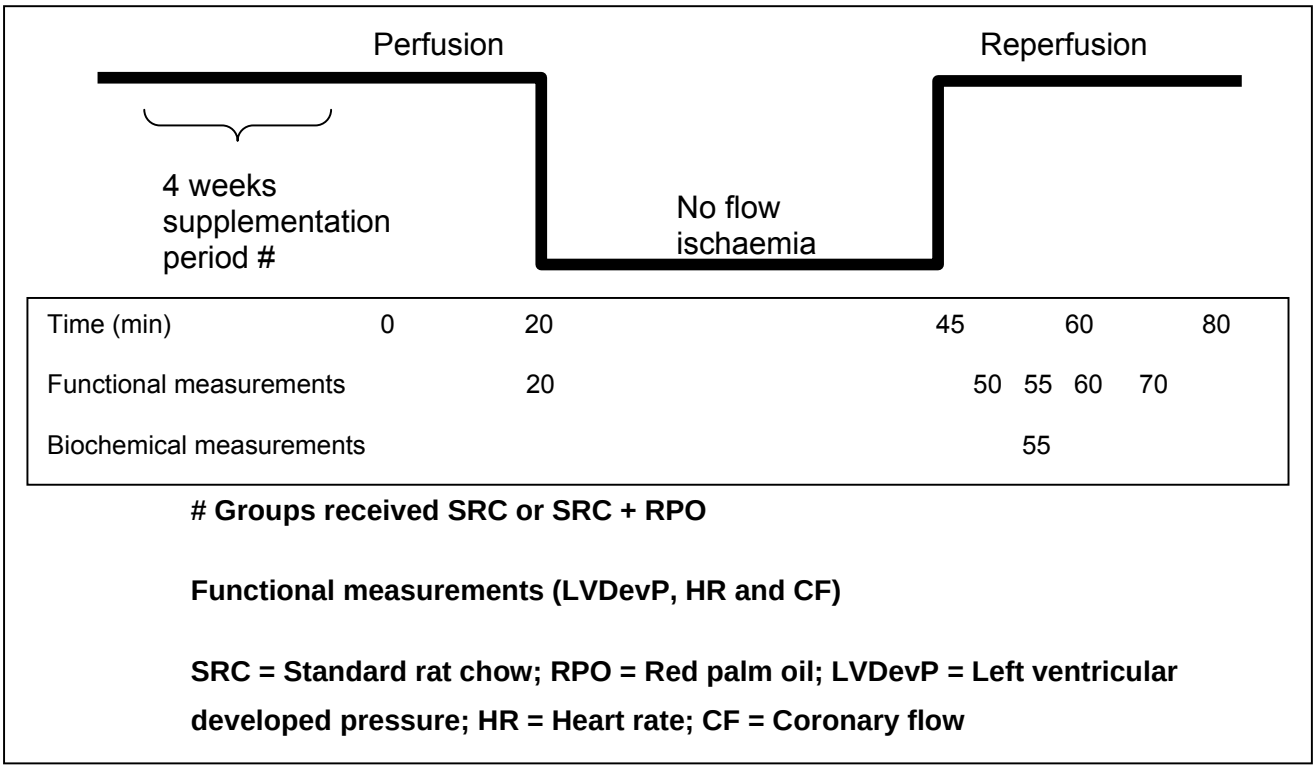

Figure 3-1: Study design

3.4.4 Rate Pressure Product (RPP)

RPP was also used to assess mechanical function of the heart. RPP was determined using the following formula:

RPP = heart rate (HR) x LVDevP

3.5 Biochemical analysis

To assess myocardial biochemical function, hearts, from all the groups, were freeze clamped 10 min into reperfusion with Wollenberger clamps precooled in liquid nitrogen. Samples were then stored at -80^{o}C until analysis were performed.

Cardiac PKB/Akt, caspase and poly-ADP-ribose-polymerase (PARP) protein were extracted with a lysis buffer containing (in mM): Tris 20, p-nitrophenylphosphate 20, EGTA 1, NaF 50, sodium orthovanadate 0.1, phenylmethyl sulfonyl fluoride (PMSF) 1m dithiothreitol (DTT) 1, aprotinin 10 μg/ml. The tissue lysates were diluted in Laemmli sample buffer, boiled for 5 min and 60 μg protein were separated by 10% PAGE-SDS gel electrophoresis (Caspase was 15%). The lysate protein content was determined using the Bradford technique (Bradford, 1976). The separated proteins were transferred to a PVDF membrane (Immobilon P, Millipore). These membranes were routinely stained with Ponceau Red for visualization of proteins. Nonspecific binding sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline – 0.1% Tween 20 (TBST) and then incubated with the primary antibodies that recognize PKB/Akt (Ser^{473} and Thr^{308}) and total PKB/Akt, PI3-K (p85), PDK1 (Ser^{241}), FKHR (Ser^{256}), GSK-3β (Ser^{9}), cleaved caspase-3 (Asp^{175}), cleaved PARP (Asp^{214}) and PTEN (Ser^{380}). Membranes were subsequently washed with large volumes of TBST (5x5 minutes) and the immobilized antibody conjugated with a diluted horseradish peroxidase-labeled secondary antibody (Amersham, LIFE SCIENCE). After thorough washing with TBST, membranes were covered with ECL detection reagents and quickly exposed to an autoradiography film (Hyperfilm ECL, RPN 2103) to detect light emission through a non-radioactive method (ECL Western blotting). Films were densitometrically analyzed (UN-SCAN-IT, Silkscience) and phosphorylated protein values were corrected for minor differences in protein loading, if required.

Antibodies were purchased from Cell Signalling Technology and all other chemicals were obtained from Sigma (St Louis, MO).

3.6 Statistics

Values are expressed as mean ± standard error of the mean (SEM). Some functional values are presented as percentage change from the baseline values. Results were compared by using a one-way ANOVA with a Bonferoni Multiple Comparison as a post hoc test. $P<0.05$ was considered as statistically significant.

Chapter 4 RESULTS

4.1 Mechanical Function data

4.1.1 Coronary flow recovery (%)

The coronary flow recoveries in all groups were similar with no significant differences (Figure 4-1).

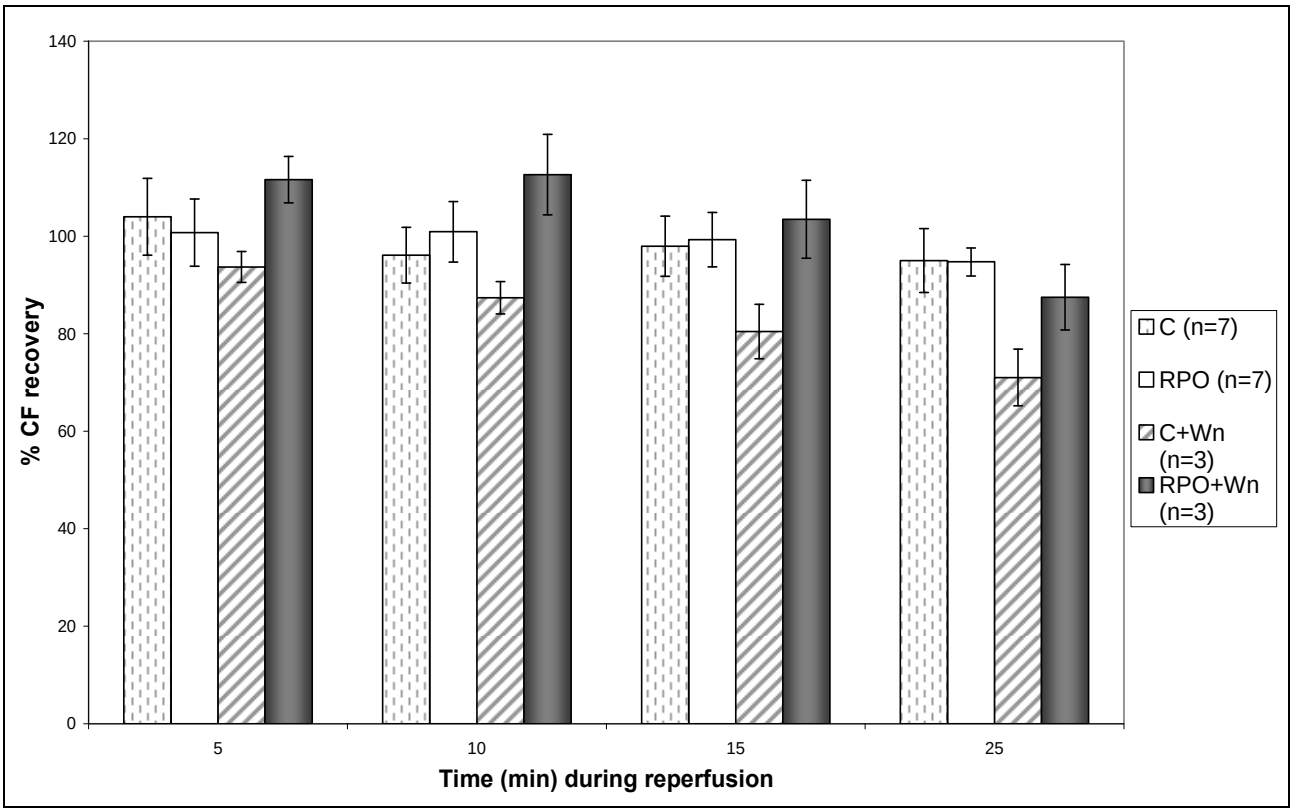

Figure 4-1: The effect of RPO-supplementation and Wortmannin administration on CF recovery during reperfusion (mean ± SEM)

CF = Coronary Flow; C = Control; RPO = Red Palm Oil; Wn = Wortmannin

4.1.2 Heart rate recovery (%)

The percentage heart rate recovery after ischaemia is shown in Figure 4-2. No differences between the groups were observed.

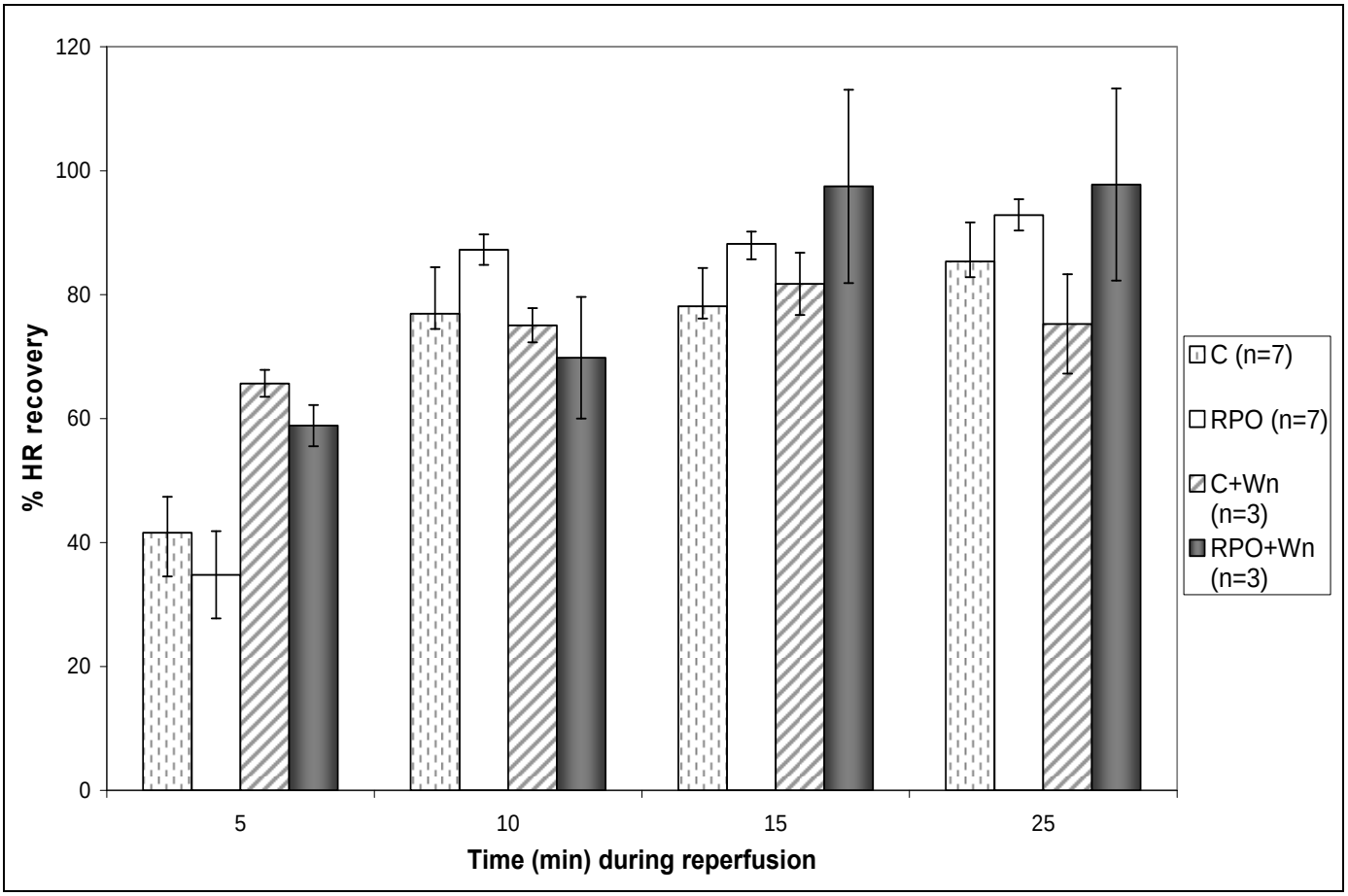

Figure 4-2: The effect of RPO-supplementation and Wortmannin administration on HR recovery during reperfusion (n=4-7 per group) (mean +/- SEM)

HR = Heart rate; C = Control; RPO = Red Palm Oil; Wn = Wortmannin

4.1.3 Left Ventricular developed pressure (LVDevP) recovery (%)

Our results show significant differences between groups in the percentage LVDevP recovery (Figure 4-3). After 5 min reperfusion, the RPO+Wn group had an increased % LVDevP recovery *vs* the control+Wn group (186.98 ± 5.21 % *vs* 81.55 ± 9.00 %; $p<0.01$). The RPO+Wn group also showed an increased LVDevP recovery after 15 min *vs* the RPO group (151.45 ± 12.02 % *vs* 107.45 ± 6.14 %; $p<0.05$). This implies that the RPO+Wn combination increased contractile functional recovery.

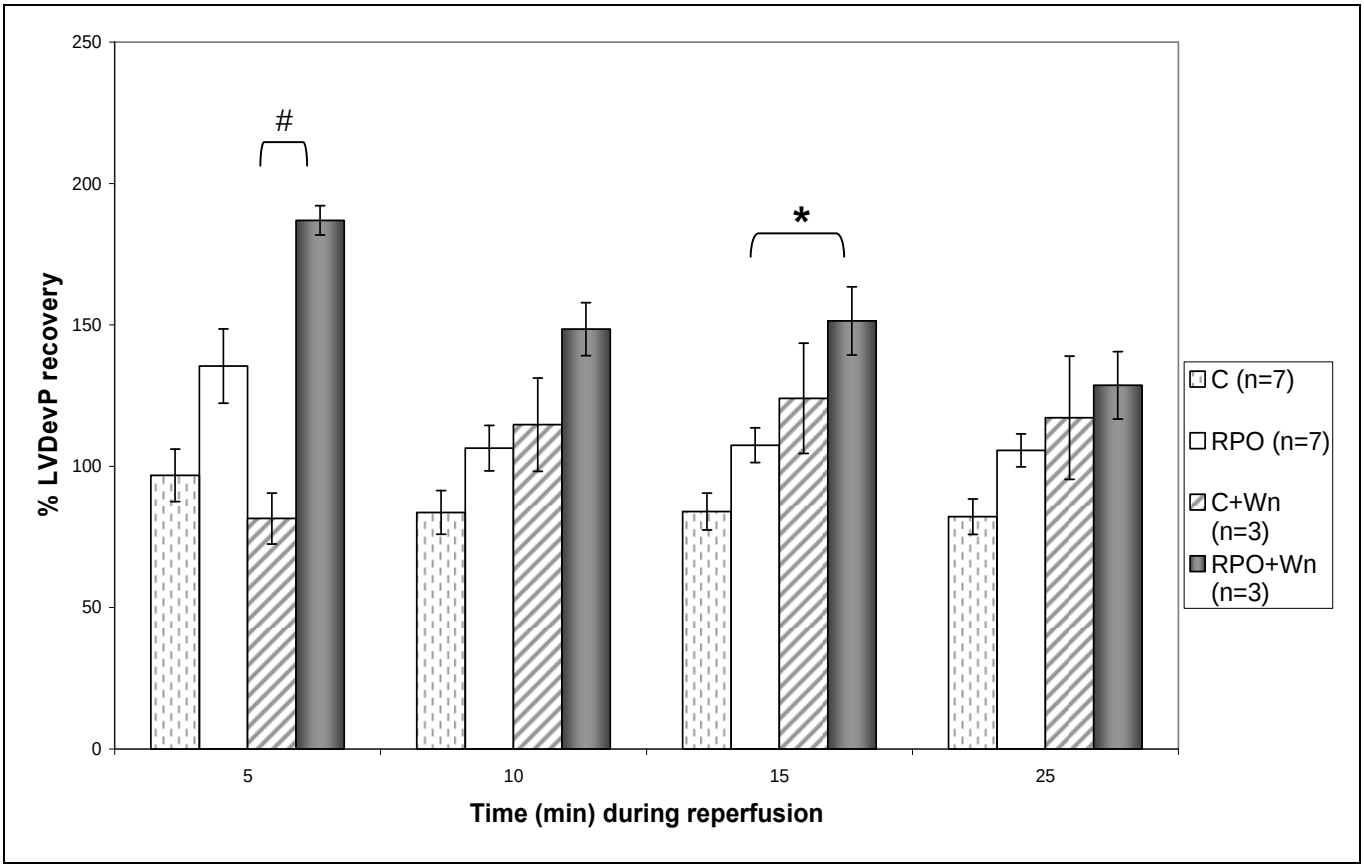

Figure 4-3: Percentage LVDevP recovery seen with RPO-supplementation and Wortmannin administration during reperfusion (n=4-8 per group) (mean +/-SEM) (*p<0.05 and # p< 0.01 for indicated groups).

LVDevP = Left Ventricular Developed Pressure; C = Control; RPO = Red Palm Oil; Wn = Wortmannin

4.1.4 Rate Pressure Product recovery (%)

RPO-supplementation caused an increase in % RPP recovery at 10 min during reperfusion when compared with the control group (63.86 ± 7.74 % in control *vs* 92.26 ± 5.89 % in RPO; $p<0.05$) confirming results in previous similar studies (Figure 4-4). Secondly, RPO+Wn also improved % RPP at timepoints 5 & 15 min (110.07 ± 7.00 % & 151.21 ± 36.67 %; $p<0.01$) *vs* control+Wn (42.52 ± 8.10 % & 81.69 ± 7.65 %; $p<0.05$). Our results also show that RPO+Wn improved % RPP at 5 min when compared with RPO alone (110.07 ± 7.00 % *vs* 43.07 ± 5.76 %; $p<0.01$).

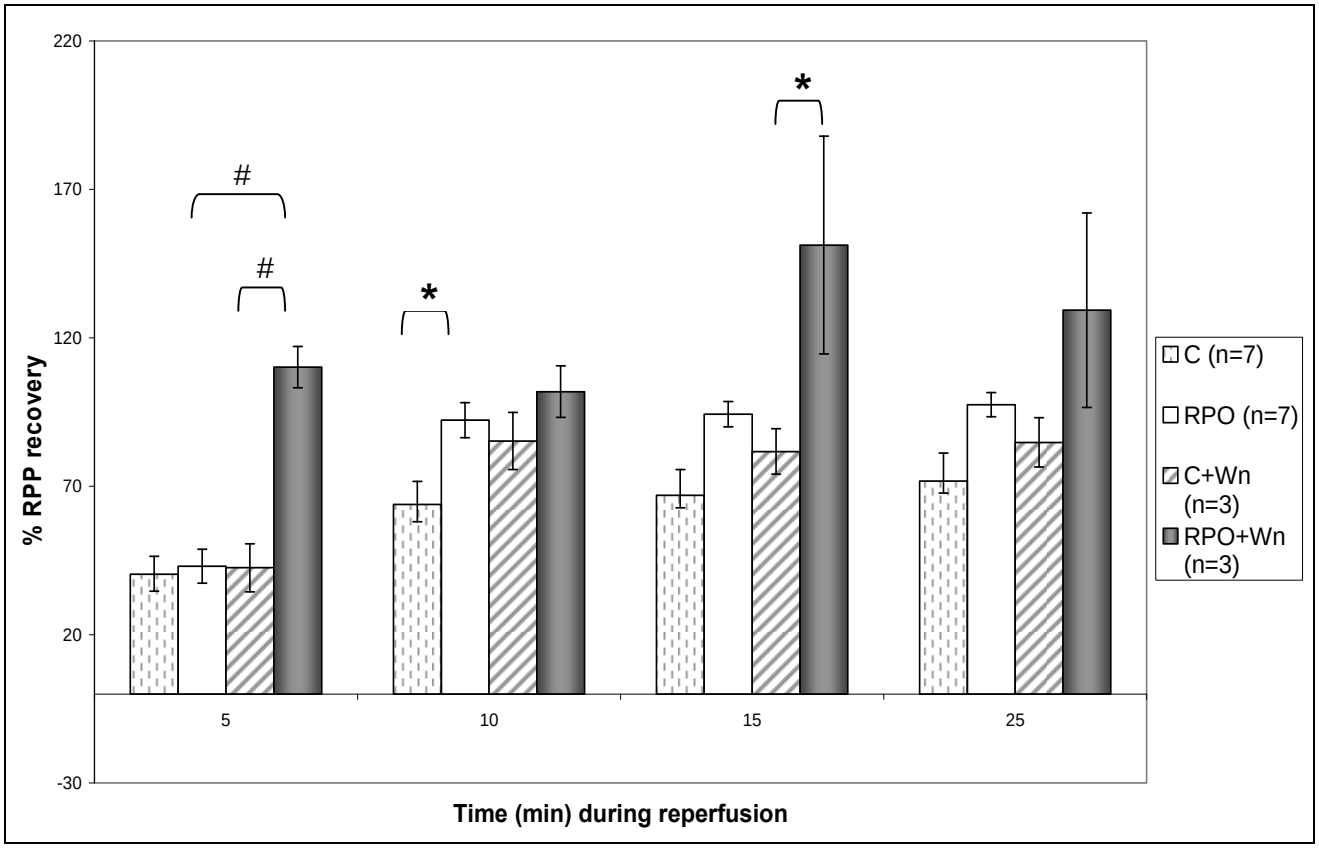

Figure 4-4: The effect of RPO-supplementation and Wortmannin administration on RPP recovery during reperfusion (n=4-8 per group) (mean +/- SEM), (* p<0.05 and # p<0.01 for indicated groups)

RPP = Rate Pressure Product; C = Control; RPO = Red Palm Oil; Wn = Wortmannin

Biochemical data

4.1.5 PI3-Kinase (p85)

The PI3-kinase (p85) activity is shown in Figure 4-5. RPO+Wn significantly decreased PI3-K (p85) activity compared to RPO alone (37.9 ± 3.3 pixels *vs* 54.5 ± 1.0 pixels; $p<0.001$).

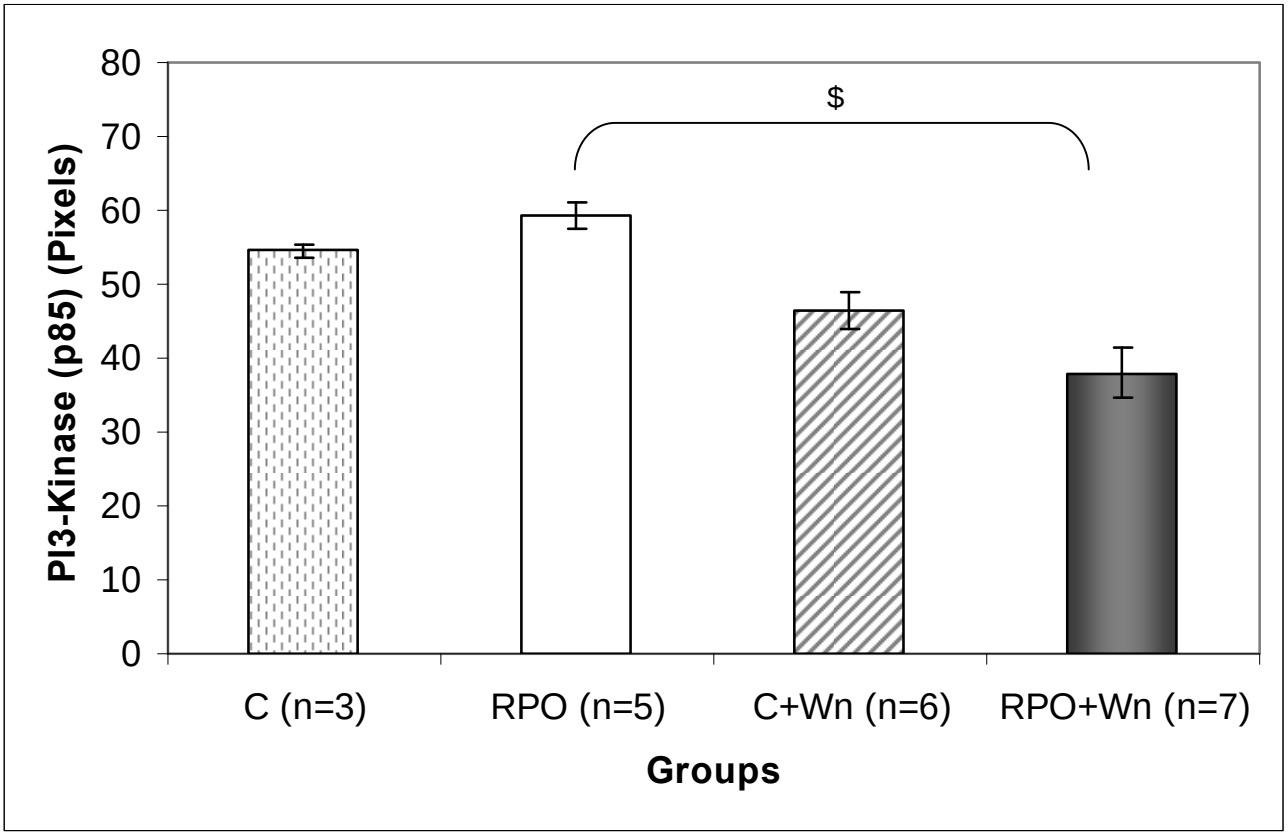

Figure 4-5: The effect of RPO-supplementation and Wortmannin administration on PI3-K (p85) activity in hearts subjected to ischaemia/reperfusion (n=3-7 per group) (mean +/- SEM), ($ p<0.001 for indicated groups)

C = Control; RPO = Red Palm Oil; Wn = Wortmannin

4.1.6 PTEN (Ser^{380})

PTEN activity (Figure 4-6) was increased in the control+Wn group when compared to the control group (72.4 ± 2.7 pixels *vs* 52.3 ± 2.8 pixels; $p<0.01$). Wortmannin administration during perfusion showed a decreased PTEN activity for the RPO+Wn group when compared with the control+Wn group (41.2 ± 3.5 pixels *vs* 72.4 ± 2.7 pixels; $p<0.001$). Wn increased the phosphorylation of PTEN, an effect which was prevented by RPO.

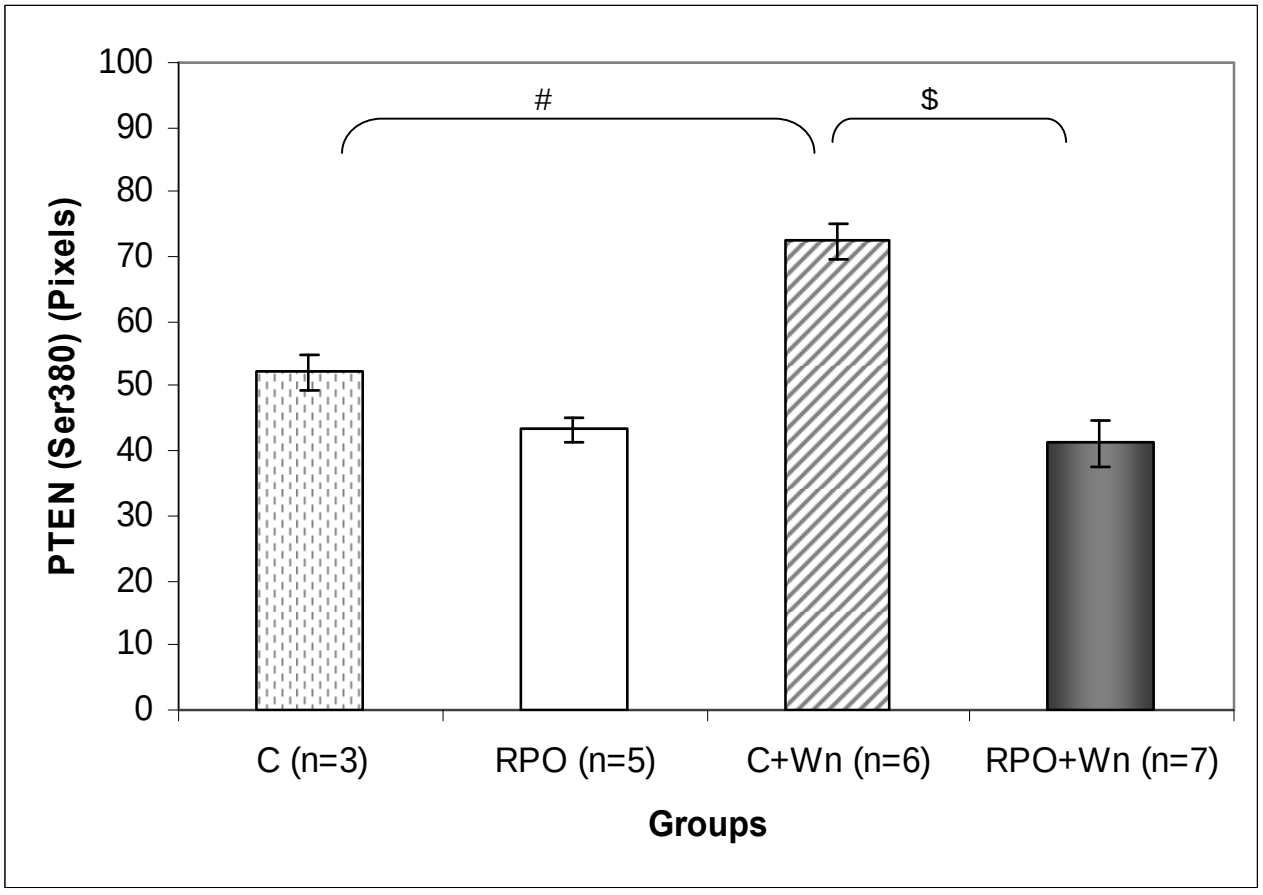

Figure 4-6: The PTEN (Ser) activity after RPO-supplementation and Wortmannin administration in hearts subjected to ischaemia/reperfusion (n=3-7 per group) (mean ± SEM) (# p<0.01 and $ p<0.001 for indicated groups)

C = Control; RPO = Red Palm Oil; Wn = Wortmannin

4.1.7 PDK (Ser^{241})

PDK phosphorylation in all four groups were similar after 10 min of reperfusion (Figure 4-7).

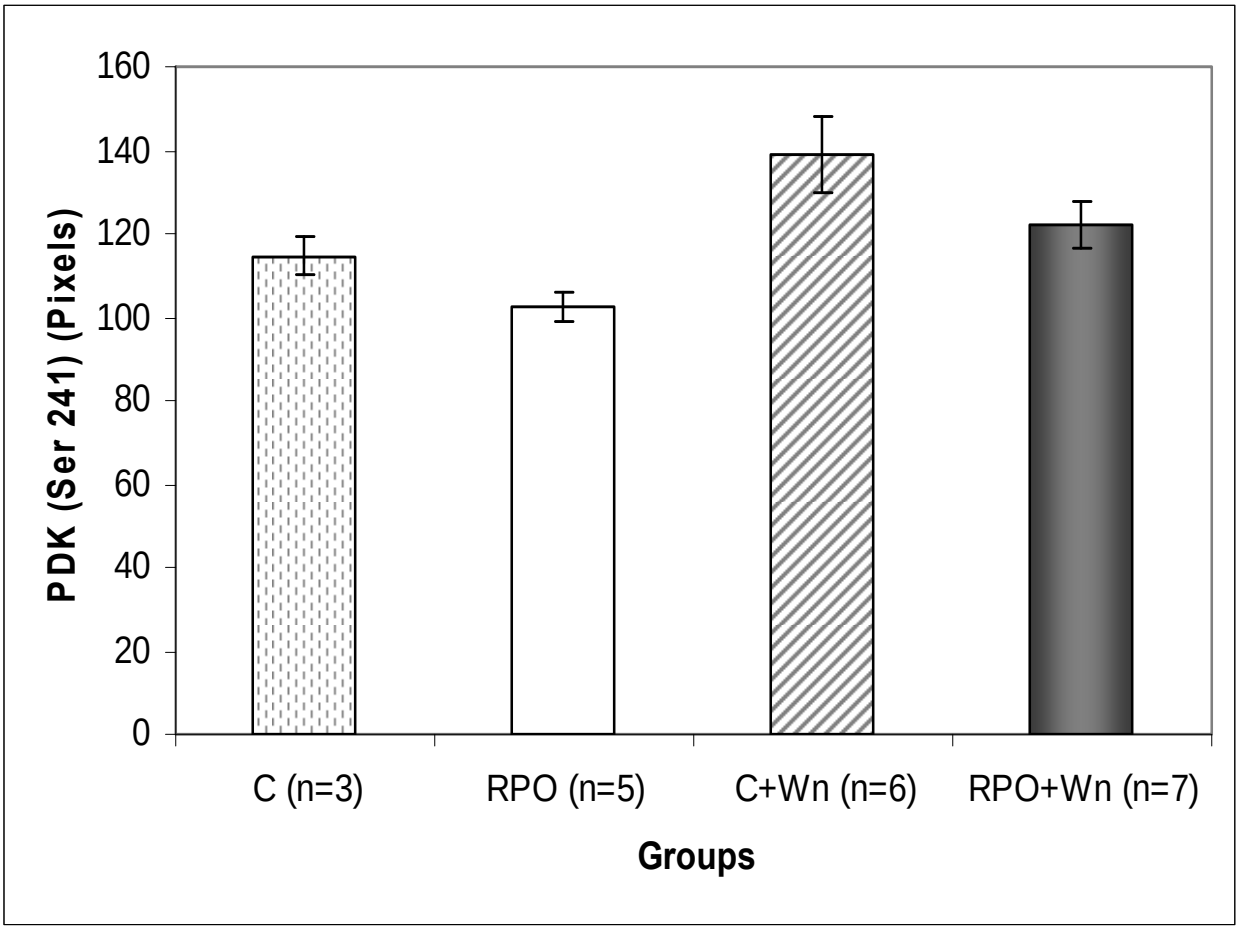

Figure 4-7: The effect of RPO-supplementation and Wortmannin administration on PDK activity in hearts subjected to ischaemia/reperfusion (n=3-7 per group)

C = Control; RPO = Red Palm Oil; Wn = Wortmannin

4.1.8 PKB/Akt (Ser^{473})

Wortmannin administration during perfusion decreased PKB/Akt (Ser^{473}) phosphorylation in the control group (33.2 ± 10.7 pixels *vs* 106.4 ± 7.8 pixels; $p<0.01$). This decrease in phosphorylation was reversed with the addition of RPO to wortmannin (33.2 ± 10.7 pixels *vs* 75.87 ± 10.3 pixels; $p<0.05$) (Figure 4-8).

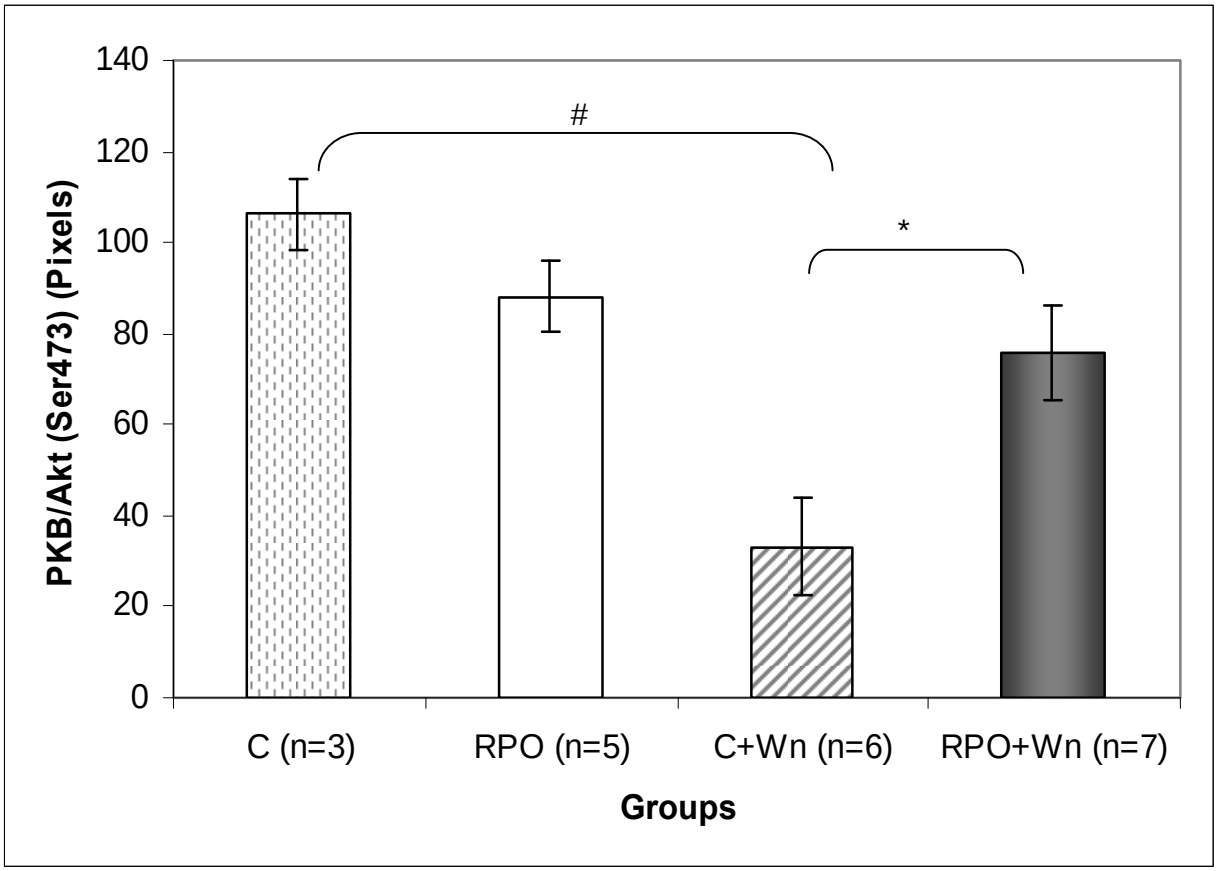

Figure 4-8: The effect of RPO-supplementation and Wortmannin administration on PKB/Akt (Ser^{473}) phosphorylation in hearts subjected to ischaemia/reperfusion (n=3-7 per group) (mean ± SEM) (* p<0.05 and # p<0.01 for indicated groups)

C = Control; RPO = Red Palm Oil; Wn = Wortmannin

4.1.9 PKB/Akt (Thr^{308})

No differences in phosphorylation between groups were found in PKB/Akt (Thr^{308}) (Figure 4-9).

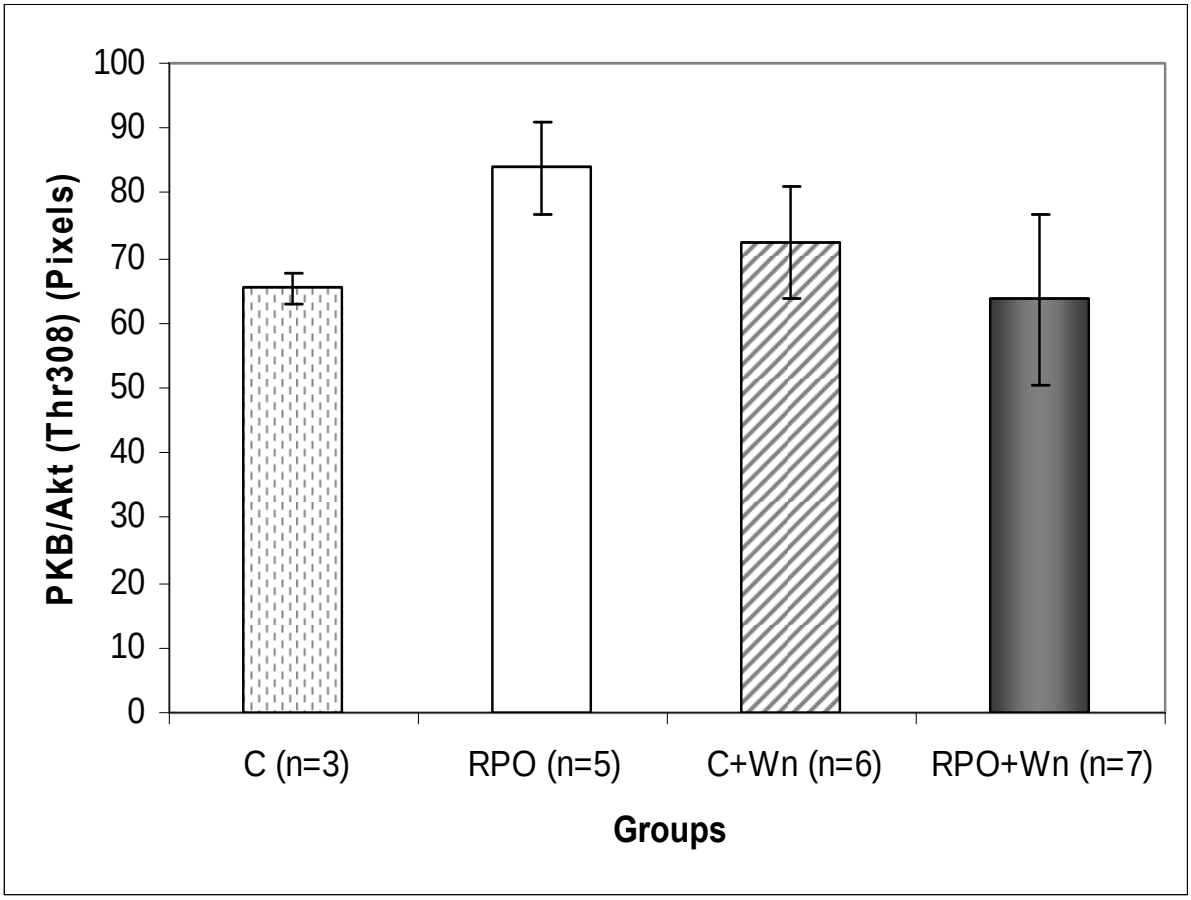

Figure 4-9: The effect of RPO-supplementation and Wortmannin administration on PKB/Akt (Thr^{308}) phosphorylation in hearts subjected to ischaemia/reperfusion (n=3-7 per group) (mean ± SEM)

C = Control; RPO = Red Palm Oil; Wn = Wortmannin

4.1.10 PKB/Akt (Tot)

The PKB/Akt (Tot) activity is given in Figure 4-10, no differences were found between the groups.

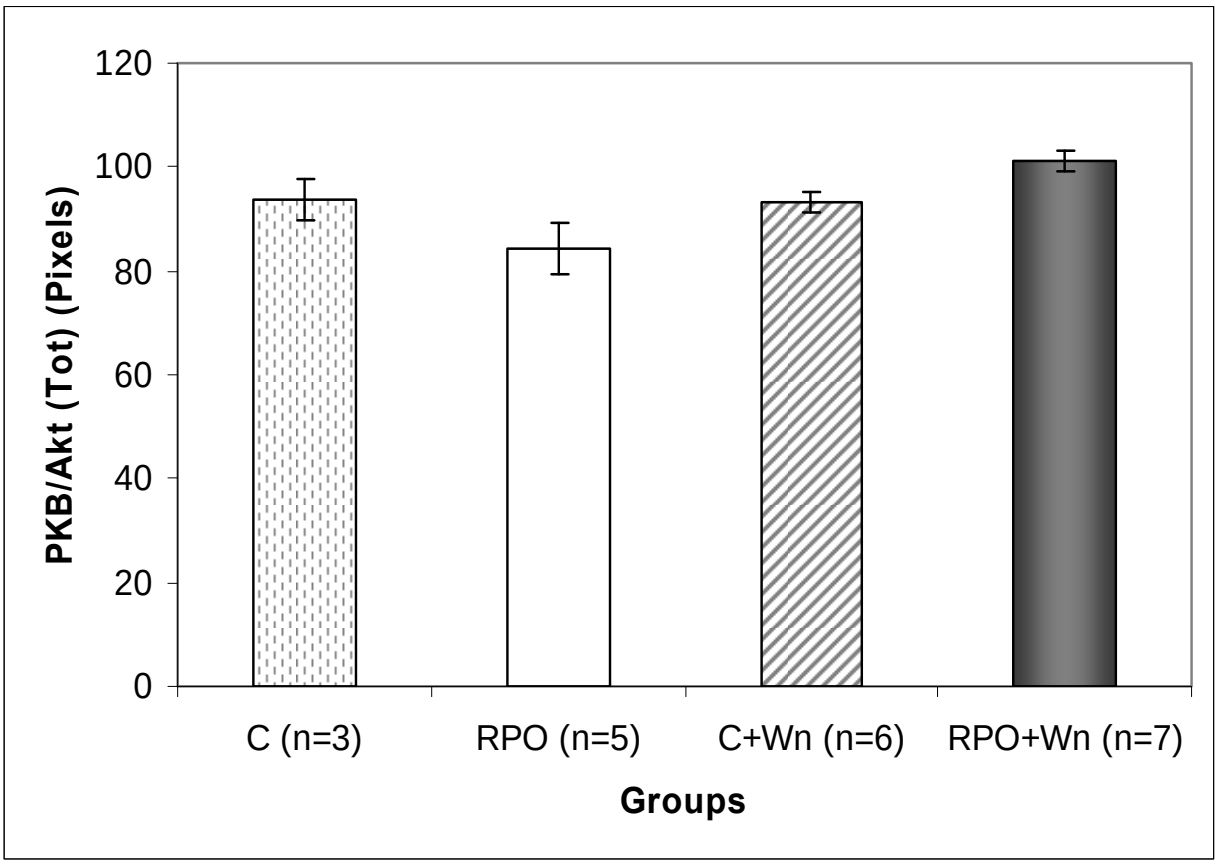

Figure 4-10: The effect of RPO-supplementation and Wortmannin administration on the total PKB/Akt activity in hearts subjected to ischaemia/reperfusion (n=3-7 per group)

C = Control; RPO = Red Palm Oil; Wn = Wortmannin

4.1.11 GSK-3β (Ser^9)

GSK phosphorylation after 10 min of reperfusion is demonstrated in Figure 4-11. An increase in GSK phosphorylation occurred in the RPO+Wn group when compared to the RPO group (115.6 ± 1.3 pixels *vs* 95.24 ± 4.5 pixels; $p<0.01$).

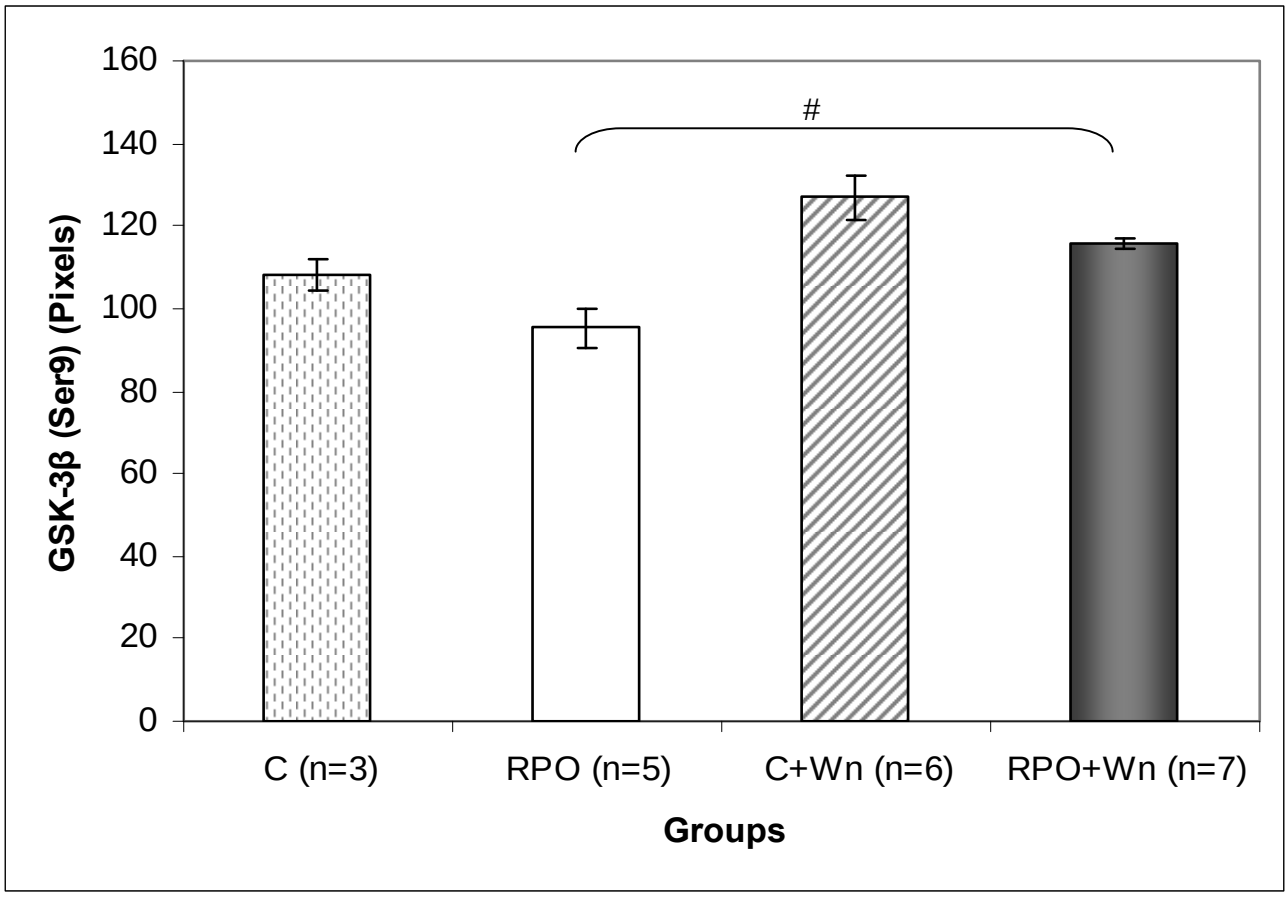

Figure 4-11: The effect of RPO-supplementation and Wortmannin administration on GSK phosphorylation in hearts subjected to ischaemia/reperfusion (n=3-7 per group) (mean ± SEM) (# p<0.01 for indicated groups)

C = Control; RPO = Red Palm Oil; Wn = Wortmannin

4.1.12 FKHR (Ser^{256})

There was a significant decrease in FKHR phosphorylation in the RPO+Wn group when compared with the RPO group (73.00 ± 14.1 pixels *vs* 130.9 ± 3.5 pixels; $p<0.05$) (Figure 4-12). The phosphorylated form of FKHR is exported from the nucleus, implying that no FKHR proteins can be transcribed. RPO caused an increase in the phosphorylation of FKHR, whereas wortmannin decreased it.

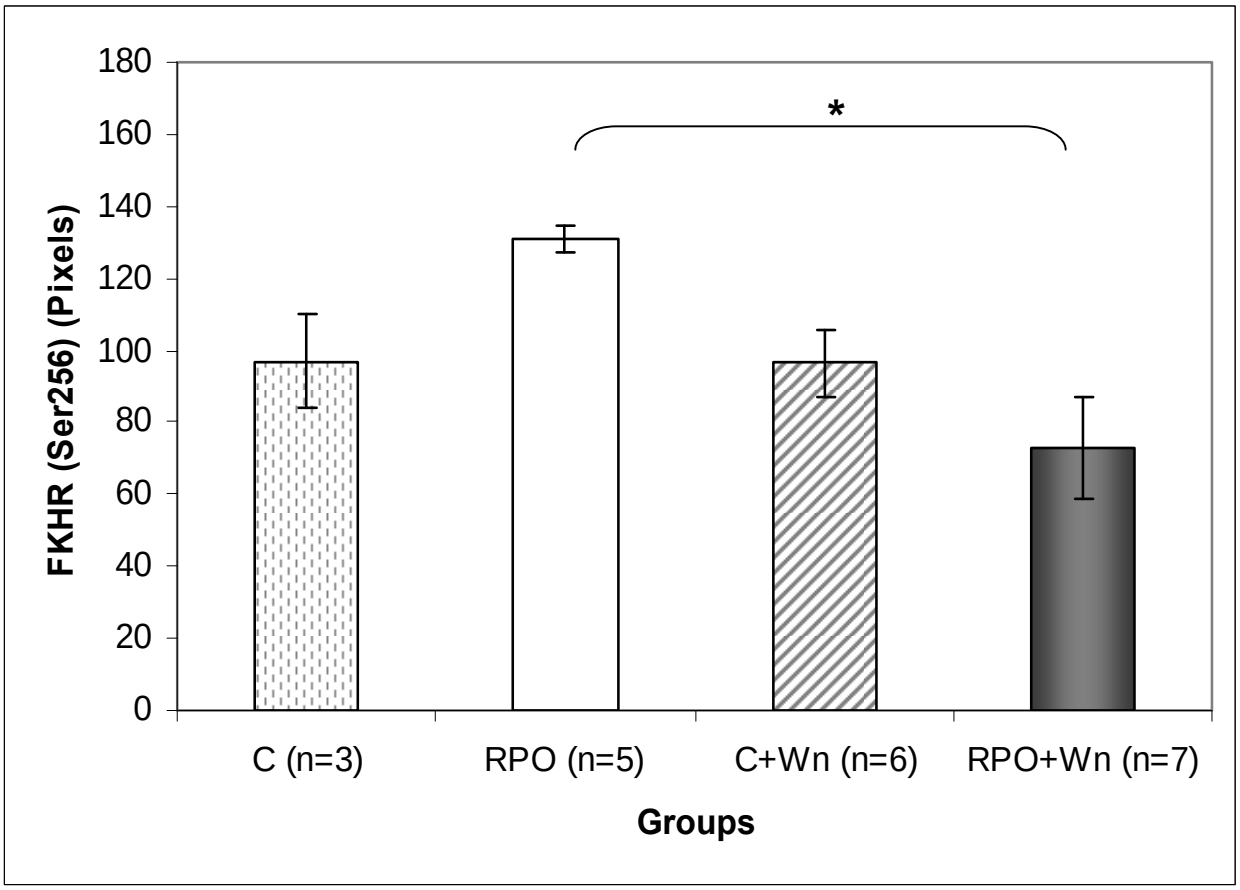

Figure 4-12: The effect exerted by RPO-supplementation and Wortmannin administration on FKHR phosphorylation in hearts subjected to ischaemia/reperfusion (n=3-7 per group) (mean ± SEM) (*p<0.05 for indicated groups)

C = Control; RPO = Red Palm Oil; Wn = Wortmannin

Cleaved Caspase-3 (Asp^{175})

Wortmannin significantly increased caspase-3 cleavage compared with the control group (56.27 ± 3.63 pixels *vs* 38.21 ± 4.14 pixels; $p<0.05$). Furthermore, wortmannin also caused a significant increase in caspase-3 cleavage in the RPO group compared with the RPO control group (59.79 ± 2.1 pixels *vs* 26.75 ± 2.6 pixels; $p<0.001$).

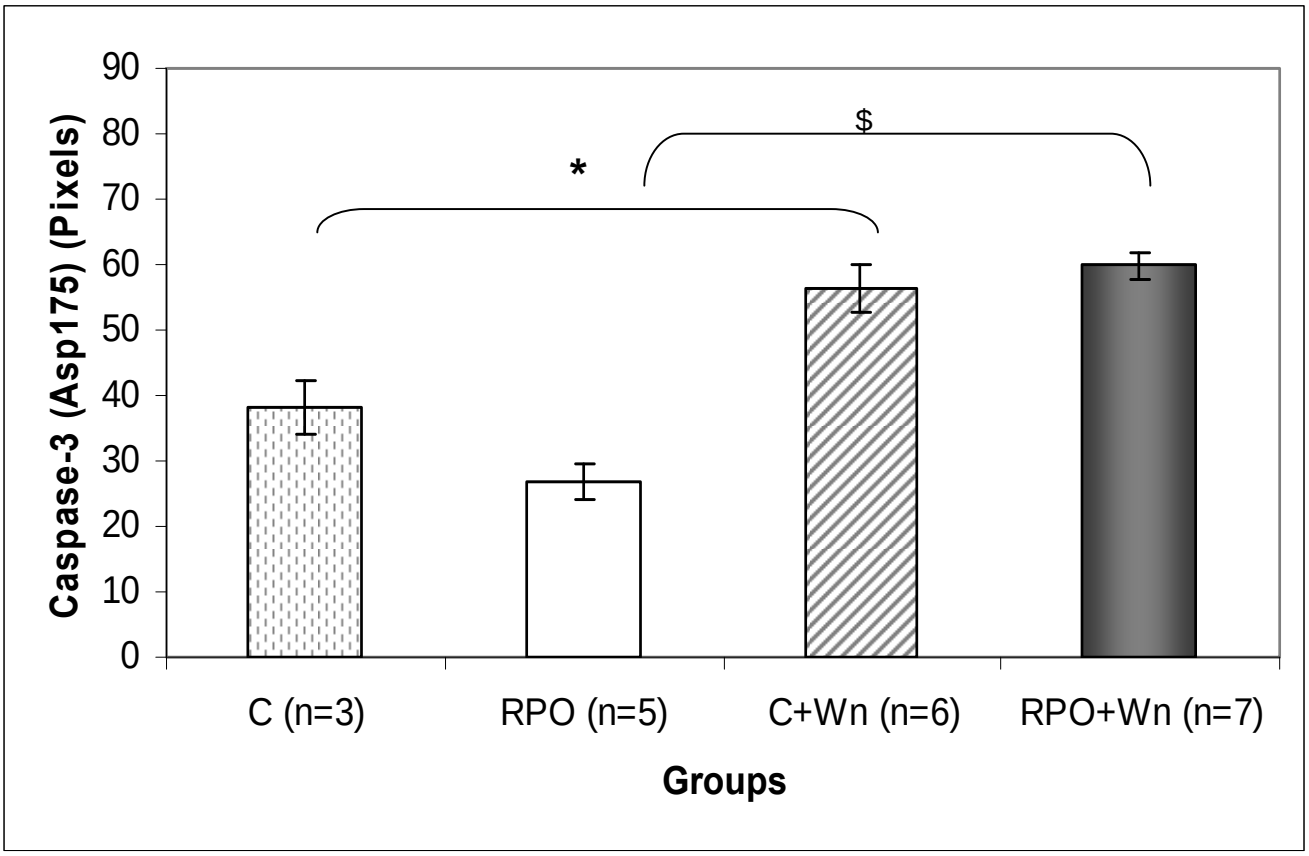

Figure 4-13: The effect exerted by RPO-supplementation and Wn administration on the cleavage of caspase-3 in hearts subjected to ischaemia/reperfusion (n=3-7 per group) (mean ± SEM) (* $p<0.05$ and $ $p<0.001$ for indicated groups)

C = Control; RPO = Red Palm Oil; Wn = Wortmannin

4.1.13 Cleaved PARP (Asp^{214})

There was an increase in cleaved PARP Figure 4-14 in the control+Wn group when compared to the control group (98.9 ± 9.3 pixels *vs* 42.12 ± 7.0 pixels; $p<0.01$). PARP cleavage was significantly reduced in the RPO+Wn group compared with the control wortmannin group (98.9 ± 9.3 *vs* 50.23 ± 6.9 pixels; $p<0.001$). Wortmannin increased the cleavage of PARP while RPO inhibited the cleavage.

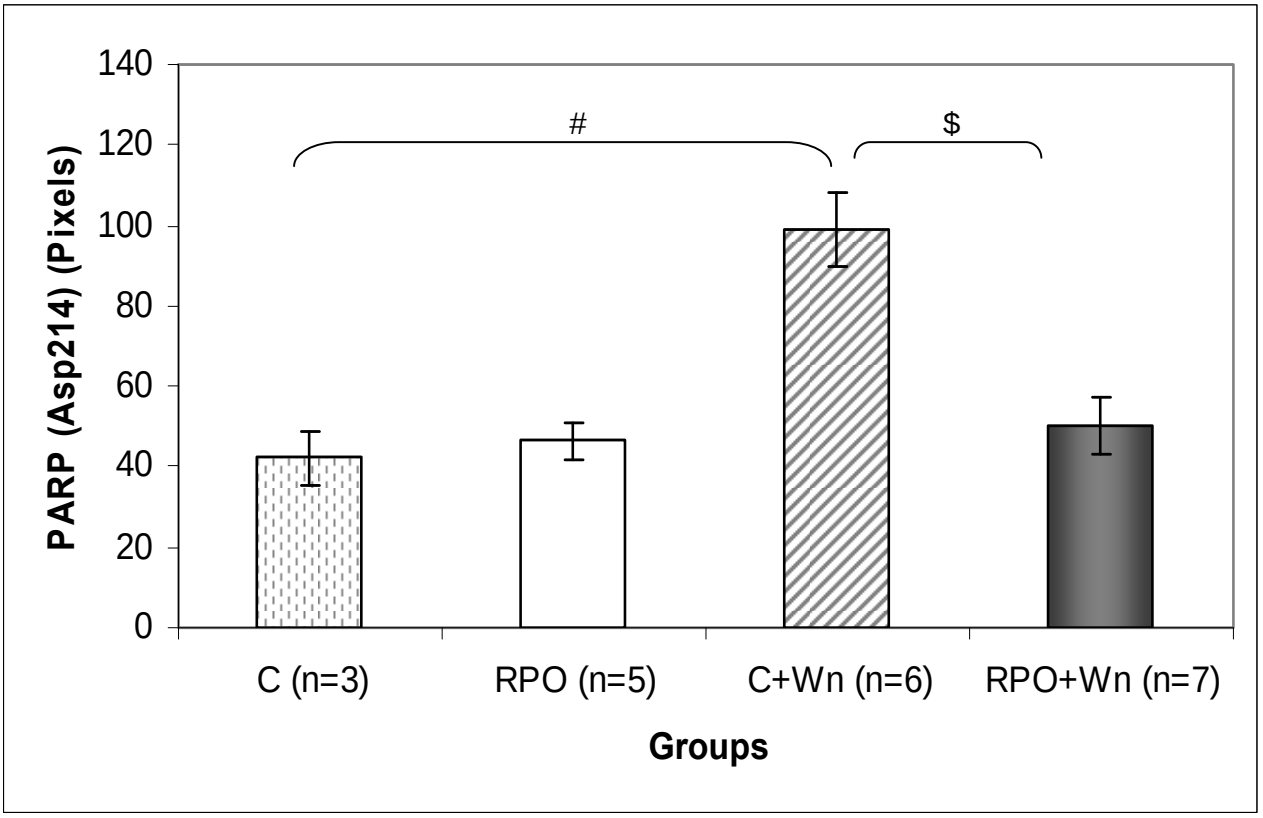

Figure 4-14: The cleavage of PARP in hearts subjected to ischaemia/reperfusion that received RPO-supplementation and Wortmannin administration (mean ± SEM) (n=3-7 per group) (# p<0.01 and $ p<0.001 for indicated groups)

C = Control; RPO = Red Palm Oil; Wn = Wortmannin

Chapter 5 DISCUSSION

Cardiovascular disease is a major cause of death in the modern world (Ho *et al.,* 1993). Although previous studies consistently showed an inverse relationship between RPO consumption and ischaemia/reperfusion injury, the mechanism of the beneficial action of RPO remains to be elucidated.

Biochemical evidence from previous studies suggests that PKB/Akt phosphorylation might induce an underlying protective mechanism (Esterhuyse *et al.,* 2005; Bester *et al.,* 2006; Engelbrecht *et al.,* 2006; Kruger *et al.,* 2007). However, the exact mechanism has not been elucidated. Therefore, the aim of this study was to determine which up and downstream signalling peptides associated with the PKB/Akt pathway are possibly involved.

5.1 Effect of RPO and Wortmannin on post-ischaemic functional recovery

RPO-supplementation did not induce a better recovery in CF, HR and LVDevP during reperfusion. The CF flow parameter was used to determine if there was a difference in bloodflow to the heart during RPO-supplementation. It was determined in this study that RPO-supplementation did not increase the bloodflow to the heart.

HR and LVDevP are used to determine the function of the heart. However, to measure the mechanical function of the heart, it must combine the speed of contraction as well as the strength of contraction. To measure this, RPP was measured, which is a scientifically excepted term for mechanical function. Individually, HR and LVDevP did not show significant differences in recoveries during reperfusion. However, when combined, the measurement of RPP showed significant differences in recoveries during reperfusion.

It was demonstrated in the current study that RPO-supplementation offered significant protection against ischaemia/reperfusion induced injury in the Langendorff-perfused rat heart model, during 10 min into reperfusion. This can be concluded from the increased percentage RPP recovery in the RPO-supplemented hearts. After 10 min, the significance of the protection was lost. This protection is in agreement with previous studies that showed dietary RPO-supplementation led to improved functional recovery after ischaemia in normal and hypercholesteroleamic diets (Esterhuyse *et al.,* 2005; Engelbrecht *et al.,* 2006; Esterhuyse *et al.,* 2006; Kruger *et al.,* 2007; Bester *et al.,* 2006).

A better functional recovery was not seen in the CF and HR of the RPO-supplemented and Wn perfused hearts. An unexpected finding was that wortmannin perfusion improved functional recovery in the RPO + Wn treated group when compared to the control and the RPO group. This was seen in the LVDevP (5 & 15 min) and the RPP (5 &15 min). The combination of dietary RPO and wortmannin given before ischaemia afforded better cardioprotection during ischaemia and reperfusion, than the RPO-supplementation alone. This is unexpected, because wortmannin is a PI3-kinase inhibitor, and activation of the PKB/Akt pathway is thought to be PI3-K-dependent. The PKB/Akt pathway is a pro-survival pathway. Therefore, inhibition of this pathway is normally associated with increased ischaemic/reperfusion injury.

It has previously been shown that hyperthemia-induced PKB/Akt activation was abrogated by wortmannin. This indicates that in the heart, hyperthermia-induced activation of PKB/Akt is predominantly PI3-kinase-dependent (Shinohara *et al.,* 2006). Therefore, when PI3-kinase is inhibited by wortmannin, the PKB/Akt pathway would be inhibited and that the function and viability of the cells should be reduced. However, our results indicate that RPO-supplementation reversed wortmannin's detrimental effect to such an extent that the myocardial functional recovery was far better than with RPO alone. To our knowledge, no literature is available on the effects of fatty acids or other components of RPO on

wortmannin. We can therefore only speculate that some component of RPO may counteract the detrimental effect of wortmannin induced PI3-K inhibition.

5.2 Effect of RPO and Wortmannin on PI3-Kinase (p85) induction

The novel mechanical results led us to study the effects of RPO and wortmannin on the signalling mechanisms that might be involved.

Although there was a decrease in PI3-K induction when wortmannin alone was added to the perfusate, it was not significant. This can be ascribed to the time the sample was taken. Since PI3-K is activated in the cell membrane it might be speculated that the activation was at a much earlier time point and returned to basal levels when the sample was taken. However, when wortmannin and RPO was administered together, there was a significant decrease in PI3-K induction.

5.3 Effect of RPO and Wortmannin on PDK phosphorylation

In our study, we observed no significant differences in PDK-1 phosphorylation due to RPO-supplementation and wortmannin administration. PDK-1 is known to phosphorylate PKB/Akt (Anderson *et al.,* 1998). PDK-1 expression does not affect PKB/Akt Ser^{473} phosphorylation, but it is involved in PKB/Akt Thr^{308} phosphorylation (Yamada *et al.,* 2001). The fact that RPO did not influence PDK-1 levels indicates that RPO does not influence the PKB/Akt pathway through PDK-1.

5.4 Effect of RPO and Wortmannin on PKB/Akt phosphorylation

Wortmannin had a significant inhibitory effect on PKB/Akt Ser^{473} phosphorylation. This is in contrast to what was found by Engelbrecht and co-workers, (2005), who reported that wortmannin inhibited PKB/Akt Thr^{308} but not PKB/Akt Ser^{473} phosphorylation. Since wortmannin is a known PI3-kinase inhibitor, we can speculate that in this model, PI3-kinase-dependent activation of PKB/Akt was achieved through the PKB/Akt Ser^{473} phosphorylation site. Interestingly, in the RPO + wortmannin group, Ser^{473} phosphorylation returned to basal levels. Wortmannin's inhibitory effects were thus overridden by RPO.

There were no differences in Thr^{308} phosphorylation with either RPO-supplementation or with wortmannin administration. This might be attributed to the lack of effect on PDK-1, as PDK-1 is known to phosphorylate PKB/Akt Thr^{308}, but not PKB/Akt Ser^{473}.

RPO did not induce an increase in PKB/Akt phosphorylation in our model. This is in contrast with the findings from a previous study which showed that RPO treatment led to an increased phosphorylation of PKB/Akt during reperfusion (Engelbrecht *et al.,* 2006). Our study suggests that the protective effects of RPO may also be mediated through another pathway. These differences may also be due to differences in study design. In our study we used a 10 times higher dose of RPO than the above mentioned study. We also used commercial available Carotino Premium RPO instead of the RPO baking fat. There was also a difference in the duration of supplementation. We supplemented rats for 4 weeks, while Engelbrecht *et al.,* (2006) fed rats with RPO for 6 weeks. In our study the Langendorff perfusion system was used while in the study of Engelbrecht *et al.,* (2006) a working heart system was used

Although RPO caused an increase in Thr^{308} phosphorylation, it was not significant. This can be ascribed to the small sample size. Most of the literature suggests that PKB/Akt pathway is regulated by phospholipids. However, there

are a few reports that suggest that mediators such as isoproteronol (Moule *et al.,* 1997) and cAMP (Sable *et al.,* 1997; Filippa *et al.,* 1999) may also be involved with PKB/Akt activation in a PI3-kinase-independent fashion. It is thus possible that there may be additional protective mechanisms other than the PI3-kinase pathway. Examination of *C. elegans* revealed a kinase that is homologous to PDK-1, but which does not have the phospholipids-binding PH domain. This PDK-1 related enzyme is capable of activating the PKB/Akt kinase in a manner similar to a PI3-kinase-independent pathway (Li *et al.,* 2001). This novel kinase, PIAK (phospholipids independent Akt/PKB kinase), is only capable of phosphorylating the Thr^{308} residue, which might also explain the increase in Thr^{308} phosphorylation in our results.

PIAK is not inhibited by wortmannin, thus the activity of PIAK kinase is independent of PI3-kinase. PIAK also induces one of the forkhead transcription factors, AFX. The mechanism through which PIAK interacts with PKB/Akt remains unresolved (Li *et al.,* 2001).

Although RPO-supplementation increased functional recovery, it was not associated with increased PKB/Akt total phosphorylation. This is contrary to previous studies that showed that RPO did indeed activate the PKB/Akt pathway (Engelbrecht *et al.,* 2005). This might be due to technical differences between the studies as mentioned earlier.

5.5 Effect of RPO and Wortmannin on GSK phosphorylation

Activated PKB/Akt phosphorylates and inactivates glycogen synthase kinase on Ser^{9} (GSK-3β) (Downward, 2004), and this inactivation leads to activation of glycogen synthase (Cross *et al.,* 1995; Van Weeren *et al.,* 1998). The activation of glycogen synthase contributes to glycogen synthesis and the

uptake of glucose from the blood. Thus, PKB/Akt is also a key regulator of glycogen metabolism.

We observed an increased GSK phosphorylation when the RPO group was supplemented with wortmannin. Since the phosphorylation of GSK actually leads to the inactivation of the enzyme, we can conclude that RPO + wortmannin inactivated GSK, which leads to an increased glycogen synthase activity, which in turn leads to increased glycogen synthesis. Glycogen is a storage form of glucose. Alterations in glycogen metabolism and glucose utilization may contribute to loss of cardioprotection in stressed hearts. It has been reported that a poor functional recovery is associated with an acceleration of glycolysis (Jaswal *et al.,* 2006). Glycogen accumulation may be beneficial and also lessens the potential for glycolysis (Henning *et al.,* 1996; Russell *et al.,* 1997). Thus, increased glycogen synthesis leads to glycogen accumulation, which may be protective to the heart. It was found that glucose from glycogen is preferentially oxidized compared with exogenous glucose (Henning *et al.,* 1996) under normoxic conditions.

The RPO + wortmannin possibly had a protective effect through GSK phosphorylation. This can either be attributed to the increased phosphorylation of PKB/Akt Ser^{473}, also seen with RPO + wortmannin, or a whole different protective pathway. Inhibitors of the protein kinase C (PKC) family of kinases have been used to demonstrate that these enzymes are likely to play a key role in GSK-3 phosphorylation (Vilimek and Duronio, 2006).

5.6 Negative regulation of PKB/Akt

PTEN exerts a negative regulatory effect on PKB/Akt. PTEN reduces the production of the messengers that leads to the activation of PKB/Akt (Haas-Kogan *et al*., 1998; Maehama & Dixon, 1998; Stambolic *et al*., 1998). PTEN exerts the same inhibitory effect as wortmannin on PI3-kinase activity (Kim *et al.,* 2002). Therefore, PTEN negatively influences PKB/Akt activity.

In our study, we found that wortmannin increased PTEN phosphorylation and inhibited PI3-K induction. These results confirm the findings of other studies (Yano *et al.,* 1993; Tong *et al.,* 2000). The action of PI3-kinase on PKB/Akt is thus opposed by PTEN.

PTEN negatively regulates PI3-kinase-dependent signaling of PKB/Akt (Barthel and Klotz, 2005). The RPO + Wn group had a PTEN phosphorylation similar to that of the control and RPO group, indicating that RPO counteracts the effect of wortmannin, and exerts a protective effect on the myocytes.

Thus, RPO exerts an inhibitory effect on PTEN in the absence of PI3-kinase, and thus lessens the negative regulatory effect that PTEN have on the PKB/Akt pathway.

5.7 Effect of RPO and Wortmannin on anti-apoptotic mechanisms

The forkhead family (FKHR and FKHRL1) is a substrate of PKB/Akt and is phosphorylated by PKB/Akt (Nakae *et al.,* 1999; Brunet *et al.,* 1999; Rena *et al.,* 1999; Guo *et al.,* 1999). Because PKB/Akt negatively regulates factors that promote the expression of death genes, like the forkhead family of transcription factors, PKB/Akt activation exerts a prosurvival effect. When PKB/Akt phosphorylates forkhead transcription factors, they are exported from the nucleus (Biggs *et al.,* 1999), accumulate and are confiscated by proteins in the cytoplasm (Brunet *et al.,* 1999).

It was observed that RPO-supplementation induced significant increased phosphorylation of FKHR. The suppressive effect of RPO on the expression of death genes may be associated with the increased post-ischaemic functional recovery observed in the RPO group. It would appear that RPO-supplementation had a negative effect on the expression of death genes, whether through PKB/Akt activation or not.

Wortmannin caused a decreased phosphorylation of FKHR in the RPO-supplemented hearts, which means that there were fewer FKHR transcription factors being exported from the nucleus, and thus that more death genes transcribed. This is to be expected with wortmannin treatment, as wortmannin inhibits PI3-kinase (Yano *et al.,* 1993; Tong *et al.,* 2000), which inhibits the pro-survival PKB/Akt pathway.

PKB/Akt also functions to promote cell survival through phosphorylation and inactivation of pro-apoptotic proteins such as Bad, caspase-9, and possibly Apaf-1 (Datta *et al.,* 1997; Del Peso *et al.,* 1997; Cardone *et al.,* 1998; Zhou *et al.,* 2000). PKB/Akt activation leads to the NF-κB activation of the transcription of anti-apoptotic genes (Ozes *et al.,* 1999; Romashkova and Makarov, 1999). It is therefore possible that RPO might also be protective through one of these pathways.

5.8 Effect of RPO and Wortmannin on Caspase cleavage

The activation (thus the cleavage) of caspases is a key step in the initiation of apoptosis. Caspases induces apoptosis by cleaving and inactivating cellular substrates, which play an essential role in maintaining cell viability (Nicholson, 1996). Apoptosis results from the activation of caspases in a cascade-like fashion (Cryns and Yuan, 1998; Thornberry and Lazebnik, 1998) and can be inhibited by increased PKB/Akt activation (Sabbatini and McCormick, 1999).

A significant increase in caspase-3 cleavage was observed in the wortmannin perfused groups compared with the control groups. Wortmannin is known to increase caspase-3 cleavage during ischaemia/reperfusion induced injury (Engelbrecht *et al.,* 2004). RPO could not counteract wortmannin's pro-apoptotic effect. Interestingly, this increased caspase-3 cleavage induced by wortmannin does not correlate with the improved functional recovery in the RPO+wortmannin group.

5.9 Effect of RPO and Wortmannin on PARP cleavage

PARP is a cellular protein that is specifically cleaved during apoptosis (Kaufmann *et al.,* 1993). The presence of cleaved PARP is thus an indication that apoptosis is already in progress. Wortmannin administration caused an increase in the PARP cleavage. This is to be expected, as wortmannin inhibits PI3-K (Yano *et al.,* 1993; Tong *et al.,* 2000), which in turn inhibits PI3-kinase-dependent PKB/Akt activation. Thus, wortmannin treatment is associated with reduced cell viability. RPO caused the PARP cleavage to return to basal levels, indicating that RPO overrides wortmannin's detrimental effects. This finding is associated with increased functional recovery in the RPO + wortmannin group.

5.10 Conclusion

RPO in conjunction with wortmannin improved reperfusion functional recovery in the reperfused rat heart. The precise manner in which RPO and wortmannin influences cellular signalling is not yet clear, but it would appear that it might be related to reduced PTEN phosphorylation and increased PKB/Akt Ser^{473} phosphorylation which was associated with decreased PARP cleavage, thus attenuating apoptosis in our model.

Although not all the cellular signalling pathways are yet clear, it is possible that RPO may also induce its protection through other pathways, such as the MAPKs or the PKC pathways. The results obtained in the present study thus suggest that RPO in conjunction with wortmannin might offer an alternative strategy to protect the heart against ischaemia/repefusion-induced injury.

The prevention of CHD in society remains a challenge and only a few drug therapies have convincingly reduced the risk of heart failure. Dietary intake of RPO in patients with a prior myocardial infarction is a low-cost and a low-risk intervention and it is time for clinicians to give increased attention to the

beneficial effects of RPO and translate this experimental evidence into clinical practice.

Chapter 6 FINAL CONCLUSION AND FUTURE DIRECTIONS

We can conclude that Carotino Premium RPO-supplementation in conjunction with wortmannin perfusion did protect against ischaemia/reperfusion induced injury in the isolated Langendorff perfused rat heart. We also conclude that RPO-supplementation and wortmannin administration influenced the PI3-K pathway; however, we are not sure through which regulatory proteins it exerted its effects. It is possible that another pathway, like MAPKs or PKC might be involved in the protection induced in this model. For future studies it would be advisable to look at RPO's affect on these pathways and other proteins involved in PKB/Akt pathway modulation, in order to further elucidate the precise mechanism through which RPO and wortmannin protects against ischaemia/reperfusion induced injury.

Chapter 7 RESERVATIONS OF THE STUDY

In our study we showed that hearts from rats supplemented with RPO did have a better functional recovery, although it was only seen at the 10 min timepoint. After 10 min, the significance of the protection was lost. An important possible explanation for this might be that the SEMs were too big in the RPO + Wn group. When individual comparisons were done between the RPO-supplemented group and the control group, significant differences were found throughout reperfusion, but because it is not scientifically correct, this measure of statistics cannot be used. This protection that was seen at 10 min in this study is similar to results from other studies (Esterhuyse *et al*., 2005; Bester *et al*., 2006). However, we found that the effect of RPO on cellular signalling was different to what these studies

found (Engelbrecht et al., 2006). These differences may be explained by the following critical issues.

A possible explanation might be that the sample sizes are too small, and if the numbers in each group were increased, a significant difference might be found. In our study, rats were supplemented with 2 ml Carotino Premium RPO per day for 4 weeks. In the above mentioned studies, rats were supplemented with 0.2 ml baking fat RPO for 6 weeks. The differences in feeding times might have had an influence on our results. The differences in types of oils might have had an influence. We used Carotino Premium RPO as it is the commercially available oil. The baking fat used in the abovementioned studies is a much more concentrated form of RPO than the Carotino Premium RPO that we used. The differences in RPO dosages between this study (the supplier suggested the use of a higher dose in this specific study) and the previous studies might explain the slight differences in outcome of results. These data imply that more is not necessarily better, and therefore our results also suggest that future studies should include a dose response study. In our study we used the Langendorff perfusion model, whereas the above mentioned studies used the working rat heart model. The working rat heart model is a more physiological system, especially when mechanical function is considered. Our study focused more on the effect of RPO on cellular signalling and therefore we used the Langendorff perfusion system.

For future studies, these aspects might be brought into consideration.

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