Effect of rooibos and red palm oil supplementation, alone or in combination, on cardiac function after exposure to hypertension and inflammation in an ischaemia/reperfusion injury model

by

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DECLARATION

I Emma Tutu Masechela Thamahane-Katengua, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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Signed **Date**

ABSTRACT

Cardiovascular disease (CVD) is without a doubt one of the most challenging health issues of our time and accounts for the highest number of deaths in both developed and developing countries. Despite the huge strides that have been achieved in the diagnosis and therapeutic intervention of CVD, the disease burden still remains enormous. Therefore, this calls for novel and innovative interventions to curb the surge of CVD. The use of plant based food with bioactive phytochemicals,has a great potential to reduce the incidence of CVD, specifically in resource-strained countries. Red palm oil (RPO) and the indigenous herbal tea, rooibos have previously been shown to exhibit potential cardioprotective effects. Their health promoting properties have largely been attributed to their antioxidant and anti-inflammatory activities and emerging evidence also showed that they have the potential to modulate cell signalling events. Substancial scientific evidence proposes oxidative stress and inflammation to play an important role in the pathogenesis of cardiovascular disease. Hence, natural plant extracts such as RPO and rooibos could be recommended as adjuvants to clinical therapy to reduce the morbidity and mortality associated with CVD.

This thesis reports on three studies investigating the cardiovascular protective effects that chronic feeding of either RPO, rooibos or their combination have on 1) antioxidant enzymes and the NO-cGMP pathway in myocardial tissue of spontaneous hypertensive rats, 2) the modulation of systemic and myocardial inflammation and 3) the myocardial ischaemic/reperfusion tolerance in a rat model of lypopolysaccharide induced inflammation.

The aim of the first study was to investigate the effect of RPO on cardiac function in sponteneously hypertensive rats. The role of the nitric oxide cyclic-guanosine monophosphate(NO-cGMP) pathway, (as determined by the nitric oxide (NOS) activity) and the antioxidant defence system (selected antioxidant enzymes) were also investigated. Cardiac function was monitored at stabilization and reperfusion using the Langendorff perfusion system. Antioxidant enzymes were determined from left ventricular tissue, while total NOS activity was determined in the aorta and left ventricular tissue. The results show that RPO offered cardiac protection as evidenced by improved left ventricular developed pressure (LVDevP), maximum velocity of pressure rise (+dp/dt) max and fall (-dp/dt) max during reperfusion in sponteneously hypertensive rats (SHR) compared to their control counterparts. Improved function in SHR was associated with increased myocardial superoxide dismutase 2 (SOD2) protein expression compared to the normotensive rats. There was differential modulation of the NOS activity by RPO, an increase in NOS activity was observed in the aorta while a reduction in the activity of NOS was observed in the left ventricular tissue of both RPO supplemented normotensive and hypertensive rats compared

to their respective control groups. These results argue a role for elevated NO production in the aorta for endothelial function maintenance. Increased SOD2 protein might lead to reduced oxidative stress. Thus, NO-cGMP pathway and antioxidant defense systems synergistically acted to restore cardiovascular function in SHR.

The aim of the second study was to investigate the effect of RPO and rooibos supplementation on the modulation of systemic and myocardial inflammation in a rat model. As RPO and rooibos contain different types of antioxidants which reside and exert their biological effects in different cellular compartments, the combination of these two natural food compounds has the potential to enhance the spectrum of available dietary antioxidants in different cellular compartments, which could result in a better protection against certain pathological conditions such as inflammation. The Langendorff system and the lypopolysaccharide (LPS)-induced inflammatory model were used to determine if RPO and rooibos could protect against the negative effect of LPS-induced inflammation on baseline cardiac function. Both inflammation and dietary supplementation did not have any effect on baseline cardiac functional parameters. Our results show that administration of LPS resulted in elevated plasma levels of IL-1β in supplemented and non-supplemented rats indicating that an inflammatory response was triggered in the LPS-treated rats. However, this increase in IL-1β was counteracted by concurrent elevation of plasma IL-10 in LPS-induced rats consuming either rooibos or RPO alone. Furthermore the combination of RPO and rooibos enhanced myocardial IL-10 levels in LPS-induced rats. This data shows a difference in response to LPS injection between the myocardium and the systemic circulation. The results indicate that the combination of these two natural food substances exhibit potential anti-inflammatory properties which could be beneficial in clinically relevant conditions where inflammation plays a role.

Having shown that dietary intervention with RPO and rooibos had the potential to modulate the inflammatory response in the model of inflammation at basal conditions, we then proceeded to the third study to specifically establish if dietary RPO when supplemented alone will improve functional recovery and reduce infarct size in LPS-treated hearts. The Langendorff perfusion system was employed for determination of cardiac function and infarct size. The roles of NFkB, p38 MAPK and the myocardial antioxidant defence systems were investigated as potential mechanisms of protection. LPS-treatment caused significant increases in myocardial IL-1 β indicating that inflammation was induced. However, the levels of myocardial IL-10 was reduced in LPS-treated hearts compared to the non-treated hearts. Intervention with dietary RPO resulted in improved functional recovery and reduced infarct size, in both healthy hearts and in the LPS-treatment group. The RPO-induced cardio-protection was associated with increases in myocardial protein expression of the antioxidant

enzymes, SOD1, SOD2, GPX1 as well as increased p38 phosphorylation during reperfusion. LPS treatment increased myocardial protein expression of NFkB p65 which was reversed by RPO supplementation. Reduction of myocardial NFkB protein expression, increased p38 phosphorylation and elevated mitochondrial antioxidant (SOD2 and GPX1) as well as cytosolic enzymes (SOD 1) are proposed as potential mechanisms underlying the RPO-induced cardio-protection in this model.

Based on these study results, for the first time, having included vasculature aspects in the cardio-protective effects of RPO we have shown that the NO-cGMP pathway and antioxidant defense systems may act synergistically to restore cardiovascular function in spontaneously hypertensive rats. Results from the second study also provide the first scientific evidence that RPO in combination with rooibos (a flavonoid rich endemic herbal tea) could have potential anti-inflammatory activities at systemic as well as myocardial level, which may be beneficial in clinically relevant conditions where inflammation plays a role. From the third study it can be concluded that dietary RPO improved myocardial tolerance to ischaemia-reperfusion injury in a model of inflammation.

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DEDICATION

This thesis is dedicated to my son, **LEHLOHONOLO KATENGUA**, for every pain and hardship that I endured during the course of this project, you were always on my mind and the thought of you gave me the strength and the determination carry on.

PREFACE

This thesis is submitted to fulfill the requirements for the degree of Doctor of Technology in the discipline of Biomedical Technology. The thesis is made up of three studies using different experimental animal models to explore and investigate the cardio-protective effects of RPO and rooibos. Chapter one, gives a brief introduction to and motivation for doing the research, while the general aims for each of the three studies are also stated. Chapter 2 gives an account of the literature highlighting and discussing concepts, which are important in understanding the significance of the research and also to help in placing the results obtained in context. The thesis is presented in an article format with three articles which will be submitted for publication in different accredited journals (chapters 3-5). Each article is prepared according to the author guidelines of the respective journals where they will be submitted. Chapter 6 provides a general discussion and conclusions of the overall thesis and a proposed way forward.

ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
BW	Body weight
cAMP	Cyclic adenosine monophosphate
CF	Coronary flow
CHD	Coronary heart disease
Co Q10	Coenzyme Q10
DNA	Deoxyribonucleic acid
(+dp/dt) max	Maximum velocity of pressure rise
(-dp/dt) max	Maximum velocity of pressure fall
EDLVP	End diastolic left ventricular pressure
ERK	Extracellular signal – regulated kinase
GPX	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HLD	High density lipoprotein
HMG Co-A reductase	3-Hydroxy-3methylglutaryl-coenzyme A
HR	Heart rate
HW	Heart weight
IL-1 β	Interleukin - 1 β
IL-1ra	Interleukin 1 receptor agonist
IL-6	Interleukin - 6
IL-10	Interleukin -10
JNK	c-Jun NH terminal kinase
GPX1	Glutathione peroxidase 1
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
LV	Left ventricle
LVDevP	Left ventricular developed pressure
LVDP	Left ventricular diastolic pressure
LVEDP	Left ventricular end diastolic pressure
LVSP	Left ventricular systolic pressure
MAPK	Mitogen Activated Protein Kinase
mRNA	Messenger ribonucleic acid
MUFA	Mono-unsaturated fatty acids
NF-κB	Nuclear factor kappa beta
NO	Nitric oxide
NOS	Nitric oxide synthase

NO-cGMP	Nitric oxide cyclic guanosine monophosphate
p38 MAPK	p38 Mitogen Activated Protein Kinase
PARP	Poly (ADP-ribose) polymerase
PI3K	Phosphatidylinositol-3 kinase
PKB/Akt	Protein kinase B
PKC	Protein kinase C
PPM	Parts per million
PUFA	Polyunsaturated fatty acids
RISK	Reperfusion Injury Salvage Kinases
ROS	Reactive oxygen species
RNOS	Reactive nitrogen oxide
RPO	Red palm oil
RPP	Rate pressure product
SOD	Superoxide dismutase
TLRs	Toll-like receptors
TLR 4	Toll-like receptor 4
SFA	Saturated fatty acids
SHR	Spontaneously hypertensive rats
SRC	standard rat chow
SOD1	Superoxide dismutase 1
SOD2	Superoxide dismutase 2
WKY	Wistar-Kyoto rats (normotensive)

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Chapter 1: General introduction

Non-communicable diseases, including cardiovascular disease, account for more than three quarters of deaths worldwide in both high and low income countries (Beaglehole and Bonita, 2008). The main pharmaceutical products currently sold by major pharmaceutical companies address cardiovascular health and inflammation (mainly arthritis). However, there is growing interest amongst consumers to shift from a high pharmacological dose to a lower dose co-recommended natural product(s) (Georgiou et al., 2011). At the same time consumers are also more informed about diseases, medication and the use of natural dietary supplements to aid their fight against aging and disease.

In today's informed and knowledgeable populations, several plant and food components are being used as natural medicines, either directly or as pro-drugs. The border between supplements and drugs is not as well-defined as decades ago. Supplements contain several bioactive compounds including functional nutrients like vitamins and antioxidants, fibres, friendly bacteria, essential fatty acids and probiotics. Modern drugs are derived from natural products. Optimal health and prevention of chronic diseases can be achieved by supplementation of certain macro- and micro-nutrients to the diet (Kotler, 2000; Argile, 2005). The classic treatment with prescribed drugs is increasingly complemented with a recommended nutritional supplement. This has caused drugs and nutrition, alone or in combination, to become an important area in health research (Visioli, 2012). The nutritional supplements industry has grown to a majestic amount of more than $100 billion per annum. The lack of scientific information on the health benefits of dietary supplements in the treatment of disease warrants research studies to validate the efficacy of this new trend in the health care sector. Furthermore, the consumers are more sensitive to the adverse effects of drugs. Pharmaceutical companies have also shown intent to follow treatment with natural substances by acquisitions of large supplements companies.

In the 90's several studies addressed the effect of palm oil on the cardiovascular system (Osim et al., 1996; Owu et al., 1997; Clandinin *et al*., 1999). Sundram and co-workers (1994) showed that palm oil did not have negatively effect on the lipoprotein profile in humans. Early in the 90's Serbinova et al. (1992) showed that palm oil vitamin E could protect the heart against the consequences of ischaemia/reperfusion injury. It was later in the 90's that some research was directed at specifically red palm oil (containing carotenes not destroyed by refinery process). A study in India showed that red palm oil could increase the retinol levels in infants when added to the diet of lactating mothers (Canfield et al., 2001). Red palm oil in the maternal diet improved the vitamin A status of lactating mothers and their infants. In

South Africa, incorporation of red palm oil into the diet of school children improved their health status (Van Stuijvenberg et al., 2000). Earlier animal model studies indicated that red palm oil also had beneficial effects on the cardiovascular system. Kritzevsky et al., (2000) showed that red palm oil could decrease the arteriosclerotic plagues in rabbits. He also showed that the position of the oleic acid and palmitic acid on the triglyceride backbone favoured unsaturated fatty acid absorption (Kritchevsky, 1988). These preliminary red palm oil studies using animal models in the 90's were followed by intensive studies on the cardiac benefits of red palm oil for the next 10 years by the group of Van Rooyen from 2003-2013. They showed in several studies that red palm oil could protect against ischaemia/reperfusion injury in healthy hearts (Esterhuyse et al., 2006; Bester et al., 2010), hypercholesterolaemic hearts (Szucs et al., 2011) and spontaneously hypertension hearts (Bacova etal., 2012). Wergeland et al., (2011) also showed that red palm oil could protect against the bad effects of chemotherapy. Red palm oil was also able to reduce infarct size in the ischaemia/reperfusion model (Bester et al., 2010; Szucs et al., 2011). The proposed cellular mechanisms of protection included a role for the NO-cGMP pathway (Esterhuyse et al., 2006), MMP-2 and attenuation of myocardial LDH release (Bester et al., 2010; Szucs et al., 2011), Akt pro-survival pathway and anti-apoptotic pathway (Engelbrecht et al., 2006; Kruger et al., 2007; Katengua-Thamahane et al., 2012). In the chemotherapy model there were evidence of anti-oxidant enzyme involvement (Wergeland et al., 2011), but this needed further clarification.

In the hypertension model red palm oil had an anti-arrhythmic effect with a reduction in blood pressure and blood glucose. Up regulation of connexian-43 was implicated as a possible mechanism for the anti-arrhythmic effect (Bacova et al., 2012). Recently Ajuwon et al. (2013) showed that red palm oil in combination with rooibos, an endemic herbal tea or alone can protect against oxidative stress-induced hepatotoxicity. Several mechanisms were implicated which included improved liver function marker enzymes (ALT, AST, LDH), prevention of lipid peroxidation (MDA and CD levels) and modulation of the activity of certain anti-oxidant enzymes. These results also confirmed the results of Alinde et al. (2012) (MTech thesis) which showed that red palm oil reduced MDA levels in plasma of oxidative stress induced rats. It is therefore clear that red palm oil showed protective effects in various experimental animal models. In all these studies some results were substantiated by the fact that different models and different research groups in different models produced the same results. The latest results implicated that the antioxidant enzymes also play an important role. This is expected since red palm contains a high concentration of antioxidants. However, the results were not conclusive and needed to be verified with further investigations.

Several studies investigated the effect of rooibos (*Aspalathus linearis*) herbal tea on oxidative stress-related conditions (Marnewick et al., 2011; Muller et al., 2012). These investigators have shown that rooibos herbal tea can improve the lipid profile and redox status in humans and can improve hypoglycaemic activity in experimental rats, respectively. Rooibos is a rich source of asphalathin, but also of a unique blend of phytochemicals, which could be responsible for these effects. Closer to our model, Pantsi et al. (2011) showed that supplementation of rooibos herbal tea could also protect against ischaemia/reperfusion injury. Evidence suggested that the flavonols in rooibos were responsible for the anti-apoptotic effect seen in the protection against ischaemia/reperfusion injury. This fact, similar to that suggested for red palm oil, initiated thought for a combination treatment. Ajuwon et al. (2013) showed that the protective effects of fat soluble antioxidant rich red palm oil, and rooibos, a water soluble antioxidant rich herbal tea, could potentially have an enhanced protective effect when supplemented together.

Hypertension is a significant public health problem world wide and it is rapidly becoming a huge public health problem on the African continent (Tesfaye et al., 2010). It is projected that 1 billion of the world adult population are currently categorize as hypertensive, and this number is expected to increase to more than 1.5 billion by 2025 (Kearney et al., 2005). The 2002 World Health report estimated that 9.2% of all total deaths in the African region resulted from cardiovascular events where hypertension was identified as an important risk factor (WHO AFRO 2005, World Health Report 2002).

There is convincing evidence showing that inflammation plays an important role in the pathogenesis of cardiovascular disease (Libby, 2006). Elevated plasma levels of inflammatory cytokines have been reported in various cardiovascular conditions such as diabetes, atherosclerosis, myocardial infarction and heart failure. This suggests the causal role for inflammation in the pathogenesis of these conditions (Nian et al., 2004). Evidence showed that systemic low-level inflammation is strongly associated with aging and is considered to be a cardiovascular risk factor.

1.1 Aims

Therefore general aims of the three studies undertaken included the following:

1. To investigate whether certain antioxidant enzymes and the NO-cGMP pathway may play a role in RPO-induced cardiovascular protection in spontaneously hypertensive rats.
2. To investigate the effect of red palm oil and rooibos supplementation on the modulation of systemic and myocardial inflammation in a rat model.
3. To investigate the effect of red palm oil supplementation on myocardial ischaemic/reperfusion tolerance in a rat model of induced inflammation.

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CHAPTER 2: Literature Review

1.1 The burden of cardiovascular diseases on health systems world wide

Cardiovascular disease (CVD) is associated with an overwhelming number of deaths in the industrialized countries and it is also becoming a significant cause of morbidity and mortality in the developing world (Leeder et al., 2004; Adeyi et al., 2007; WHO, 2008b; WHO 2009e; Gaziano et al., 2010). The prevalence of CVD imposes a huge health and economic burden to governments across the globe and in developing countries this problem is exacerbated by the concomitant surge in infectious diseases (Mayosi et al., 2005). Coronary heart disease (CHD) is the most important cause of death among the different forms of CVD (Callow 2006; Gaziano et al., 2010). Projections from the WHO estimated that by 2015, 20 million deaths will result from CVD (WHO, 2005). It has also been estimated that by 2030 non-communicable diseases including CVD will account for more than three quarters of deaths worldwide of which a large proportion of CVD deaths will occur in the low income countries (Beaglehole and Bonita, 2008; Fig 1). Non-communicable diseases such as CVD, cancers, chronic respiratory diseases and diabetes are considered to be the biggest threat of our time to human health and development. It has been projected that these diseases are the leading killer diseases contributing to approximately 60 % of deaths globally with 80 % of these deaths occuring in the developing countries (WHO 2008-2013 Action Plan for Global Strategy in Non-communicable disease).

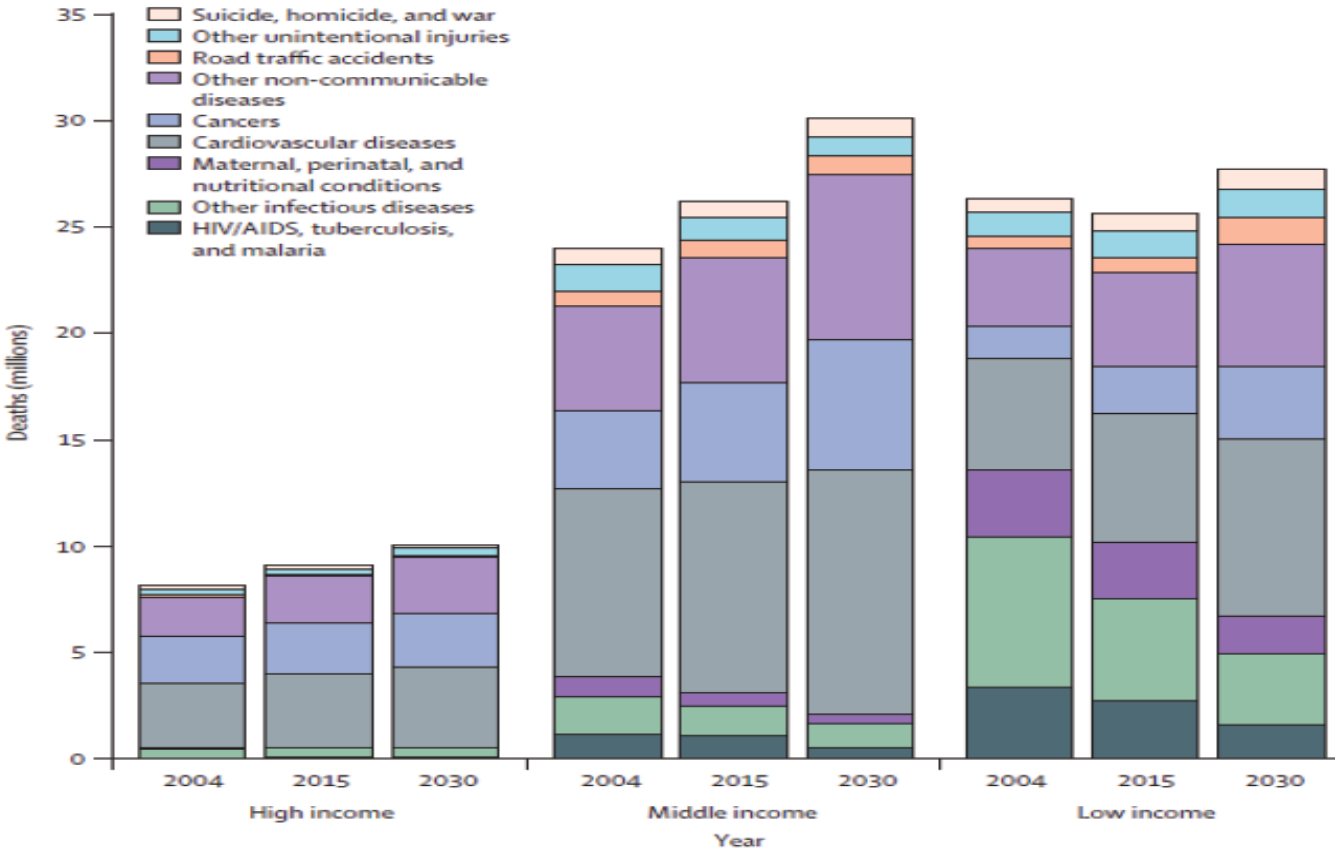

Figure 1: Projected global deaths by cause (Beaglehole and Bonita 2008).

CVD is no longer the disease of the affluent, but it is increasingly becoming a huge problem in the low and middle income countries (Beaglehole and Bonita, 2008). Mayosi and co-workers (2009) reported that mortality rates for CVD and diabetes are rising in South Africa. It is estimated that in South Africa, 41% of deaths in the age bracket of 35 - 64 years occur as a result of CVD (Leeder et al., 2004). The surge of CVD in developing countries has a huge economic impact with detrimental consequence at both individual and national level (Mclntyre et al., 2006; Xu et al., 2007; WEF, 2009).

1.2 Risk factors associated with cardiovascular disease

Primary prevention of CVD commences with control of cardiovascular risk factors, therefore, early detection and aggressive interventions are imperative in reducing the rise in cardiovascular disease. CVD is a complex and multifaceted disease involving a variety of cardiac and vascular pathologies characterised by multiple risk factors (Opie et al., 2006). Several cardiovascular risk factors have been identified such as elevated blood pressure, left ventricular hypertrophy, increased markers of inflammation, smoking, diabetes, increased

blood cholesterol, abdominal obesity, age, sex, psychosocial factors, reduced consumption of fruits and vegetables and lack of exercise (Anderson et al., 1991; Yusuf et al., 2004; Opie et al., 2006). The WHO 2008-2013 Action Plan on Non-communicable Diseases places special emphasis on the importance of promoting interventions which are targeted at reducing the main shared modifiable risk factors for non-communicable diseases which include smoking, diet and physical inactivity. Another important emerging cardiovascular risk factor is childhood obesity which has both immediate and long-term consequences. Childhood obesiy is one of the major predisposing risk factors in developing cardiovascular diseases later in adult life (Feedman et al., 2001; Bridger, 2009). 170 million children under the age of 18 are estimated to be overweight and the prevalence rates of childhood obesity are increasing at an alarming rate in lower to middle-income coutries (WHO, 2012)

1.3 A potential role for dietary phytochemical intervention in CVD

Modification of dietary patterns is one of the most important factors, which, if given attention, has the potential to significantly reduce the risks of developing CVD, especially in the developing countries where the burden of CVD is emerging at an alarming rate. A considerable, amount of evidence from independent research groups has shown that plant based dietary food sources such as polyphenols and phytochemical compounds have a great potential to be explored as preventive or therapeutic agents for cardiovascular disease (Aviram et al., 2004; Grassi et al., 2005; Sumner et al., 2005; Faridi et al., 2008). Several lines of evidence from random control trials indicates that increased consumption of fruits and vegetables results in substantial improvements in cardiovascular risk factors such as blood pressure, serum lipid profile, endothelial dysfunction, and inflammation markers (Esposito et al., 2004; Elmer et al., 2006; McCall et al., 2009; Jenkins et al., 2009). Consumption of a basic Mediterranean diet has been shown to reduce the rate of complications after myocardial infarction (de Lorgeril et al., 1999). Estruch and co-workers (2013) recently reported that a Mediterranean diet supplemented with extra-virgin oil or nuts resulted in a considerable reduction in the risk of major cardiovascular events among high risk individuals. This diet had the same effect as taking statins, which is a lipid-lowering drug that has been proven to reduce the risk of major cardiovascular events by 25% to 30% (Estruch et al., 2013). Based on available evidence it has been suggested that the beneficial effects of an increased intake of fruits and vegetables is mainly attributable to the phytochemicals and the complex form of micronutrients, which may exhibit a potentially enhanced bioavailability when they are in their natural state (Miller et al., 2006; Al-Solaiman et al., 2009; Mozaffarian et al., 2011).

1.4 Role of oils and cardiovascular health

For many years the fatty acid composition of edible oils has been a subject of major interest owing to the direct relationship that exists between the oils and the incidence of cardiovascular disease. The fatty acid composition of the diet is particularly important because it influences the serum lipoprotein profile and the ratio of LDL to HDL, which is an important determinant of atherosclerosis (Perona et al., 2010). Dietaty fatty acids also play a critical role in determining the fatty acid composition of cell membranes, which in turn influences the way in which membrane-bound protein interact (Clandinin et al., 1991; Clandinin et al., 1992;Carrié et al., 2000; Andersson et al., 2002). Membrane proteins play a vital role in various cellular signalling events, hence, changes in the composition of membrane fatty acids can cause alterations in cellular communication and function, ultimately leading to changes in gene expression (Jahangiri et al., 2006). The myocardial sarcolemma plays a critical role in regulating movement of ions across the membrane and changes in the physical properties of the myocardial cells have huge implications, because it can affect both the electrophysiological and biochemical properties of the cardiomyocytes (Charnock *et al*., 1994; Jahangiri et al., 2006).

Consumption of different types of oils has been shown to have beneficial effects on cardiovascular health (Pepe and McLennan 2002; Stine et al., 2011; Boon et al., 2013). Jahangiri and co-workers (2006) reported that dietary fish oil supplementation attenuates the susceptibility of cardiomyocytes to ROS-induced injury. Proposed mechanisms included changes in the fatty acid composition of the membrane and increased antioxidant defences. Dietary supplementation of fish oils, rich in omega 3 PUFAs has been shown to reduce the risk of CHD and to reduce vulnerability of the myocardium to arrhythmias (Pepe et al., 1996; Daviglus et al., 1997; Hu et al., 2002; Marchioli et al., 2002). Several mechanisms have been attributed to the anti-arrhythmic effects of omega 3 PUFAs, including its ability to be incorporated into myocardial cell membranes (Harris et al., 2004) and potential alteration of eicosanoid production and ion channel function (Leaf et al., 2003). Bacova and colleagues (2012) recently demonstrated that dietary RPO (which has considerable amount of monounsaturated fatty acids) protected spontaneously hypertensive rats against life-threatening arrhythmias.The anti-arrhythmic action was linked to up-regulation of connexin-43 and suppression of PKCε activation. Consumption of fish oil has been linked to anti-hypertensive effects and some of the proposed mechanisms for the blood pressure lowering effects included modulation of the composition of membrane phospholipids with potential increase in systolic arterial compliance (Nestel et al., 2002) and improvement of endothelial function (Wang et al., 2012). Other cardiovascular benefits of dietary fatty acids, especially

omega 3 includes anti-inflammatory effects and anti-platelet aggregation, both of which have been shown to play an important role in the pathogenesis of CVD (Calder, 2004;Arita et al., 2005; Micallef and Garg 2009).

Krill oil obtained from the Antarctic krill (*Euphausia Superba*) represents another viable alternative source of marine PUFAs. The overall fatty acid composition of krill oil is comparable to that of fish oil albeit with a higher EPA content. Moreover, metabolic effects of fish oil and krill oil have been shown to be essentially similar. However, in addition to the abundant EPA and DHA, krill oil also contains a potent antioxidant known as astaxanthin which may also contribute to its health benefits (Tou et al., 2007; Stine et al., 2011).

1.5 Red palm oil and health benefits

RPO, an edible oil produced from *Elaesis guineesis*, is obtained from crude palm oil by the process of raffination (Nagendran et al., 2000; Hariharan et al., 1996; Sundram et al., 2003). RPO contains 11% poly-unsaturated fatty acids, 38% mono-unsaturated fatty acids and a range of micronutrients such as carotonoids and pro-vitamin E substances; tocopherols and tocotrienols (Nagendran et al., 2000; Sundram et al., 2003). RPO retains up to 80% of carotenes and vitamin E originally present in crude palm oil after processing (Nagendran *et al*., 2000). It is hence the richest food source of caroteniods and its characteristic colour is due to the high content of these carotenoids in the oil (Cottrell, 1991). Tocopherols and tocotrienols can also act as potent physiological antioxidants *invivo* (Goh *et al*., 1985; Sundram *et al* 1994). Evidence from previous studies showed that tocotrienols have a cholesterol lowering effect via inhibition of 3-hydroxy-3-methyl-gluaryl-COA (HMG-COA) reductase, the rate-limiting enzyme in the biosynthesis of cholesterol (Qureshi *et al.,* 2001). Yuen and colleagues (2011) reported that supplementation of mixed tocotrienols at a dose of 300mg/day for five months resulted in lowering of serum total and LDL-cholesterols.

In addition to a wide spectrum of antioxidants, RPO also has a unique composition of fatty acids that allows it to behave like a monounsaturated fatty acid even though it has a high content of saturated fatty acids (Sundram et al., 2003). RPO is unique from other vegetable oils as it contains almost equal proportions of saturated to unsaturated fatty acids (in a ratio of 1:1). Palm oil is essentially devoid of lauric acid and myristic acid and appears to have sufficient oleic acid and linoleic acid to counteract the most deleterious effects of palmitic acid present in palm oil (Khosla and Hayes 1992; Pronczuk *et al*., 1994). The high content of antioxidants especially, tocopherols and tocotrienols also add to the uniqueness of palm oil as these micronutrients can act as potent antioxidants which contribute to the stability of the

oil protecting it from oxidation. The lipophilic nature of vitamin E allows easy incorporation into cellular membranes where it can act as an antioxidant protecting membranes from oxidative damage. Vitamin E has been shown to prevent the oxidation of LDL cholesterol by ROS in the arterial wall, thereby blocking this crucial step in the development of atherosclerosis (Esterbauer et al., 1993).

The effects of dietary RPO have been demonstrated in various experimental models using both normal and high cholesterol diets. Both working heart model and Langendorrf models have been used to investigate the effect of dietary RPO on ischaemia-reperfusion injury. Previous studies have shown that RPO, when used as a single dietary supplement could provide protection against the detrimental effect of ischaemia/reperfusion injury (Esterhuyse *etal*., 2006; Engelbretch et al., 2006; Engelbretch et al., 2009; Van Rooyen et al., 2008; Bester *et al*., 2010). Serbinova and co-workers (1992) showed that the α-tocotrienol preparations from palm oil protected more efficiently against ischemia/reperfusion injury in a Langendorff perfused rat heart when compared to the tocopherols.

Studies have shown that RPO concentrate has protective effects against ischemia/reperfusion-induced injury (Esterhuyse et al., 2006; Engelbretch et al., 2006). Esterhuyse and co-workers (2006) demonstrated that RPO concentrate supplementation improved functional recovery in hearts subjected to ischaemia/reperfusion injury. Engelbrecht and co-workers (2006) hypothesized that RPO concentrate supplementation may confer protection via the mitogen activated protein kinases (MAPKs) and protein kinase B (PKB/Akt) signalling pathways during ischemia/reperfusion induced injury. These authors demonstrated that RPO concentrate supplementation increased phosphorylation of PKB/Akt and decreased phosphorylation of c-Jun NH2-terminal protein kinase (JNK) during ischemia/reperfusion. The changes in phosphorylation were associated with improved functional recovery and reduced cleavage of an apoptotic marker Poly (ADP-Ribose) Polymerase (PARP). In another study Kruger et al. (2007) demonstrated that RPO concentrate supplementation caused a significant cardio-protection against the adverse effects of a high cholesterol diet through mechanisms that involve the MAPK-signalling pathway. Dietary supplementation of RPO concentrateto rats which were fed a cholesterol enriched diet resulted in increased ERK phosphorylation while phosphorylation ofp38 MAPK and JNK was decreased compared with the cholesterol-supplemented group.The differential phosphorylation of MAPKs was thought to contribute to the decrease in apoptosis that was observed. Evidence from recent studies confirmed that phosphorylation of PKB/Akt plays an important role in the RPO-induced cardioprotection. In this regard Katengua-Thamahane and co-workers (2012) reported that inhibition of PKB/Akt was associated with attenuated functional recovery in RPO supplemented rats. Infarct size is an important functional end point and a reliable predictor of

prognosis after myocardial infarction. Studies from our group have shown that dietary RPO supplementation reduced infarct size in normal and in hypercholesterolaemic rats (Bester et al., 2010; Szucs et al., 2011).

1.6 Polyphenol rich-foods and cardiovascular health

There is over 8000 polyphenols found in food and beverages derived from plant based food stuffs consumed by humans (Crozier et al., 2009;Tsao et al., 2010). Plant based foods and beverages such as wine, rooibos herbal tea, cocoa and resveratrol, which are rich in polyphenols, have been shown to offer cardio-protection and lead to reduction of cardiovascular risk factors (Renaud and debrgeril 1992; Mukamale et al., 2002; Persson et al., 2010). Numerous experimental and clinical studies have investigated and elaborated on potential cardio-protective mechanisms of dietary polyphenols. The mechanisms by which polyphenols have been proposed to offer cardio-protection include antioxidant activity, anti-inflammatory effect, anti-coagulation effect, improvement of endothelial dysfunction and activation of various signal transduction pathways which may lead to improved cell survival (Demrow et al., 1995; Hodgson et al., 2001; Freedman et al., 2001; Hirata et al., 2004; Thirunavukkarasu et al., 2008; Monagas et al., 2009; Xi et al., 2009). Excellent reviews have been written, elaborating on potential anti-inflammatory and cardioprotective mechanisms of polyphenols (Santangelo et al., 2007; Lecour and Lamont 2011).

1.7 Rooibos and health benefits

Rooibos herbal tea, made from the leaves and stems of the shrub-like leguminous bush, *Aspalathus linearis,* is native to Cedarberg mountains in the Western Cape in South Africa (Mckay and Blumberg, 2007). It is rich in polyphenols which include the dihydrochalcones, aspalathin and nothofagin (Mckay and Blumberg, 2007). Aspalathin is a unique phenolic compound to rooibos with other flavonoids including luteolin, quercetin, isoqurcitrin and hyperoside (Joubert et al., 2008). Rooibos has become popular among local people due to the fact that it is caffeine free and contains negligible amounts of tannin (Standlely et al., 2001; Marnewick, 2009). Studies have shown that rooibos has potent antioxidant properties, immune modulating actions and antimutagenic properties among others (Mckay and Blumberg, 2007, Marnewick et al., 2000; 2003; 2004; Marnewick, 2009). Rooibos also demonstrated potential vasodilating properties. In this regard Persson and co-workers (2006) demonstrated that incubation of cultured endothelial cells with rooibos resulted in increased NO production in a dose-dependent manner after 24 hours. In another study Persson et al. (2010) demonstrated that consumption of rooibos was associated with inhibition of

angiotension-converting enzyme (ACE) in humans.This enzyme plays a critical role in the development of cardiovascular pathologies, specifically hypertension. Therefore, the results of these studies present a great potential for further research to elucidate the anti-hypertensive properties of rooibos. Marnewick et al. (2011) reported that consumption of six cups of traditional/fermented rooibos a day for a period of six weeks by adults at risk for developing heart disease, resulted in attenuation of certain cardiovascular disease biomarkers (modulation of lipid profile), enhanced the endogenous antioxidant, glutathione level, resulting in an increased redox status and resultant decrease in lipid oxidative damage (measured as conjugated dienes and thiobarbituric acid reactive substances on HPLC). Rooibos has also been shown to have potential preventive and therapeutic effects on diabetic vascular complications (Ulicna et al., 2006). Pantsi et al. (2011) demonstrated that rooibos consumption offered protection against ischaemia-reperfusion injury when compared to green tea via partial anti-apoptotic mechanism combined with an improved myocardial glutathione status.The beneficial effects of rooibos have been mostly attributed to its polyphenolic content (Liu et al., 2008; Duthie et al., 2003). Aspalathin is not only the unique but also the major phenolic constituent found in both fermented as well as green rooibos and it has been shown to improve glucose uptake in muscle cells, which was associated with increased secretion of insulin and improved glucose tolerance in animal studies (Kawano et al., 2009). Experimental evidence has also demonstrated the immune modulating properties of rooibos. It has been shown that rooibos extracts were associated with stimulation of antigen-specific antibody, associated with enhanced production of IL-10 (Kunishiro et al., 2001; Ichiyama et al., 2007). Addition of rooibos extract to unstimulated cells resulted in induction of IL-6 and IL-10 (Heindricks and Pool, 2010). The antioxidant properties of rooibos has also been linked to its potential anti-inflammatory and DNA protective effects (Baba et al., 2009).

1.8 Hypertension: An overview

Hypertension refers to long term elevation of arterial blood pressure which, if left untreated, can cause end-organ damage which can significantly increase morbidity and mortality associated with cardiovascular diseases (Vaughan and Delanty 2000; Zhou et al., 2001). Several classifications have been devised to diagnose hypertension (Giles et al., 2005). However, the most used diagnostic criteria for defining hypertension in a clinical setting is a blood pressure of 140/90 or more, based on the readings obtained on at least two separate occasions (Carretero and Oparil, 2000). Hypertension can be either primary (essential) with the cause largely unknown or it can be secondary resulting from underlying pathological conditions. Essential hypertension is the most prevalent form resulting in over 90% of all hypertension cases (Carretero and Oparil, 2000). Even though this criterion is routinely used

to start treatment for hypertensive patients, there is significant evidence to show that the risk of death from CHD and stroke increases substantially from blood pressure levels of as low as 115 mmHg systolic pressure and 75 mmHg diastolic blood pressure (Lewington et al., 2002). Hypertension is a significant health problem world wide as well as in Africa (Tesfaye et al., 2009). It is projected that 1 in 4 (1 billion) of the world adult population are currently classified as hypertensive, and this number is expected to increase to more than 1.5 billion, which is 30% of the global population by 2025 (Kearney et al., 2005). The 2002 World Health Report estimated that 9.2% of all deaths in the African region resulted from the cardiovascular events where hypertension was identified as an important risk factor, accounting for 25% to 35% in the adult population (WHO AFRO, 2005; World Health Report, 2002). Cerebrovascular disease, coronary artery disease, heart failure and renal failure constitute a significant cause of mortality in hypertensive patients (MacMahon et al., 1990; Klag et al., 1996; Baigent et al., 2000; Lawes et al., 2003; Woodward et al., 2006; Perkovic et al., 2007). Even though hypertension poses a considerable risk in CVD for millions of people around the globe, it is also a major modifiable risk factor. This implies that aggressive treatment of hypertension has the potential to significantly reduce morbidity and mortality associated with CVD (Carretero and Oparil 2000; Staessen et al., 2001; Mehler et al., 2003).

1.8.1 Regulation of blood pressure

Blood pressure is an important prognostic factor in cardiovascular disease and studies have shown that both systolic and diastolic blood pressures are important determinants of the cardiovascular function and disease (Benetos et al., 1997; Stergiopulos and Westerhof 1998; Assmann et al., 2005; Wong et al., 2011). The control of blood pressure is complex and is subjected to homeostatic regulation by physiological, neurological and endocrine mechanisms. However, cardiac output and total peripheral resistance are key determining factors in the regulation of blood pressure (Fig 2). Both cardiac output and total peripheral resistance are in turn regulated by complex interplay between various physiological mechanisms designed to regulate short term and long term variations in blood pressure (Mayet and Hughes, 2003; Foëx and Sear, 2004).The mechanisms which regulate blood pressure in the short term include the endothelial mechanisms, which mainly involves vasoconstriction and vasodilatation of the small arteries and arterioles, heart rate and contractility of the heart muscles (Griffith et al., 1987; Jackson, 2000). The long term regulatory mechanisms of blood pressure include those factors thar are involved with regulation of blood volume which is mainly regulated by the kidney. In this context the renin-angiotension-aldosterone system plays a pivotal role in the long term maintenance of blood pressure, as such it is regarded as one of the main targets in the effective treatment of hypertension (Bakris, 2007;Dorrington and Pandit, 2009). Anything which may have a

negative effect in anyone of the factors regulating blood pressure will subsequently affect either cardiac output or total peripheral resistance and consequently have effect on normal blood pressure regulation which may result in hypertension.

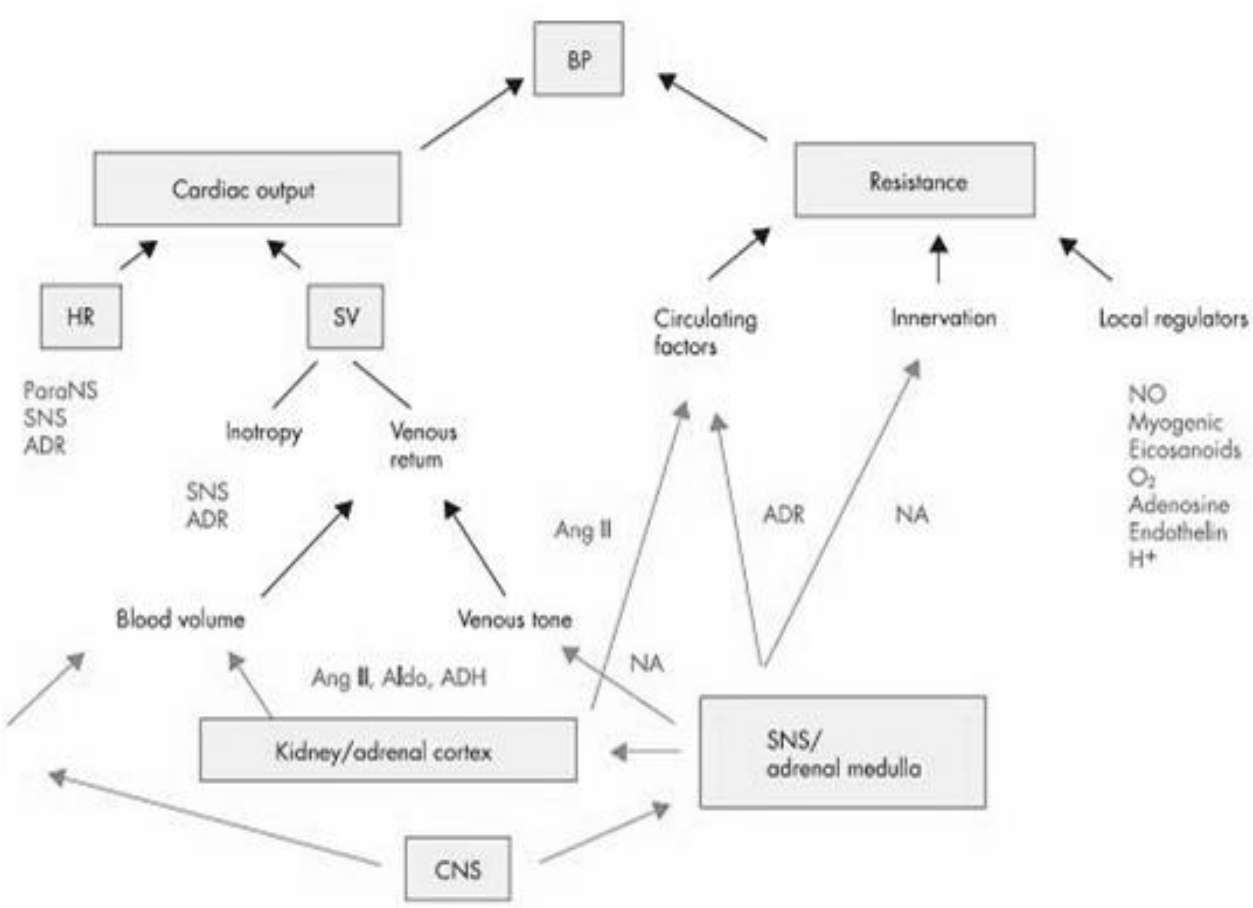

Figure 2: Physiological mechanisms involved in the regulation of mean arterial blood pressure (Mayet and Hughes 2003).

1.8.2 Pathophysiology of hypertension

The aetiology and pathogenesis of essential hypertension is complex and multi-factorial and poorly understood. However, evidence shows that genetic factors and environmental factors may play an important role (Williams et al., 1991; Jeunemaitre et al., 1997). Fetal programming factors such as low birth weight have also been shown to increase the risk of developing hypertension in adulthood among twins, independently of other risk factors (Bergvall et al., 2007).

The hallmark of hypertension is excessive and prolonged elevation of arterial blood pressure. The prolongation in blood pressure elevation induces structural changes in the vasculature

and on the heart. As a result of these alterations in vascular structure, there is a subsequent increase in the thickness of the vessel wall and reduction in the diameter of the vessel lumen ultimately resulting in increased peripheral resistance (Intengan and Schiffrin 2001). The amplification in total peripheral resistances increases the work load against which the heart has to pump blood and this in turn triggers physiological compensatory mechanisms in the heart such as cardiac remodelling. However, as the disease progresses the compensatory mechanisms are overwhelmed and they become pathophysiological (Mayet and Hughes 2003). The adult cardiomyocytes lack the capacity to proliferate and as a result they respond to stress by enlarging their size through increased protein synthesis or reduced protein degradation (Muslin 2008). In hypertension the exposure of the myocardium to prolonged pressure overload is associated with abnormal gene expression and increased deposition of extracellular matrix material (Heineke and Molkentin 2006). Enlargement of individual cardiomyocytes, which is caused by desregulated cardiac gene transcription and the deposition of extracellular matrix, ultimately result in development of cardiac hypertrophy (Lorell, 1995; Bishop and Lindahl 1999; Heineke and Molkentin 2006). Pressure overload-induced ventricular hypertrophy is a very poor diagnostic indicator in humans. It increases the vulnerability of the myocardium to arrhythmias and also contributes to the development of left ventricular diastolic dysfunction and congestive heart failure (Okin et al., 2004). The long term complications of untreated hypertension can affect and cause damage to virtually every major organ in the body including the heart, kidney, brain and the vasculature (Giles et al., 2005), Fig 3.

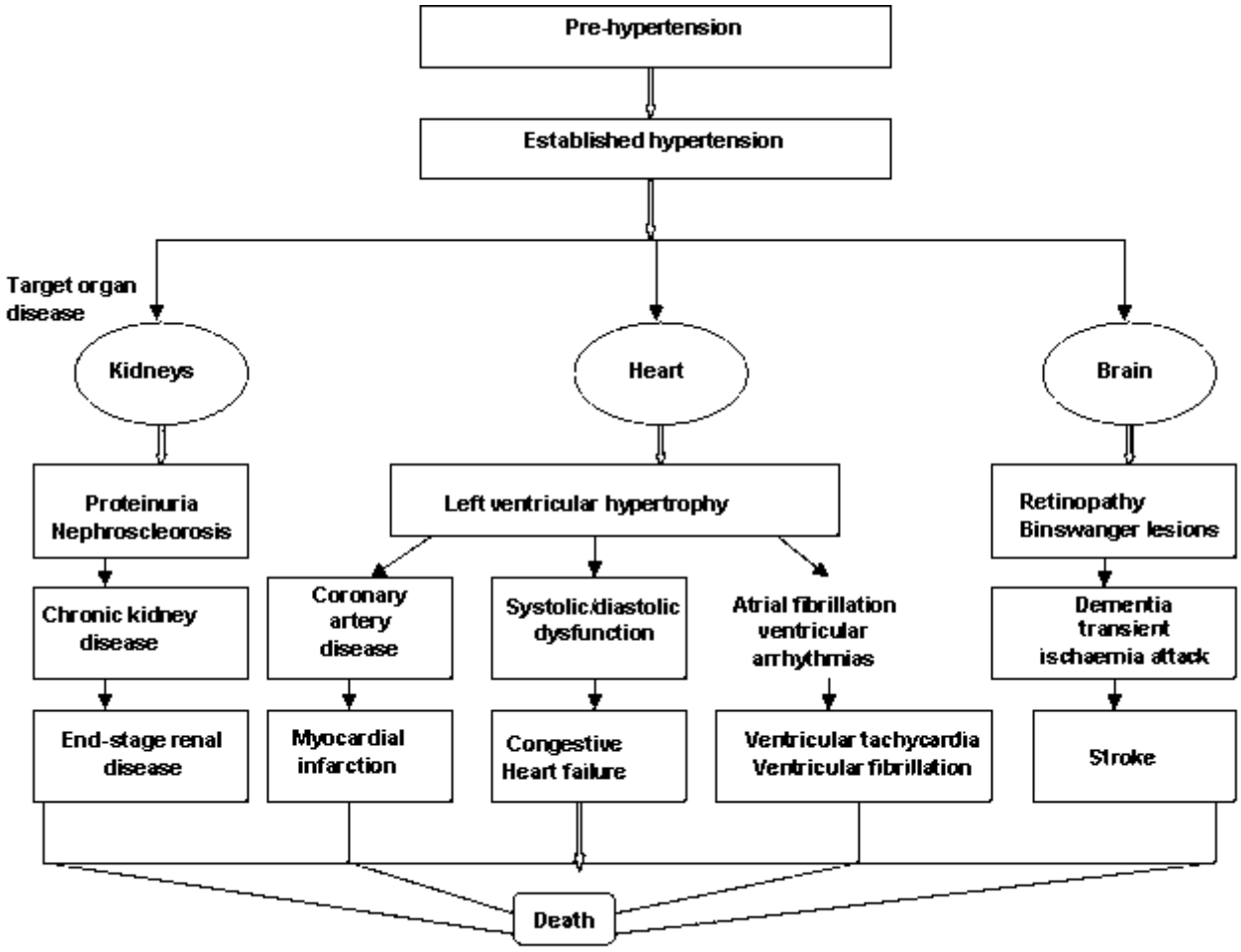

Figure 3: Range of hypertensive cardiovascular disease from pre-hypertension to target organ damage and end-stage disease (adapted from Messerli et al., 2007).

1.8.3 Role of NO-cGMP signalling in hypertension

The endothelium plays a crucial role in the regulation of blood pressure by mainly releasing vasoconstrictors and vasodilators which modulate the vascular smooth muscle tone (Ergul et al., 1996; Puddu et al., 2000). Nitric oxide is one of the most important and potent vasodilators produced by the endothelial cells in response to various stimuli such as changes in blood pressure and shear stress (Oparil et al., 2003). Under normal physiological conditions the balance between vasoconstrictors and vaso-relaxing factors released by the endothelial cells is tightly regulated. However, pathological conditions such as hypertension, diabetes and atherosclerosis can lead to disruption of this balance ultimately resulting in endothelial dysfunction (Oparil et al., 2003; Sainani and Maru, 2004). The resulting changes

can have deleterious effects in hypertensive individuals as they can lead to reduced nitric oxide bioavailability and impairment of endothelium-dependent vasodilation (Kobayashi and Uesugi, 1995, Puddu et al., 2000). Hypertension can also cause fundamental modifications in key signalling pathways such as the NO-cGMP pathway (Bauersachs et al., 1998; Kojda et al., 1998). The importance of NO-cGMP signalling in regulation of blood pressure and in maintenance of vascular tone has been demonstrated by studies showing that deficiency of eNOS and cGMP kinase I in mice resulted in develop hypertension(Huang et al., 1995; Pfeifer et al., 1998; Schlaich et al., 2004). Impairment of the NO-cGMP signalling pathway plays an important role in the pathogenesis of most cardiovascular diseases including hypertension and some of the components of the NO signalling pathway such as the guanylate cyclase are attracting great interest as therapeutic targets (Stasch et al., 2011).

1.8.4 Oxidative stress and hypertension

Hypertension is associated with increased production of ROS which can lead to depletion and reduction of endogenous antioxidant mechanisms (Pedro-Botet et al., 2000). Cells of the vascular system are capable of producing various types of reactive oxygen species (ROS) and reactive nitrogen oxide species (RNOS) to varying magnitudes. These are tightly regulated by cellular antioxidant defence systems under normal physiological conditions (Halliwell 1999; Channon and Guzik 2002; Guzik et al., 2002). However, in conditions of increased ROS production such as it may be the case in hypertension, the normal antioxidant defense mechanisms become overwhelmed leading to a condition of oxidative stress with subsequent oxidative damage to the vascular system (Griendling et al., 2000a; Landmesser and Harrison 2001). Several potential sources of ROS generation in hypertension have been proposed which include enzymatic and non-enzymatic sources, but of particular interest in the pathophysiology of hypertension are NAD(P)H oxidase and the Renin Angiotensin System (RAS) (Berry et al., 2000; Touyz et al., 2002a; Landmesser et al., 2003). Oxidative stress and increased activity of the RAS have been implicated in the pathogenesis of hypertension together with the presence of reduced NO bioavailability (Griendling et al., 1994; Pueyo et al., 2000; Cheng et al., 2005).

Oxidative stress can lead to structural modification of endothelial nitric oxide synthase (eNOS) which can cause loss of its catalytic activity (Förstermann and munzel 2006). Another important consequence of oxidative stress in hypertension is oxidation of tetrahydrobiopterin, a cofactor for eNOS which results in eNOS uncoupling with consequent reduction in NO production and increase in superoxide production (Milstien and Katusic 1999; Xia et al., 1998; Laursen et al., 2001; Landmesser et al., 2003). There is evidence to suggest that oxidative stress may contribute to the pathogenesis of hypertension by altering

the normal homeostasis of calcium regulation. In this regard, it has been shown that oxidative modification of specific amino groups on the calcium channels in the vascular membrane muscle, can result in increased influx of calcium into the cytosol which can lead to increased intracellular calcium overload (Suzuki and Ford 1991; Suzuki and Ford 1999; Viner et al., 1999). Thus oxidative stress can potentially lead to hypertension by the increase in intracellular calcium which can lead to increased peripheral vascular tone (Gordeeva et al., 2003; Tabet et al., 2004; Vasdev et al., 2011). There is comprehensive evidence showing that oxidative stress plays an important role in the pathogenesis of hypertension (Schnackenberg and Wilcox 1999; Vaziri et al., 2000; Chen et al., 2001; El Midaoui and de Champlain 2002). Evidence from these studies demonstrated that administration of antioxidants lead to mitigation of oxidative stress and hypertension.

1.8.5 Inflammation and hypertension

Oxidative stress and inflammation usually occur together in most pathological conditions and the presence of oxidative stress can lead to overstimulation of ROS-mediated signaling pathways. The activated redox sensitive signaling pathway may lead to activation of transcription factors and ultimately resulting in induction of genes that regulate inflammation (Chiarugi and Cirri 2003; Touyz and Schiffrin 2004). Abnormal changes in the immune system including altered profile of pro-and ant-inflammatory cytokines have been identified in patients with essential hypertension (Peteers et al., 2001). It has been shown that hypertension is associated with activation of monocytes and endothelial cells with subsequent increases in pro-inflammatory cytokines (Liu et al., 1996). Inflammatory pathways have been reported to be activated even in the early stages of hypertension, highlighting the significant role of inflammation in the pathogenesis of hypertension (Chae et al., 2001; Vanhala et al., 2008). Rodriguez-Itube and co-workers (2003) demonstrated increased renal infiltration by lymphocytes and macrophages which significantly correlated with systolic blood pressure. Endothelial injury associated with hypertension may cause sustained inflammatory response with release of pro-inflammatory cytokines. This can lead to migration and activation of leukocytes, resulting in injury of the vascular tissue by the infiltrating immune cells into the vascular tissue (Harrison et al., 2011). Based on evidence from both experimental and clinical studies it is clear that alterations in the vascular structure and associate functional changes that usually accompany hypertension play a key role in the pathogenesis of hypertension (Touyz, 2003). Thus antioxidant-rich foods have been shown to have a positive modulatory effect on endothelial function. Ignarro and colleagues (2006) reported improved biological actions of NO by pomegranate juice, which is rich in polyphenolic compounds such as tannins and anthocyanins. The enhancement of NO activity was attributed to the ability of pomegranate to protect NO from oxidative destruction.

1.8.6 Pharmacological intervention in hypertension

There are several pharmacological anti-hypertensive agents available for effective treatment of hypertension, including diuretics, β blockers, calcium blocker, vasodilators and ACE inhibitors (Bakris 2007). Thiazide diuretics which have been shown to possess both blood pressure and lipid lowering effects are usually considered as the first-line therapy for treatment of hypertension in the general population (ALLHAT-LLT trial 2002). For patients with other confounding factors such as diabetes, the first-line treatment of choice is the ACE inhibitors aimed at lowering blood pressure as well as reducing short-term to long-term risks of microvascular complications (Blood Pressure Trialist Collaboration 2005; The HOPE Study 2000; Dahlöf et al., 2002). ACE inhibitors have been linked to significant reduction of overt nephropathy in patients with type 2 diabetes and also significant reduction in the risk of new onset of diabetes in patients at high risk of cardiovascular events (The HOPE Study 2000; Dahlöf et al., 2002). This is mainly due to safety considerations, as long term use of thiazide is associated with increased incidence of diabetes (Franse et al., 2000; ALLHAT-LLT trial 2002). Other treatment choices include calcium channel blockers and β blockers. However, the use of calcium channel blockers is contraindicated in patients with systolic heart failure and proteinuric kidney disease (Tuomilehto et al., 1999).

1.8.7 Non-pharmacological intervention in hypertension

Modification of lifestyle in hypertensive and normotensive individuals is an important intervention to curb hypertension and other cardiovascular risk factors. Adoption of the dietary approach to stop hypertension (DASH diet) has been shown to be an effective intervention having multiple beneficial effects on the cardiovascular risk factors and also on lowering blood pressure. The DASH diet has also been shown to be effective as a first-line therapy in individuals with stage 1 isolated systolic hypertension (Apple et al., 1997; Moore et al., 2001). Adherence to the traditional Mediterranean diet has also been associated with a reduction of the concentration of inflammatory markers in myocardial infarction survivors. The finding of that study implicated the Mediterranean diet in the modulation of low-grade systemic inflammation, which has been shown to play a role in the pathogenesis of endothelial dysfunction and hypertension (Demosthenes et al., 2009).

1.9 Atherosclerosis: A chronic inflammatory disease

Atherosclerosis is a chronic and multifaceted disease whose pathophysiology involves disruption of vascular endothelium, accumulation of atherogenic lipoproteins, macrophages and vascular smooth muscle cells in the sub-endothelial space (Steinbrecher et al., 1984; Ludmer et al., 1986; Berliner et al., 1993; Sorensen et al., 1994; Kinlay et al., 2001; Frazaneh-Far et al., 2001). Even though the pathogenesis of atherosclerosis is complex, evidence suggests that endothelial dysfunction, increased atherogenic LDL and inflammation are important triggering factors (Berliner et al., 1993; Sorensen et al., 1994; Gu et al., 1998; Ross 1999).The integrity of the endothelium can be disrupted by several injurious factors such as hypertension, altered serum lipid profile, hyperglycaemia, smoking and inflammation (Celermajer et al., 1993; Shebuski and Kilgore, 2002; Davignon and Ganz, 2004; Yang et al., 2008; Versari et al., 2009). Damage to the endothelium leads to increased permeability to plasma lipoproteins, causing them to accumulate in the subendothelial space (Frazaneh-Far et al., 2001). The presence of accumulated lipids in the intimal wall is perceived as foreign invaders and hence an inflammatory response is mounted against it in order to get rid of the potential source of injury.

There is credible evidence showing that inflammation plays a critical role in the pathogenesis of atherosclerosis, from the initiation, growth, progression and complications associated with atherosclerotic process (Pearson et al., 2003; Libby, 2006). The early stages of atherosclerosis are characterised by up-regulation of leukocyte adhesion molecules which play an important role in recruiting inflammatory cells into the atherosclerotic lesion (Cybulsky and Gimbrone, 1991; Marui et al., 1993; Libby and Okamoto 2010). The presence of cytokines and other inflammatory mediators in the sub-endothelial space can lead to increased oxidative modification of LDL (Cybulsky and Gimbrone, 1991). Oxidation of LDL is believed to be the major cause of endothelial injury in the process of atherosclerosis and it has been shown to have pro-inflammatory properties (Steinberg et al., 1989; Berliner et al., 1993; Berliner et al., 1995). The macrophages which have been recruited in the intimal wall have increased expression of scavenger receptors and as a result they exhibit higher affinity for accumulated oxidized LDL, leading to their internalization by macrophages to form foam cells (Steinbrecher et al., 1984). The oxidized LDL particles in the arterial wall create a vicious cycle of inflammation which leads to further oxidation (Frazaneh-Far et al., 2001; Berliner et al., 1995). Therefore, scientific evidence suggests that oxidation of LDL together with increased inflammation and endothelial dysfunction play a fundamental role in the

pathogenesis of atherosclerosis (Steinbrecher et al., 1984; Steinberg et al., 1989; Harrison, 1997).

1.9.1 Atherosclerosis and acute ischaemic events

The rupture of the atherosclerotic plaque and subsequent thrombosis is the cause of most cases of fatal myocardial infarction (Kume et al., 2009; Kashiwagi et al., 2009). Atherosclerosis is also a common cause of a wide spectrum of clinically related diseases including stroke, abdominal aneurysms and lower limb ischaemia and all of these pathologies are the leading cause of morbidity and mortality in the Western world (van der Wal and Becker, 1999). The common pathogenic mechanisms underlying most complications of atherosclerosis are fracture in the protective fibrous cap of the plaque and thrombosis, which can ultimately lead to precipitation of the occlusion of blood vessels (Falk 1989; Eliasziv et al., 1994; Falk, 1999; Libby, 2001). Certain plaques are particularly more vulnerable to disruption and thus are more likely to cause manifestations of acute coronary syndromes such as myocardial infarction or unstable angina (Libby, 1995). The integrity of the fibrous cap underlying the lipid-rich core plays a critical role in determining the stability of the atherosclerotic plaque and this in turn is determined by the cellular composition of the fibrous cap (Geng et al., 1997; Libby and Okamoto, 2010). Other important factors which can lead to destabilization of the plaque include apoptosis and increased production and activity of pro-inflammatory extracellular matrix metalloproteinases (MMP) especially in advanced lesions (de Nooijer et al., 2006; Nabata et al., 2008).

Numerous studies have highlighted the crucial role played by both acute and chronic inflammation in coronary artery disease patients presenting with acute myocardial infarction (Ridker et al., 2000; Asanuma et al., 2003; Maradit-Kremers et al., 2005, Klingenberg and Hansson, 2009). Targeting inflammation with pharmacological intervention in atherosclerotic cardiovascular disease has been shown to have a beneficial role in prevention of cardiovascular events (Bustos et al., 1998; Ridker et al., 2008; Ridker et al., 2009). In this regard, initiation of rosuvastatin in the JUPITER trial was associated with reduction of LDL cholesterol together with C-reactive protein, a known marker of inflammation. These findings were paralleled with reduced cardiovascular events (Ridker et al., 2009). ACE inhibitors normally used for their blood pressure lowering effect have been shown to have potent anti-inflammatory properties in a rabbit model of atherosclerosis (Hernandez-Presa et al., 1997). Dietary interventions have been shown to reduce the vulnerability of atherosclerotic lesions to rapture and thrombosis by reducing the expression and activity of MMP thus leading to lowering of coronary events and strokes (Aikawa et al., 1998; Libby et al., 2001). Evidence

presented above shows that a comprehensive strategy aimed at reducing cardiovascular morbidity and mortality should include a cardio-protective diet in association with pharmacological interventions targeting multiple pathways.

2.0 Myocardial infarction

Myocardial infarction is the commonest complication of ischaemic heart disease encountered in clinical cardiology. Ischaemic heart disease occurs as a result of mismatch between the myocardial blood flow and its metabolic demands which usually results from rupturing of progressive and unstable atherosclerotic plaques and its associated complications (Anversa and Sonnenblick, 1990; Anversa et al, 1995). The occlusion of one or more coronary arteries can cause an ischaemic episode resulting in reduced oxygen supply to the myocardium and accumulation of metabolic waste products. After sustained periods of myocardial ischaemia, cells may undergo irreversible injury leading to necrosis of cardiac tissue causing the normal contractile cardiac tissue to be replaced by non-functional scar tissue (Sabine et al., 2009). Pathological remodelling of the myocardium can result from myocardial cell loss following acute myocardial infarction as well as a consequence of chronic exposure of the myocardium to pressure overload in hypertension. In both instances there is associated myocardial hypertrophy with ultimate deterioration to congestive heart failure and vulnerability to arrhythmias (Frey and Olson 2003; Diwan and Dorn 2007). Restoration of coronary blood flow after acute myocardial infarction is currently the only established and clinically approved method to limit infarct size and this can be achieved by percutaneous coronary interventions, thrombolic agents or coronary by-pass surgery (Takayuki et al., 2012). The main aim of treatment of acute myocardial infarction is restoration of the disrupted coronary flow as soon as possible with the ultimate goal of reducing myocardial infarct size in the long run and also to optimize cardiac repair following myocardial infarction (Sabine et al., 2009). Various studies have shown that early reperfusion therapy can salvage myocardium at risk from injury (Panes et al., 1999, Tsujita et al., 2004; Ovize et al., 2010). As a result of this observation it is now a clinically established standard that reperfusion therapy for ischaemic heart disease patients should be established within 3 to 6 hours after the onset of ischaemia (Simonis et al., 2012). Even though timely reperfusion of the myocardium after myocardial infarction is the prerequisite to salvage the viable myocardial cells, it has been shown to contribute to lethal injury following prolonged periods of ischaemia (Przyklenk, 1997).

2.1 Ischaemia-reperfusion injury

Ischaemia-reperfusion injury is a clinically relevant occurrence associated with diverse clinical disorders such as organ transplantation and reperfusion after thrombotic events (Ovize et al., 2010). Numerous mechanisms are involved in the induction of cardio-myocyte necrosis by ischaemia-reperfusion injury. These may include depletion of intracellular ATP, intracellular calcium and sodium ion overload and cell membrane fragility and the opening of the mitochondrial permeability transition pores (MPTP) (Halestrap, 2010; Takayuki et al., 2012,). Ischaemia-reperfusion injury is associated with increased ROS production and inflammation both of which can contribute to myocardial cell death following ischaemia-reperfusion (Mukherjee et al., 2002; Cailleret et al., 2004). However, ROS can also play an important role in stimulating signal transduction leading to induction of inflammatory cytokines. Induction of inflammatory cytokines in the ischaemic-reperfused myocardium can serve as a host response to injury and thus representing a cell survival regulatory mechanism (Nian et al., 2004). ROS and inflammatory cytokines have a cardio-depressant effect which is associated with altered intracellular calcium homeostasis (Waypa et al., 2002). The intracellular calcium overload mediated by ROS can lead to myocardial necrosis via enhanced opening of the MPTP, while inflammatory cytokines can lead to apoptotic cell death through the TNF-α signaling pathway (Mukherjee et al., 2002; Cailleret et al., 2004). The importance of oxidative stress and inflammation in ischaemia-reperfusion injury has also been shown by dietary intervention with α-linolenic acid which resulted in attenuation of ischaemia-reperfusion injury by exerting anti-inflammatory and antioxidantive effects (Xie et al., 2011).

3.0 Inflammation and CVD

Inflammation is a complex and well orchestrated response of immune cells to tissue injury which can result from physical agents or invasion by microbial pathogens. Induction of inflammation is normally aimed at protecting the organisms against the spread of injury or infection. However, failure of the inflammation to resolve can have detrimental consequences as the affected tissues can fail to be restored to their normal structure and function. Hence, the inflammatory response which started off as an acute response can result in chronic inflammation (Nathan and Ding 2010).

There is credible evidence implicating inflammation and increased activation of the coagulation system in the pathogensis and development of CHD (Yarnell et al., 2005; Libby et al., 2006). Elevated plasma levels of inflammatory cytokines have been reported in various cardiovascular conditions such as diabetes, atherosclerosis, myocardial infarction and heart

failure suggesting the causal role for inflammation in the pathogenesis of these conditions (Nian et al., 2004). Inflammatory mediators have dual roles in the setting of ischaemia-reperfusion injury, in acute reperfusion injury and also in cardiac repair. The inflammatory response may also contribute to adverse remodelling of the ventricle by triggering degradation of extracellular matrix (Steffens et al 2009). Some of the beneficial effects of CVD medications and the life style medification interventions such as diet are at least in part attributable to their anti-inflammatory properties (Aikawa et al., 1998; Bustos et al., 1998; Hernandez-Presa et al., 1998).

Myocardial infarct size is the major prognostic determinant in ischaemic heart disease patients (Takayuki et al., 2012). The predominant mode of cell death during myocardial infarction is necrotic cell death as opposed to apoptosis. The intra-cellular contents of necrotic cells which are released into the extracellular matrix can serve as danger signals and result in inititiation of an inflammatory response (Rock and Kono, 2008). Moreover, infarcted myocardium is associated with increased production of ROS which can also act as inflammatory signals resulting in production of pro-inflammatory cytokines which may have deleterious effect on the myocardium (Hori and Nishida 2009). Postconditioning intervention strategies with anti-inflammatory agents applied during early reperfusion, have been shown to attenuate ischaemia-reperfusion injury (Xiong et al.,2012). This shows that addition of adjunct reperfusion therapies at the onset of reperfusion have the potential to salvage more myocardial cells over and above that which can be salvaged by reperfusion alone (Piper, 1998; Ovize et al., 2010). Therefore, development of novel interventional strategies aimed at limiting myocardial infarction represent great clinical therapeutic target.

3.1 Pro-inflammatory cytokines and anti-inflammatory cytokines

Cytokines represent a diverse group of secreted low-molecular weight polypeptides which facilitate communication between immune cells and play a crucial role in coordinating inflammatory responses (Borish and Steinke, 2003; Mak and Saunders, 2006). Pro-inflammatory cytokines have been implicated in exerting a negative inotropic effect on the myocardium (Guillen et al., 1995; Oral et al., 1997; Stangl et al., 2002). There is evidence to suggest that induction of pro-inflammatory cytokines following ischaemia-reperfusion injury could represent the myocardial adaptation to oxidative stress (Maulik et al 1993). The role of cytokines in inflammation is complex and is determined by various factors such as the magnitude of cytokine induction, the presence of receptors to cytokines and also by the presence of antagonist mediators such as anti-inflammatory cytokines. Thus, the net physiological or pathological effect of cytokines at any given inflammatory site is greatly determined by the tightly regulated balance between pro and anti-inflammatory mediators

(Feldmann et al., 1996). Inflammatory response and subsequent cytokine release constitutes an integral component of the body's normal response to myocardial injury following myocardial infarction (Nian et al., 2004). Acute induction of cytokines can confer cellular survival mechanism or it can be detrimental depending on the magnitude of cytokines released and the degree of the inflammatory response elicited after myocardial infarction (Nian et al., 2004).

3.1.1 IL-1β

The IL-1β family of cytokines is the main mediator of inflammatory reactions (Stangl et al., 2002). There are two isoforms of IL-1 cytokines, namely: IL-1α and IL-1β which share structural similarities and bind to the same receptor, therefore elicit similar biological functions (Guillen et al., 1995). IL-1α is primarily associated with the membrane while IL-1β is secreted in the blood and has been shown to be the main pro-inflammatory cytokine that appears in plasma after myocardial infarction (Guillen et al., 1995). IL-1β is one of the initial pro-inflammatory cytokines to be released in response to the invading microbial pathogens (specifically the lipopolysaccharide, an endotoxin embedded within the bacterial membrane) and it plays a crucial role in the initiation of inflammation (Kadokami et al., 2001). IL-Iβ plays a critical role in mediating inflammatory processes through induction and expression of inflammatory genes such as cyclooxygenase-2 and and iNOS which can ultimately lead to eicosanoids (White et al., 2008). IL-1β has been shown to increase production of NO in the rat myocardium by inducing iNOS expression possibly via increased de-novo protein synthesis of iNOS (Pinsky et al., 1995). The TLR4 signalling during ischaemia-reperfusion has been shown to contribute to cardiac dysfunction via production of TNF-α and IL-1β (Cha et al., 2008).

3.1.2 IL-6

IL-6 is classically characterized as a pro-inflammation cytokine, however, it has also been shown to have both pro-inflammatory and anti-inflammatory features (Damas et al., 1992; Steensberg et al., 2003; Wu and Schauss 2012). Studies have shown that IL-6 can evoke an anti-inflammatory environment in some instances by inducing the production of anti-inflammatory cytokines, such as IL-10 and IL-1ra in humans (Steensberg et al., 2003). Xing and co-workers (1998) showed that endogenous IL-6 plays an anti-inflammatory role in both local and systemic acute inflammatory responses in mice. This mechanism acts by controlling the level of pro-inflammatory, but not anti-inflammatory cytokines (Tilg et al., 1994; Yasukawa et al., 2003). Others have also shown that blockade of IL-6 in patients with

rheumatoid arthritis led to enhanced cholesterol and plasma glucose levels, indicating a role for IL-6 in modulation of glucose and lipid metabolism (Choy et al., 2002; Nishimoto et al., 2004). IL-6 acts through activation of the gp130 to activate various signaling pathways including the JAK-STAT in the setting of ischaemia-reperfusion. It has been shown to play an obligatory role in late preconditioning via the JAK-STAT signaling and upregulation of iNOS and COX-2 (Dawn et al., 2004)

3.1.3 IL-10

IL-10 is one of the predominant and well studied cytokines in the family of anti-inflammatory cytokines. Although it has been shown to be released by various cells of the immune system in response to a variety of cell injury and shock, substantial evidence indicates that it is mainly produced by T-cell regulatory cells (Perretti et al., 1995; Standiford et al., 1995; Shibata et al., 1997; Vignali et al., 2008). The presence of an inflammatory stimulus such as LPS evokes the production of pro-inflammatory cytokines which is initially released as a response to the danger. However, the body has also evolved regulatory systems to maintain the balance between the levels of pro-inflammatory mediators and anti-inflammatory mediators in order to sustain cellular homeostasis and immune system integrity. IL-10 is a potent ant-inflammatory cytokine whose role is to counteract the effects of pro-inflammatory mediators in various forms of shock and inflammation (Rennick et al., 1997; Vignali et al., 2008). Elevated myocardial levels of IL-10 have been shown to have cardio-protective effects (Jones et al., 2001). The importance of IL-10 in maintaining myocardial integrity has also been shown by studies which showed that genetic deletion of IL-10 was associated with enhanced inflammation and increased myocardial infarction and necrosis (Yang et al., 2000). The importance of endogenous IL-10 in ischaemia-reperfusion has been reported in other tissues. In this regard Zingarelli and colleagues (2001) demonstrated that IL-10 deficient mice experienced increased mortality and more severe tissue injury compared to wild-type mice after subjection to ischaemia and reperfusion. Based on the evidence obtained, the authors suggested that endogenous IL-10 exerted an anti-inflammatory effect during reperfusion injury. Further more the levels of plasma IL-10 has been shown to be decreased in patients with heart failure (Stumpf et al., 2003).

4.0 The role of NFkB in inflammation and regulation of cell survival

NFkB is a ubiquitously expressed, redox sensitive transcription factor which plays important roles in the regulation of a number of genes involved in inflammatory response, cell survival and cell death (Mustapha et al., 2000; Misra et al., 2003). The activation of NFkB can be

triggered by numerous cellular stresses such as oxidative stress, ischaemia-reperfusion, LPS and cytokines (Valen et al., 2001; Valen, 2004). In unstimulated cells, NFkB normally remains in the cytoplasm bound to its inhibitor protein (IkB). Upon stimulation, IkB becomes phosphorylated which then lead to its degradation by proteasomes. The release of NFkB from IkB causes a conformational change in the structure of NFkB facilitating its activation and translocation to the nucleus where it induces transcription of numerous genes which either confer protection or serve as stress alert (Valen, 2004). NFkB has been shown to regulate cardiac myocyte survival through inhibition of apoptosis in ventricular myocytes (Mustapha et al., 2000). In another study Misra and colleagues (2003) reported that transgenic mice expressing a cardiac specific inhibitor of NFkB alpha displayed a greater infarct size and increased apoptosis. One of the ways by which NFkB is believed to regulate cell survival is through induction of anti-apoptotic protein genes such as Bcl2 and SOD2 (Catz and Johnson, 2001). NFkB plays an important role in controlling cellular homeostasis by regulating transcription of inducible genes in response to cellular stress such as oxidative stress and ischaemia-reperfusion injury (Perkins 1997). Thus acute activation of NFkB plays a vital role in the regulation of cell survival, however, chronic activation of NFkB has been shown to have detrimental effect (Mustapha et al., 2000).

5.0 p38 MAPK signalling and regulation of cell function

p38 mitogen-activated protein kinases (MAPK) are serine/threonine kinases which transduce signals from the cell membrane to the nucleus in response to a variety of stimuli, resulting in a wide range of cellular effects including gene expression, cell division, apoptosis, inflammatory response and metabolic changes (Behrend et al., 2003; Baines and Molkentin, 2005; Cleark and Sugden, 2006). p38 MAPK and other MAPKs can be activated by a variety of cellular stresses including oxidative stress, ischaemia-reperfusion, heat shock and inflammatory cytokines (Weinbrenner et al 1997; Anu Punn et al., 2012). Upon activation MAPKs phosphorylate their substrates at specific serine/threonine residues and the phosphorylation of the substrates results in either a positive or negative regulation of the substrates (Behrend et al., 2003). Some of the well characterised or studied substrates of p38 are the transcription factors, AP-1, Nrf1 and NFkB which when activated, can translocate to the nucleus and bind to their specific DNA binding sequence and cause changes in gene transcription (Wada et al 2004). p38 MAPK has been shown to play an important role in response to ischaemia-reperfusion injury (Weinbrenner et al., 1997; Mocanu et al., 2000; Schulz et al., 2002; Bassi et al., 2008). The role of p38 MAPK in ischaemia-reperfusion has been a subject of great debate: there are two schools of thought concerning its roles in the setting of ischaemia-reperfusion, with some evidence showing that increased phosphorylation of p38 is protective, while others believe that it is detrimental (Wang et al.,

1998; Mocanu et al., 2000). Wang and co-workers (1998) demonstrated that the two p38 isoforms expressed in the heart displayed opposing cellular effects with p38 α implicated in apoptosis while p38 β was associated with anti-apoptotic effects. Mocanu and co-workers (2000) showed that inhibition of p38 MAPK phosphorylation was associated with abrogation of ischaemic preconditioning and they also showed that timing of activation and inhibition of p38 MAPK play a critical role in determining the outcome of p38 activation in the heart. p38 α has been shown to play a protective role against myocyte apoptosis and cardiac remolling (Nishida et al., 2004). Another study reported that activation of p38 in the heart resulted in phosphorylation of small heat-shock protein which was associated with enhanced protection against ischaemia-reperfusion (Martendale et al., 2005).

6.0 Antioxidant enzymes as cardiac therapeutic targets

Antioxidant enzymes play an important role in regulating the redox status of the cell by protecting the cells from oxidative stress. Under normal conditions the protein level and the activity of antioxidant enzymes are subjected to tight regulation at transcriptional, translational and post translational levels. Antioxidants have also been shown to affect translational and transcriptional regulation of antioxidant enzymes (Sadi and Guray 2009, Sadi et al., 2013). SOD2 is found in the mitochondrial matrix where it catalyses the conversion of superoxide to hydrogen peroxide and oxygen. It has been shown that delayed ischaemic preconditioning lead to upregulation of SOD2 and reduction of ROS (Bolli et al., 2007). One of the ways by which organisms protect themselves against oxidative stress is through enhanced production of endogenous antioxidant defence systems (Polisak, 2011). Reperfusion of the ischaemic myocardium results in an increased production of ROS generating a condition of oxidative stress with potential depletion of myocardial antioxidants. It has been shown that the mitochondria are the major generators and the main targets of ROS during oxidative stress conditions (Assalyn et al., 2012). SOD2 and GPX1 are antioxidant enzymes which are mainly located in the mitochondrial, as such they play a key role in protecting the myocardial mitochondria from oxidative damage (Okado-Matsumoto and Fridovich 2001), Fig 3. Emerging evidence suggests that blockade of mitochondrial ROS is a promising therapeutic target for inhibiting pro-inflammatory disease in atherosclerosis (Aon et al., 2010; Tschopp 2011; Handy and Loscalzo, 2012).

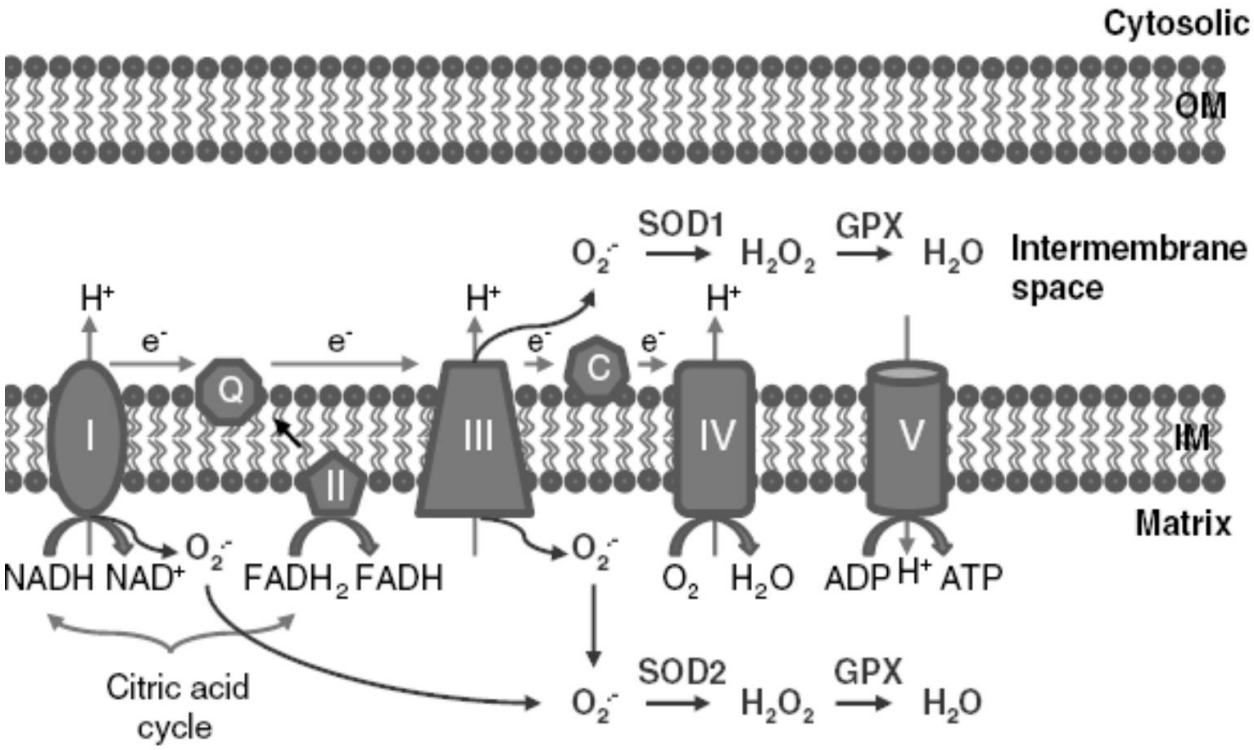

Figure 4: Production and disposal of mitochondrial ROS (Li et al., 2013)

References chapter 2

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CHAPTER 3

Effect of red palm oil on cardiac function in genetically hypertensive rats: role of NO and antioxidant defence enzymes

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Abstract

Red palm oil (RPO) protects the heart in various pathological states, including hypertension. We used a genetic model of experimental hypertension to investigate the role of the antioxidant enzyme system in cardiac protection. Male 3 months old spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY) were fed standard rat chow plus or minus RPO (200ul/day) for 5 weeks. Antioxidant enzymes were determined from left ventricular tissue while total NOS activity was determined in the aorta and left ventricle. Cardiac function was monitored at stabilization and reperfusion using the Langendorff system. RPO improved cardiac function in SHR by improving reperfusion LVP, maximum velocity of pressure rise +dp/dt max and fall -dp/dt max compared to their controls. The +dp/dt max was increased by 26% in SHR+RPO (SHRrpo) vs SHR+control diet (SHRc) $^{x}p<0.05$. Improved function was associated with increased SOD2 protein expression by 39% vs SHRc ($p<0.01$). NOS activity increased in the aorta but reduced in the heart of both WKYrpo and SHRrpo vs. their control. These results argue a role for elevated NO production in the aorta for endothelial function maintenance. Increased SOD2 protein might lead to reduced oxidative stress. Thus, NO-cGMP pathway and antioxidant defense systems synergistically acted to restore cardiovascular function in SHR.

1.0 Introduction

Hypertension is an enormous public health burden which poses significant cause of morbidity and mortality associated with cardiovascular diseases, in both the developing and developed countries. It is also an important independent risk factor for cardiovascular diseases [1, 2.] The pathophysiology of essential hypertension is complex and poorly understood but may result from genetic and environmental factors [3, 4, 5]. It has been shown that increased cardiovascular response to sympathetic nerve activity, which is usually associated with obesity, could also play an important role in the pathogenesis of hypertension [6]. The hallmark of hypertension is excessive and prolonged elevation of blood pressure, which if left untreated, could have serious implications such as end stage renal failure and increased incidences of heart failure [1, 7]. Some of the most serious complications of hypertension include left ventricular and vascular hypertrophy, endothelial dysfunction, compromised ventricular function and increased susceptibility of the myocardium to ventricular arrhythmias and sudden cardiac death [8]. Cardiac hypertrophy is one of the most deleterious consequences of hypertension and it has been shown to be an important predictor of mortality in hypertensive heart disorders [9, 10, 11].

A growing body of scientific evidence suggests that oxidative stress may play a pivotal role in the pathogenesis of hypertension [12, 13, 14, 15, 16]. Evidence from these studies indicates that increased production of reactive oxygen species (ROS) in hypertension may lead to impairment of endogenous antioxidant defense systems. Therefore, oxidative stress has been implicated in either the pathogenesis or exacerbation of hypertensive heart disorders [17]. One important mechanism by which oxidative stress is thought to play an important role in the pathophysiology and development of hypertension is through increased production of superoxide and hydrogen peroxide which may lead to decreased nitric oxide bioavailability [18, 19, 20, 16].

Studies have shown that RPO improves reperfusion functional recovery in healthy rat hearts subjected to ischaemia reperfusion injury [21, 22, 23]. More studies reported cardioprotective effects of RPO in hyperlipedemic rats exposed to ischaemia-reperfusion [24, 25]. Esterhuyse and co-workers (2006) [24] proposed that one of the possible mechanisms underlying RPO-induced cardioprotection could be via modulation of the NO-cGMP signalling pathway during the ischaemic period with a potential to increase nitric oxide bioavailability. Szucs *et al*., (2011) [25] showed that RPO reduced myocardial infarct size in hearts of rats fed a high cholesterol diet. In another study RPO supplementation protected hearts against anthracycline-induced cardiac toxicity via attenuation of both mRNA and protein levels of SOD1 [26]. Evidence from this study suggested that the cardioprotective effects of RPO in the model of anthracycline induced-cardiac toxicity could be partly related to the antioxidant

activity of RPO and its ability to modulate the oxidative sensitive MAPK signalling pathways. Bačová and co-workers (2012) [27] recently demonstrated, for the first time, that dietary RPO supplementation reduced blood pressure and blood glucose in spontaneously hypertensive rats (SHR) compared to non-supplemented control rats. They further showed that RPO significantly reduced the incidence of lethal arrhythmias in spontaneously hypertensive treated with RPO compared to their control counterparts. This anti-arrhythmic effect was linked to up-regulation of connexin 43.

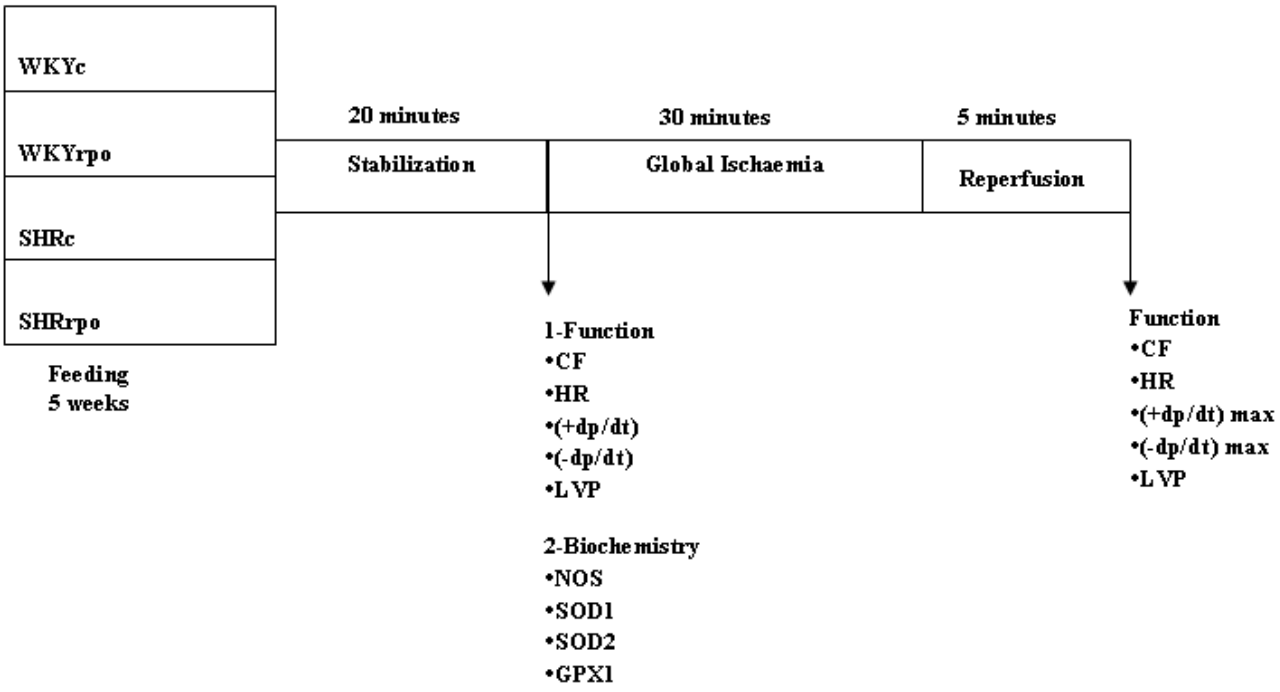
There is substantial evidence from experimental models showing that dietary RPO is effective in protecting the heart against ischaemia-reperfusion injury [21, 22, 23, 24, 25, 26]. The findings from these studies indicate that the beneficial effect of RPO on cardiovascular health may involve its ability to improve endogenous antioxidant defence mechanisms and modulation of the redox sensitive signalling pathways. Most importantly recent evidence show that dietary RPO exhibited cardioprotection in SHR [27].

In view of this evidence the aim of this study was to investigate whether the antioxidant enzymes and the NO-cGMP pathway may play a role in RPO induced cardiovascular protection in spontaneously hypertensive rats. For the first time, this study would attempt to include vasculature aspects in the cardio-protective effect of red palm oil.

2.0 Materials and Methods

2.1 Animal care and ethical consideration

All animal experiments were performed in accordance with the rules issued by the State Veterinary Administration of the Slovak Republic, legislation No 289/2003 and they conform to the 'European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985). Male, three-month-old spontaneously hypertensive rats (SHR) and non-hypertensive Wistar-Kyoto rats (WKY) fed a standard rat chow plus RPO (200ul/day) for 5 weeks were compared with untreated controls. Hypertension in animals was confirmed as mentioned in Bacova *et al.,* (2012) [27]. The hearts were rapidly excised into ice-cold saline from the anaesthetized rats, whereby whole heart and left ventricular weights were registered. Left ventricular tissue was used for analysis of myocardial antioxidant enzymes and isolated Langendorff-perfused rat hearts were used for registering heart function. Total NOS activity was determined immediately in fresh tissue of the aorta and left ventricle.



- **CF- coronary flow**
- **HR- heart rate**
- **(+dp/dt) max- maximum velocity of pressure rise**
- **(-dp/dt) max- maximum velocity of pressure fall**
- **LVP- left ventricular pressure**
- **NOS- total nitric oxide synthase**
- **SOD1- Cu/Zn superoxide dismutase**
- **SOD2- Mn superoxide dismutase**
- **GPX1- glutathione peroxidase 1**

Figure 1: Study design showing experimental groups, feeding period, perfusion protocol and the arrows indicating where functional parameters were documented and also where biochemical samples were collected.

2.2 Experimental protocol of isolated Langendorff-perfused heart

The heart of 6 rats from each group was perfused via canulated aorta in Langendorff mode with oxygenated Krebs-Henseleit solution at constant pressure of 80mmHg and temperature of 37^0 C. Left ventricular pressure (LVP) was measured isovolumetrically by a water-filled latex balloon, which was introduced into the left ventricle through the mitral orifice and connected to a pressure transducer. LVP, Heart rate, and the coronary flow (CF) were continuously monitored during experiment for the evaluation of heart function. Left ventricular systolic function was assessed by recording LVP and the positive and negative first derivatives of LVP, +dp/dt (mmHg/s), -dp/dt (mmHg/s). The latter are sensitive indices of contractile function with respect to the rate of increase and rate of decrease of intraventricular pressure, respectively. Upon 20 min of equilibration the heart was subjected

to 30 min global ischemia followed by 5 min reperfusion to examine early reperfusion-induced changes in heart function.

2.3 Measurement of Nitric oxide synthase activity

NO synthase activity was measured in tissue homogenates of the aorta and left ventricle. Tissues (200 mg/ml) were collected to ice cold homogenisation buffer. Homogenisation buffer contained 1% Protease inhibitor cocktail (104 mmol/l 4-(2-aminoethyl)benzenesulfonyl fluoride, 80 µmol/l Aprotinin, 4 mmol/l Bestatin, 1.4 mmol/l E-64, 2 mmol/l Leupeptin, 1.5 mmol/l Pepstatin A, purchased from Sigma-Aldrich) in 0.05 mol/l Tris-HCl, pH 7.4. Then the tissues were immediately homogenised using an Ultra-Turrax homogeniser at 4°C and centrifuged (4°C, 15 min, 9500 g). After centrifugation, total NO synthase activity was determined in the supernatants by conversion of [^{3}H]-L-arginine (MP Biomedicals, USA, 50 Ci/mmol) to [^{3}H]-L-citrulline as described previously [28] and expressed as pmol/min/mg of proteins.

2.4 Analysis of myocardial SOD1, SOD2 and GPX1 using Western blot analysis

Analysis of basal myocardial antioxidant protein expression levels was carried with the use of western blot protein analysis. Cardiac proteins 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 equal amounts of protein concentration were loaded per lane and subjected to PAGE-SDS gel electrophoresis (Bio-RAD Mini Protein Tetra cell 552BR). 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. Non-specific binding sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline – 0.1% Tween 20 (TBST). Membranes were then probed with primary antibodies which recognise SOD1, SOD2 and GPX1 and incubated overnight. SOD1 and GPX1 were diluted 1:1000 and SOD2 was diluted 1:2000. Membranes were subsequently washed with large volumes of TBST (5x3 minutes), and incubated with the secondary antibody conjugated with alkaline-phosphotase for one hour with continuous shaking at room temperature. After thorough washing with TBS-T, membranes were covered with a chromogenic substrate (Protein Detector^{TM}, Western Blot Kit, BCIP/NBT System, KPL Inc) for chromogenic detection and visualization of membrane-bound proteins. Protein loading was normalized with beta actin to ensured equal protein loading for different lanes.

Antibodies were purchased from Cell Signalling and abcam. Other chemicals were obtained from BIO-RAD and Sigma (St Louis, MO).

2.5 Statistical analysis

The data are expressed as means ± SEM. A two-way ANOVA and Bonferroni post hoc test were used to analyze statistical significance between means. Values were considered to differ significantly when was $p<0.05$.

3.0 Results

3.1 Effect of dietary RPO supplementation on coronary flow (CF) in WKY and SHR during stabilization and reperfusion.

RPO significantly increased CF during stabilization in WKYrpo compared to SHRrpo during stabilization, there was no significant difference observed in normotensive controls compared to hypertensive control rats at stabilization. RPO improved coronary flow by approximately 60% in WKYrpo compared to their control counter parts during the first five minutes of reperfusion ($^{*}p<0.05$). A significant increase in coronary flow was also observed in SHRrpo compared to their control counterparts: (8.50±1.02 vs 14.94±1.31 ml/min, $^{x}p<0.05$) Fig 2.

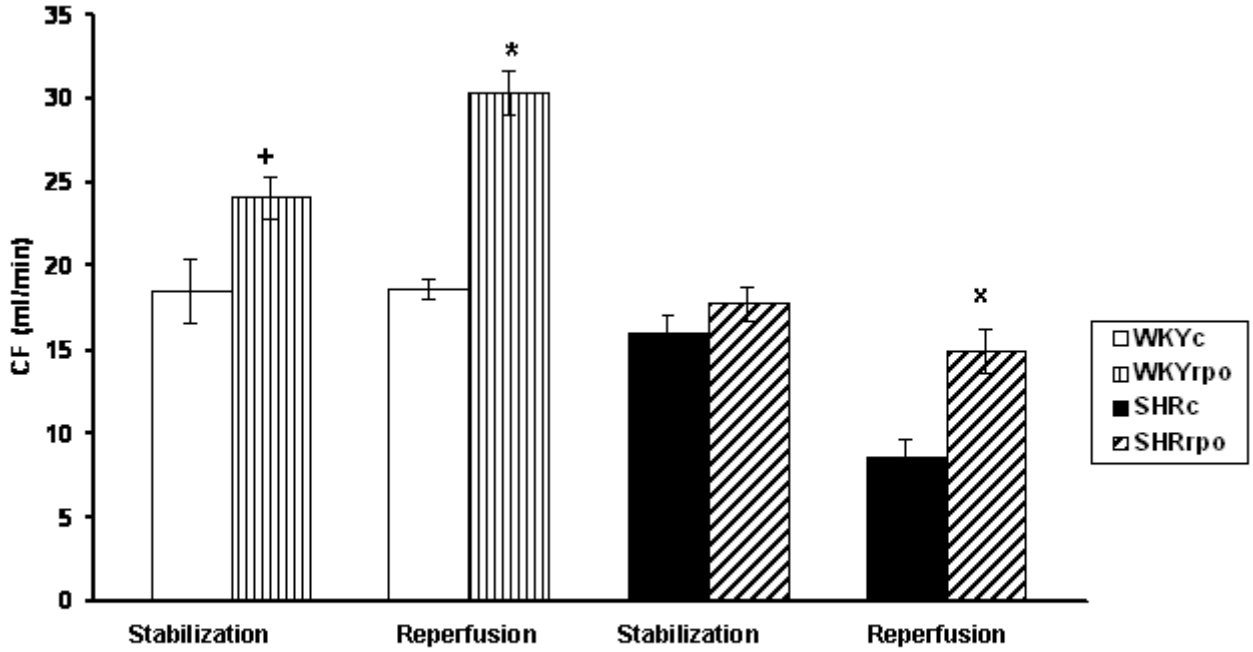


Figure 2: Coronary flow in untreated and RPO-treated SHR and WKY rat hearts during stabilization and reperfusion. Abbreviations: WKYc – untreated WKY rats, WKYrpo - WKY rats treated with red palm oil, SHRc – untreated SHR, SHRrpo - SHR treated with red palm oil. Results are expressed as mean±SEM (n=6/group). $^{+}p<0.05$ for WKYrpo vs SHRrpo at stabilization. *p<0.05 for WKYc vs WKYrpo at reperfusion. $^{x}p<0.05$ for SHRc vs SHRrpo at reperfusion.

3.2 Effect of dietary RPO supplementation on heart rate (in WKY and SHR during stabilization and reperfusion

RPO significantly reduced heart rate in WKYrpo compared to their respective control hearts at stabilization (285.28±14.29 vs 217.91±3.38, $^{\#}p<0.05$). Heart rate was also significantly reduced in SHRc hearts compared to WKYc during stabilization: 202.70±14.29 vs 285.28±14.29 beats/min, $^{*}p<0.05$. There were no significant differences observed in heart rate during stabilization in RPO treated hearts of both phenotypes. The heart rate was significantly reduced in SHRrpo compared to SHRc during reperfusion phase: 156.68±11.88 vs 269.00±14.08 beats/min, ($^{x}p<0.05$) Fig 3.

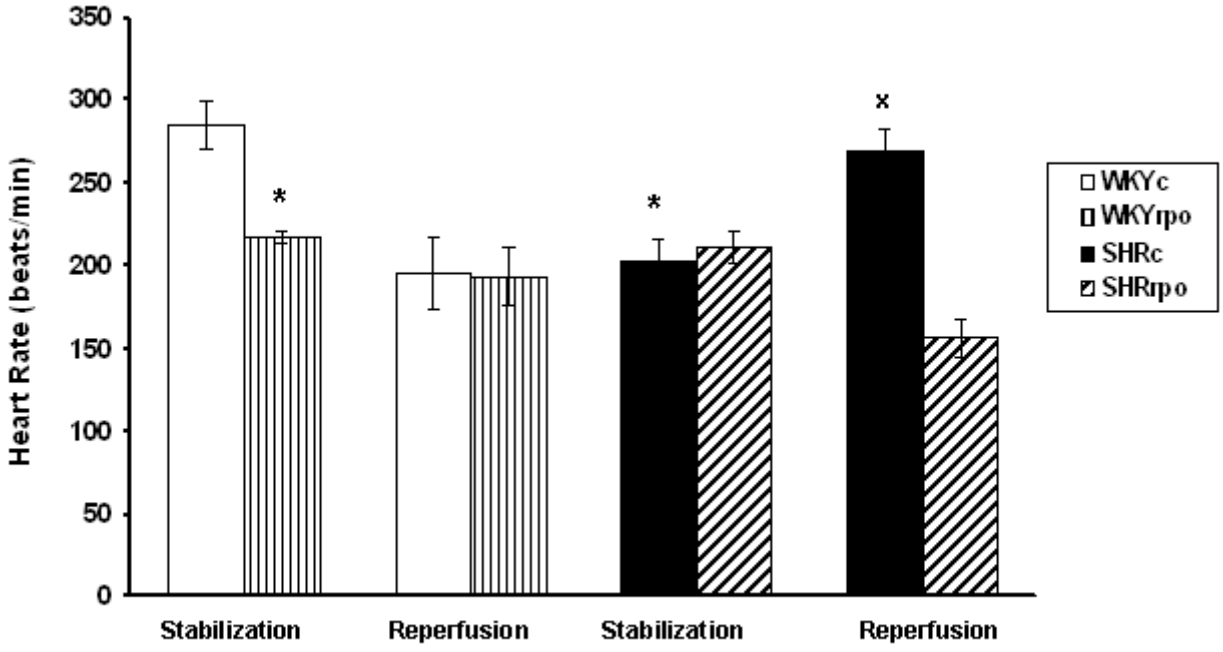


Figure 3: Heart rate in untreated and RPO-treated SHR and WKY rat hearts during stabilization and reperfusion. Abbreviations: WKYc – untreated WKY rats, WKYrpo - WKY rats treated with red palm oil, SHRc – untreated SHR, SHRrpo - SHR treated with red palm oil. Results are expressed as mean±SEM (n=6/group). $^{\#}p<0.05$ for WKYc vs WKYrpo at stabilization. *p<0.05 for WKYc vs WKYrpo and vs SHRc at stabilization significant difference. $^{x}p<0.05$ for SHRc vs SHRrpo during reperfusion.

3.3 Effect of dietary RPO supplementation on left ventricular pressure SHR and WKY rat hearts during stabilization and reperfusion

Effect of dietary RPO supplementation on LVP in WKY and SHR RPO treated and their respective control counterparts at stabilization and reperfusion. LVP was significantly increased in SHRc compared to WKYc during stabilization (153.92±4.90 vs 130.51±5.19, *p<0.05). This increase in LVP was again observed in SHRrpo compared to WKYrpo during the same time point (144.91±4.94 vs 120.64±5.19, $^{\#}$p<0.05). There was no significant difference in post-ischaemic LVP of RPO treated normotensive rats compared to their control counterparts. LVP was significantly increased in SHRc compared to WKYc during reperfusion, the same trend of results were observed when SHRrpo hearts were compared to WKYrpo hearts. RPO significantly improved LVP in SHRrpo compared to SHRc during reperfusion (123.63±6.14 vs 90.85±5.31 mmHg), $^{+}$p<0.05 (Fig 4).

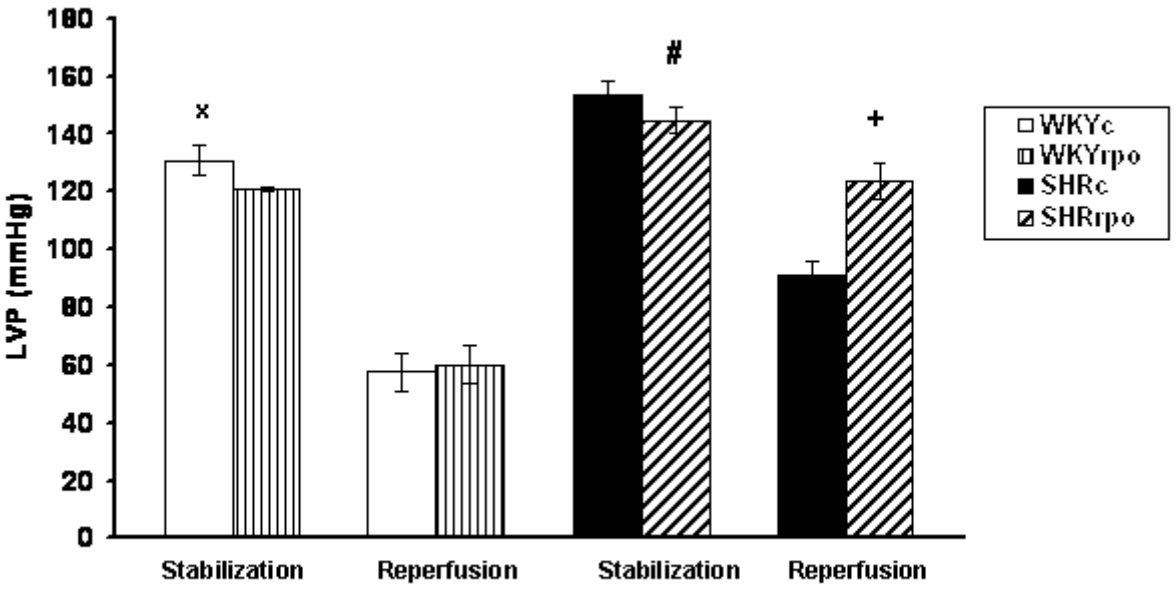


Figure 4: Left ventricular pressure in untreated and RPO-treated SHR and WKY rat hearts during stabilization and reperfusion. Abbreviations: WKYc – untreated WKY rats, WKYrpo - WKY rats treated with red palm oil, SHRc – untreated SHR, SHRrpo - SHR treated with red palm oil. Results are expressed as mean±SEM (n=6/group). ^{x}p<0.05 for WKYc vs WKYrpo at stabilization. $^{\#}$p<0.05 for WKYrpo vs SHRrpo at stabilization and $^{+}$p<0.05 for SHRc vs SHRrpo at reperfusion.

3.4 Effect of dietary RPO supplementation on LVP in WKY and SHR during stabilization and reperfusion

3.4.1 Contractile function assessed by +dp/dt max in untreated and RPO-treated SHR and WKY rat hearts during basal condition and postischemic reperfusion

There was reduction of +dp/dt max in wild type RPO treated hearts compared to their control counterparts at stabilization period, WKYrpo vs WKYc: (1847.88±84.11 vs 3619.60±202.62 mmHg/sec, $^{*}p<0.05$). However, at the same time point, RPO increased +dp/dt max by approximately 26% in SHRrpo compared to their control counterparts ($^{\#}p<0.05$). RPO improved +dp/dt max in WKY by approximately 46% compared to their control counter parts: (2074.81±146.24 vs 1411.86±105.37 mmHg/s, $^{+}p<0.05$). A significant increase in +dp/dt max was also observed in SHRrpo hearts compared to their control group: (3293.76±277.55 vs 2407.73±134.72 mmHg/sec, $^{x}p<0.05$) Fig 5 A.

3.4.2 Contractile function assessed by -dp/dt max in untreated and RPO-treated SHR and WKY rat hearts during basal condition and postischemic reperfusion

There was significant reduction in -dp/dt max in WKYrpo hearts compared to WKYc during stabilization: (1073.78±130.21 vs 2147.45±66.00 mmHg/sec, $^{*}p<0.05$). There were no differences observed between SHRc and SHRrpo at the same time point. However RPO increased -dp/dt max in SHRrpo by approximately 45% compared to SHRc during reperfusion: (1429.49±124.01 vs 982.95±61.24 mmHg/sec), $^{x}p<0.05$ (Fig 5 B).

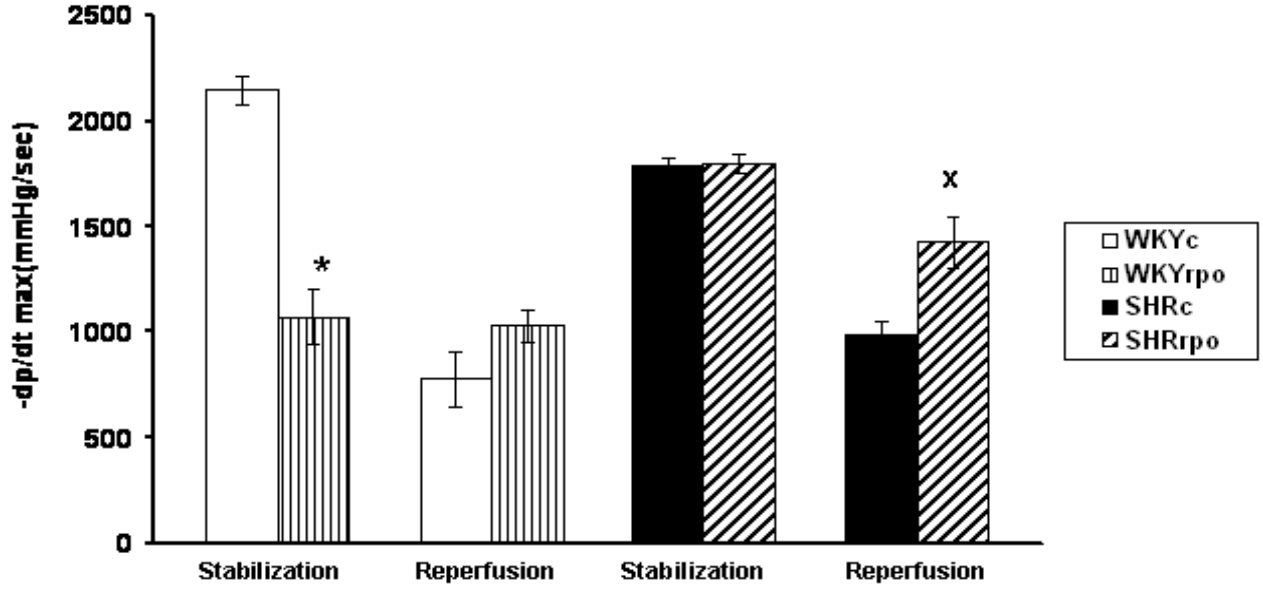


Fig 5 A

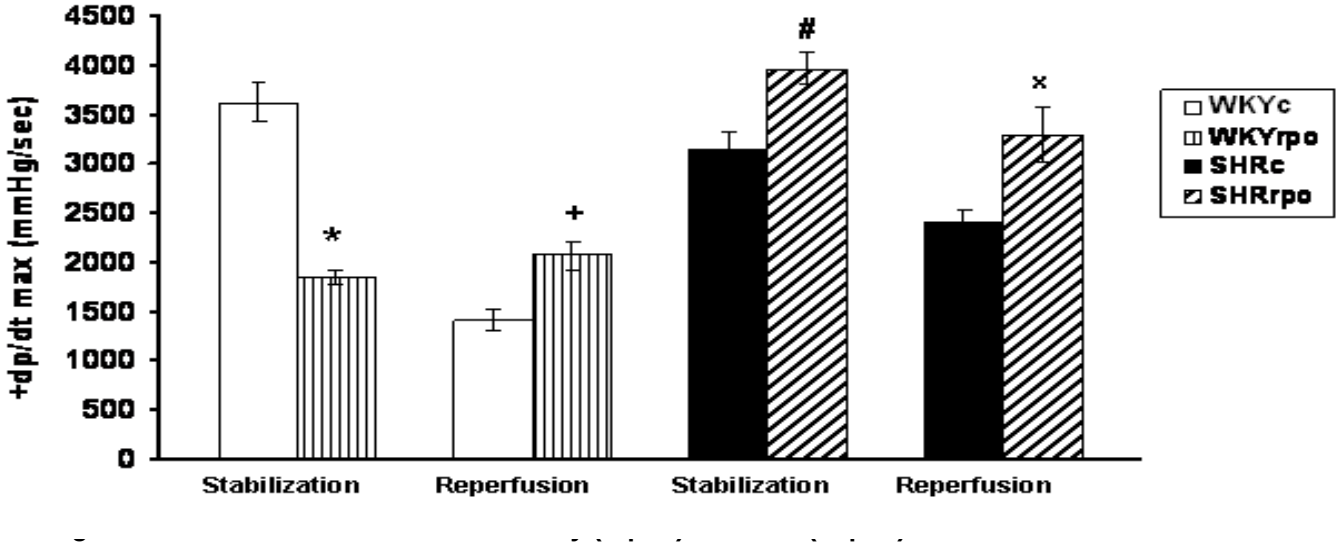


Fig 5 B

Figure 5A: Contractile function assessed by -dp/dt max in untreated and RPO-treated SHR and WKY rat hearts during basal condition and postischemic reperfusion. Abbreviations: WKYc – untreated WKY rats, WKYrpo - WKY rats treated with red palm oil, SHRc – untreated SHR, SHRrpo - SHR treated with red palm oil. Results are expressed as mean ± SEM (n=6/group). *p<0.05 for WKYc v WKYrpo at stabilization, ^{x}p<0.05 for SHRc vs SHRrpo during reperfusion. Fig 5 B: Contractile function assessed by +dp/dt max in untreated and RPO-treated SHR and WKY rat hearts during basal condition and postischemic reperfusion. Abbreviations: WKYc - untreated WKY rats, WKYrpo - WKY rats treated with red palm oil, SHRc – untreated SHR, SHRrpo - SHR treated with red palm oil. Results are expressed as mean±SEM (n=6/group). *p<0.05 for WKYc vs WKYrpo at stabilization. $^{\#}$p<0.05 for SHRc vs SHRrpo at stabilization. $^{+}$p<0.05 for WKYc vs WKYrpo during reperfusion and ^{x}p<0.05 for SHRc vs SHRrpo at stabilization.

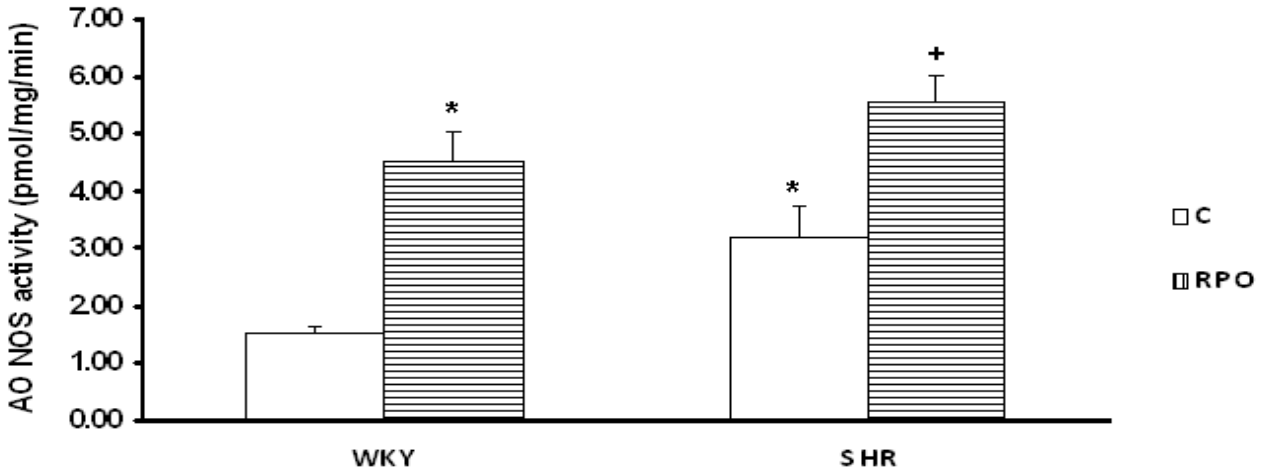


Fig 6 A

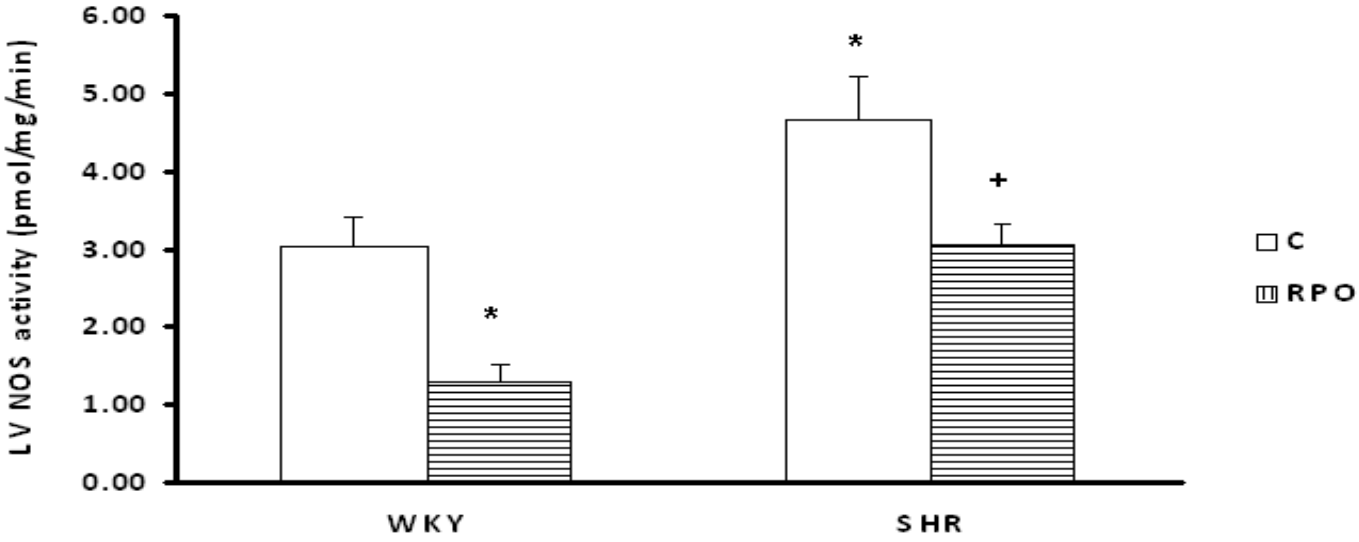


Fig 6 B

Figure 6: Effect of dietary RPO on aortic (A) and left ventricular (B) nitric oxide synthase activity. Abbreviations: RPO-red palm oil, AO-Aortic, LV-Left ventricular. Results are expressed as mean±SEM (n=6/group). *p<0.05 vs control WKY, $^{+}$p<0.05 vs control SHR.

3.6 Effect of dietary RPO on myocardial GPX1 protein expression in normotensive and hypertensive rats.

There were no significant differences observed in GPX1 proteins expression in all investigated groups (Fig 7).

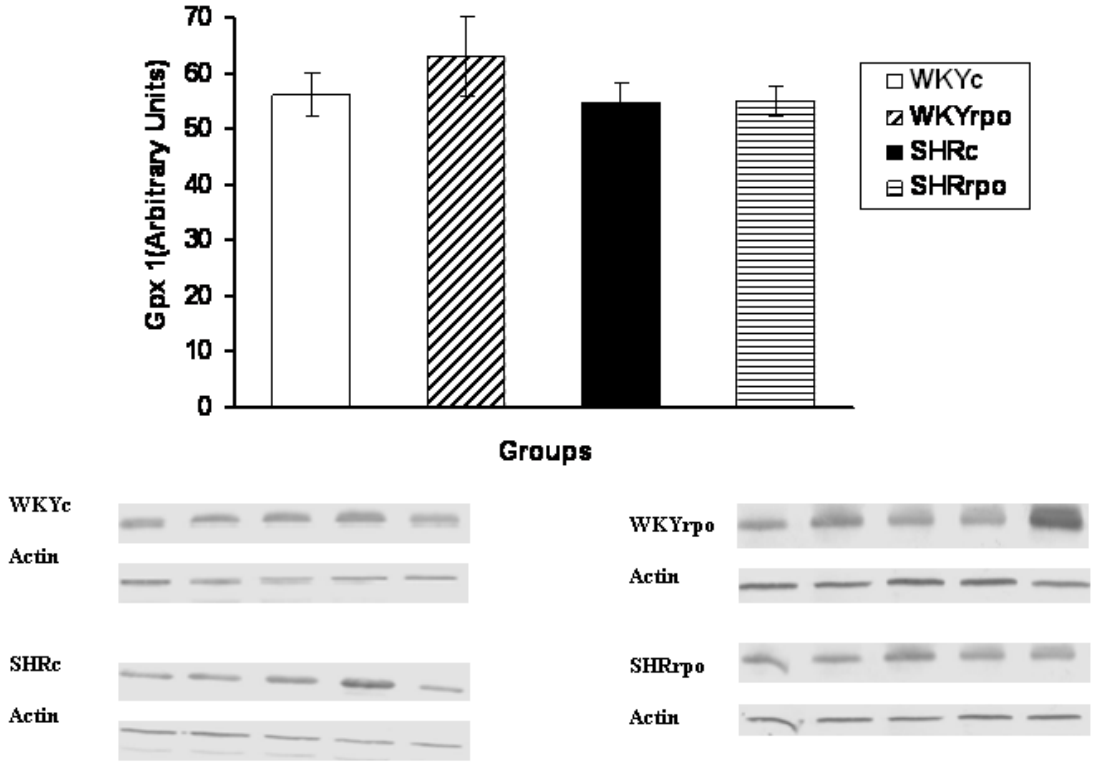


Figure 7: Effect of dietary RPO on myocardial GPX1 protein expression in normotensive and hypertensive rats. Results are expressed as means±SEM (n=5/group).

3.7 Effect of dietary RPO on myocardial SOD1 and SOD2

There were no significant differences observed in SOD1 proteins expression in all groups (Fig 8 A). RPO significantly increase SOD2 level in SHRrpo compared to SHRc: (77.95±5.22 vs 56.06±3.99 Arbitrary units) $^{\#}p<0.01$ (Fig 8 B).

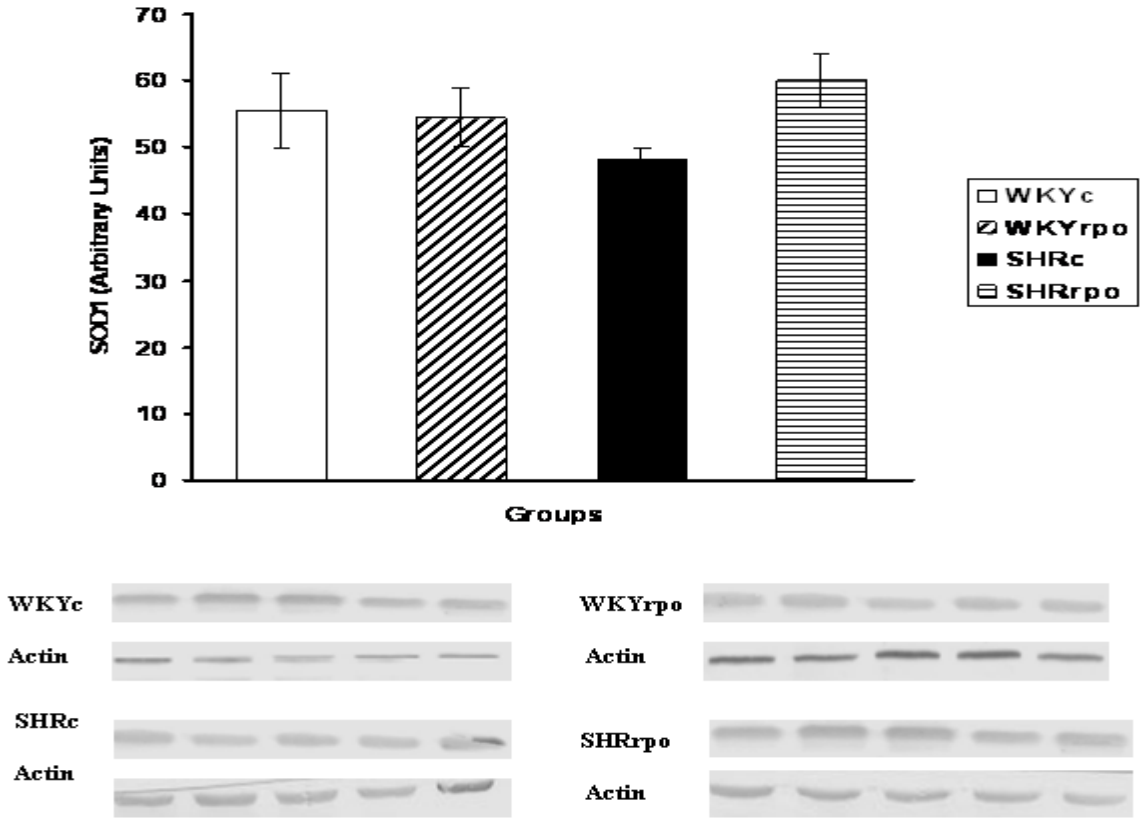


Fig 8 A

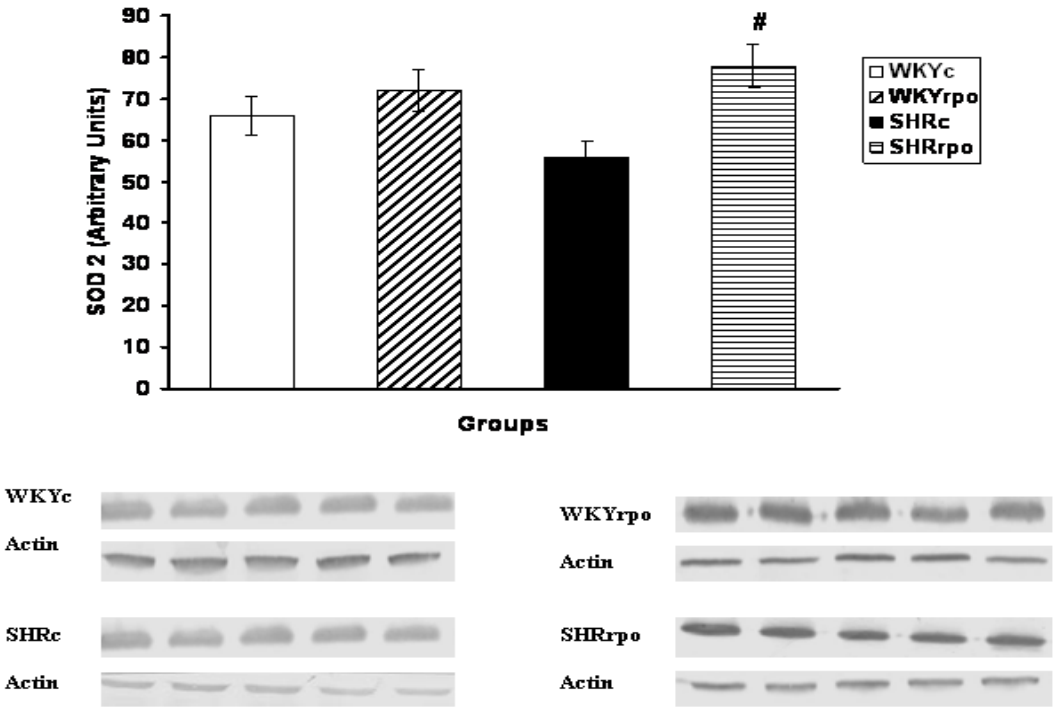


Fig 8 B

Figure 8: Effect of dietary RPO on myocardial SOD1 (A) and SOD2 (B) protein expression in normotensive and hypertensive rats. Results are expressed as means±SEM (n=5/group) $^{\#}p<0.01$ for SHRc vs SHRrpo.

4.0 Discussion

This study investigated the effect of RPO on cardiac function in spontaneously hypertensive and normotensive rats. In addition potential mechanisms of RPO-action was investigated by determination of NO production in the aorta and left ventricle and expression of selected antioxidant enzymes (SOD1, SOD2 and GPx1) in the heart. The main finding of this study was that, dietary RPO supplementation resulted in improved ventricular function and increased coronary flow in SHR. Although RPO offered protection in normotensive rats the cardioprotective effects were more pronounced in SHR rats. The improved cardiac function in SHR rats was associated with elevated protein expression of SOD2 in the heart while SOD1 and GPX1 were unchanged. However, tissue specific changes in NO production were observed: the reduction in the left ventricle and elevation in the aorta.

4.1 Effect of dietary RPO supplementation on cardiac function in spontaneously hypertensive

RPO significantly improved left ventricular systolic and diastolic function in spontaneously hypertensive rats as shown by improved maximum velocity of pressure rise +dp/dt max and maximum velocity of pressure fall -dp/dt max compared to their control counterparts. In control hearts treated with RPO a reduction in +dp/dt max and -dp/dt max was observed at baseline. This may be due to the fact that both the maximum velocity of pressure rise and fall are dependent on the workload against which the heart has to contract and pump blood. Both +dp/dt max and -dp/dt max are determinants of ventricular contraction and relaxation. The -dp/dt max represents the maximum rate of pressure fall during ventricular relaxation and +dp/dt max represent the pressure rise during isovolumic contraction. Amongst other factors, +dp/dt max is largely dependent on the structure of the myocardium and the ability of the myocardial cells to stretch in response to varying pressure loads [29]. In hypertension the myocardium is chronically exposed to excessive high pressure load which in time lead to maladaptation, resulting in ventricular hypertrophy [30, 31]. RPO conferred cardioprotection in spontaneously hypertensive as evidenced by improved LVP during reperfusion in the SHRrpo group compared to their control counterparts. This was also associated with a reduction in heart rate and improved coronary flow during reperfusion phase. Hypertension is associated with coronary insufficiency which usually results from associated long term complications [32, 33, 34]. Therefore, the increased coronary flow during reperfusion maybe particularly important in improving post-ischaemic function in SHR. Collectively the functional results in the current study indicate that RPO supplementation improved ventricular function and coronary circulation in hypertensive rats. The results further show that the cardio-protective effect of RPO are more pronounced in hypertensive rats than in normotensive rat hearts. This results support the hypothesis by Bačová *et al*., (2012) [27] who suggested that,

there might be differences in the effective RPO preventive and therapeutic doses. However it could also be speculated that RPO demonstrates protective characteristics when the heart is challenged by a pathological condition.

4.2 Effect of dietary RPO supplementation on SOD2 protein expression in SHR

The improved function in SHR treated with RPO was associated with increased mitochondrial superoxide dismutase (SOD2) protein expression. SOD2 is an important mitochondrial antioxidant enzyme involved in dismutation of superoxide to hydrogen peroxide and oxygen [35]. All cells including myocardial cells have intrinsic antioxidant systems to combat deleterious effects of excessive production of ROS. These antioxidant defense systems include enzymatic and no-enzymatic antioxidants [36, 37]. Myocardial cells are highly specialized aerobic cells with a large number of mitochondria. The large amount of ROS produced by the myocardial mitochondria renders the heart more prone to oxidative stress in conditions of cellular stress [38]. Our results show that hypertensive rats without RPO supplemetantion had reduced protein level of SOD2 compared to those supplemented with RPO. Reduced expression or activity of SOD2 is a good indicator of mitochondrial oxidative stress and a sensitive indicator for myocardial oxidative stress [39, 40, 41]. Therefore we can argue that the cardioprotective effect of RPO in hypertensive rats is at least in part mediated by up-regulation of SOD2 protein expression with potential attenuation of oxidative stress. There is convincing scientific evidence showing the indispensable role of SOD2 in maintaining cardiovascular health, in this regard Melov and colleagues (1999) reported that mice exhibiting partial SOD2 deficiency had increased mitochondrial oxidative damage [42]. It has also been shown that complete SOD2 deficiency in mice was associated with dilated cardiomyopathy with increased neonatal mortality [41]. Another study demonstrated that over expression of SOD2 decreased mitochondrial superoxide and restored NO bioavailability in hypertension [35]. SOD mimetic agents were associated with amelioration of oxidative stress and hypertension in spontaneously hypertensive rats [43, 44].

The effect of RPO on myocardial SOD has previously been investigated using different scientific and pathological models [24, 26]. Esterhuyse and co-workers (2006) found no effect on myocardial SOD activity in hearts exposed to ischaemia-reperfusion injury [24]. However it is worth mentioning that these investigators determined the effect of RPO on the activity and not the protein level of SOD and the analysis were not isoform specific. In another study Wergeland *et al.,* (2011) reported increased mRNA and proteins levels of SOD1 as possible mechanisms by which RPO conferred cardioprotection in anthracycline

treated hearts [26]. In the current study we have specifically shown that RPO supplementation increased protein expression of SOD2 in SHR. This finding is important because it indicates that RPO increased the pool of available SOD2 which is a key mitochondrial antioxidant. Our results suggest that the effect of RPO on myocardial SOD is isoform specific. In the current study RPO did not have an effect on SOD1 protein level, which is contrary to the result of [26]. This argues that the efficacy of RPO protection maybe model dependent.

Interventions aimed at combating oxidative stress should be specifically targeted at the primary site of ROS production such as the mitochondria, in order for them to be effective. Chronic exposure of the mitochondria to increased ROS production in conditions such as hypertension can have negative consequences, which may include mitochondrial DNA damage, functional decline, exaggerated ROS production and cellular injury [45]. Therefore targeting the modulation of mitochondrial antioxidant enzymes, which may lead to alleviation of mitochondrial oxidative stress, may be considered a viable therapeutic target.

4.3 Effect of dietary RPO supplementation on NOS activity

NOS production was elevated in SHR as compared to WKY in the both tissues investigated. Similar findings of elevated NOS activity or expression were shown by others [46, 47, 48]. On the other hand, there are studies showing reduced NO production in hypertensive rats [59, 50]. The reason for these discrepancies is unclear. It might result from quick modulation of NO production by negative feedback regulation [51] due to actual tissue-specific metabolic demands or from exhaustion of essential NOS cofactors under specific experimental conditions. In this study RPO supplementation was associated with increased aortic NOS activity in both normotensive and hypertensive rats compared to their control counterparts, which is in agreement with observation of elevated coronary flow after reperfusion in both genotypes. Moreover, elevated aortic NOS production can be implicated in reduction of blood pressure of RPO-treated rats, as it was shown previously by Bacova *et al.* (2012) [27]. In contrast, reduced NO production was seen in the LV of both RPO-treated rat strains. Tissue specific differences in the effect of RPO were observed previously. Esterhuyse *et al.* (2006) observed elevated aortic output recovery in Wistar rats, implicating elevated vascular NO production, while basal myocardial nitrate/nitrite level and NOS activity were unchanged [24]. However, cardiac NOS activity might be quickly increased in RPO-treated rats during ischemia which may provide protective mechanism during reperfusion. One of the possible mechanisms by which RPO might confer cardiovascular protection during ischemia/reperfusion is via increased phosphorylation of Akt [21, 23]. This activation might be especially important in the SHR rats as their basal NOS protein expression was shown to

be elevated in the heart [47]. In such conditions a direct activation of the NOS protein can occur very fast, resulting in sudden increase of NO bioavailability associated with cardioprotection. Although we observed reduced basal (i.e. determined before induction of ischemia/reperfusion) NOS activity in the LV of RPO-treated rats in this study, on the basis of previous results we can assume increased NO bioavailability at the end of reperfusion in SHR. Furthermore, increased level of SOD2 protein can be another mechanism improving NO level in the heart of SHR [35]. NO is an important regulator of cardiac function and vasodilator produced by the endothelial cells [52]. It has been shown to play an important role in regulating cardiovascular homeostasis. However, if not regulated it may be deleterious to cells. SOD enzymes play a key role in regulating NO bioavailability as they can degrade superoxide leading to improvement of NO bioavailability [53]. However, oxidative stress specific studies will be needed to determine the specific effect of RPO as both SOD1 and GPx1 protein levels were unchanged, indicating that the effect of RPO might be isoform specific.

5.0 Conclusion

In conclusion, in light of the evidence presented in this study we propose that the increased SOD2 protein expression in the heart and NOS activity in the aorta could be considered as possible mechanisms for RPO induced cardiovascular protection. While elevated NO production in the aorta can play a role in restoration of endothelial function, and thus in reduction of blood pressure, the increase in SOD2 protein could result in reduced oxidative stress. Thus, the NO-cGMP pathway and antioxidant defense systems could have acted synergistically, leading to restoration of cardiac and vascular function in SHR. This hypothesis is conceivable because endothelial dysfunction and oxidative stress are common features in hypertension. To our knowledge this is the first evidence implicating the beneficial effect of RPO through augmentation of SOD2 and improved endothelial function in the model of SHR.

6.0 Conflict of interest

The authors declare no conflict of interest

7.0 Acknowledgements

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Chapter 4

The combination of Red Palm Oil and Rooibos show anti-inflammatory effects in rats.

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Abstract

Red palm oil (RPO) and rooibos have been shown to exhibit cardioprotective properties. RPO is rich in essential fatty acids and fat soluble antioxidants while rooibos contains polyphenolic compounds with a unique composition of flavonoids. They exert their biological effects in different cellular compartments. Male Wistar rats weighing 150-200g were supplemented with RPO, rooibos or their combination for 28 days. The Langendorff system and the LPS-induced inflammatory model were used to determine if RPO and RB could protect against the negative effect of LPS-induced inflammation on baseline cardiac function. LPS treatment and dietary supplementation did not have effect on baseline cardiac functional parameters. LPS-induced systemic inflammation, as evidenced by increased IL-1β, was associated with elevated plasma levels of anti-inflammatory cytokine, IL-10 in LPS-induced rats consuming either rooibos or RPO alone. The combination of RPO and rooibos enhanced myocardial IL-10 in LPS-induced rats. This argues for potential protection against inflammation at the myocardial level.

1.0 Introduction

Natural food substances have the potential to alter biological functions of cellular and molecular components' mechanisms by either enhancing the endogenous antioxidant system or through altering the redox signalling status of the cell [1]. This could be beneficial in pathological conditions where oxidative stress and inflammation play an important role. Previous studies have shown that rooibos and red palm oil (RPO) protected the heart against the detrimental effects of ischaemia/reperfusion injury when supplemented individually to rats [2, 3, 4, 5, 6, 7]. Experimental evidence has also shown that RPO has potential anti-hypertensive and hypoglycaemic properties [8]. Recent evidence showed that RPO alone or in combination with rooibos can alleviate oxidant-induced hepatotoxicicity in male rats [9].

Red palm oil is a product from the fruits of the oil palm tree, *Elaeis guineensis* (Family *Arecaceae*) which has been shown to have protective effects against hypercholesterolemia and atherosclerotic plaque formation, despite being high in saturated fatty acids [10,11]. In addition to the various fatty acids that RPO contains, it is also a rich source of a wide spectrum of different lipid soluble antioxidants such as tocopherols, tocotrienols, carotenoids, lycopene and co-enzyme Q10, among others [12, 13, 14]. The health benefits of RPO have been attributed to its unique composition of fatty acids and a high content of natural antioxidants [15, 13]. RPO is one of the richest sources of natural vitamin E, especially tocotrienols [16] It has been shown that vitamin E molecules regulates specific cell signalling pathways independent of their antioxidant properties, therefore some of its beneficial effects have been attributed to its ability to modulate signal transduction pathways [17, 18]. There is also credible evidence showing that palm oil vitamin E have potential anti-inflammatory properties [19, 20, 21, 22]. Rooibos is a uniquely South African herbal tea made from the leaves and stems of the shrub-like leguminous bush, *Aspalathus linearis* (Brum.f) Dahlg (Fabaceae, Tribe Crotalarieae). It's flavonoids are unique in that it contains the *C*-*C* linked dihydrochalcone glucoside, aspalathin which is oxidized to the flavanones dihydro-iso-orientin and dihydro-orientin during fermentation, the cyclic dihydrochalcone, aspalalinin, the rare 3-dehydroxy dihydrochalcone glucoside, nothofagin, the *C*-glycosyl flavones orientin, isoorientin, vitexin, isovitexin, and the flavones hemiphlorin and chrysoeriol, luteolin and luteolin-7-*O*-glucoside and flavonols quercetin and its *O*-linked glycosides quercetin-3-robinobioside, hyperoside, isoquercitrin and rutin, [23, 24, 25]. The health effects of rooibos have been proposed to be mostly attributed to the unique polyphenolic composition and its related antioxidant activities [26, 27, 28, 29, 30]. Animal and recent human studies have shown that consumption of rooibos or its phenolic components had positive effects on cardiovascular health and inflammation [31, 32, 33, 34, 35, 36, 37, 38]. Studies have shown that rooibos may have potential preventive and therapeutic effects against vascular complications in diabetic rats [39]. Aspalathin, the main and unique polyphenol in rooibos,

has been shown to positively modulate glucose homeostasis in type 2 diabetes [30], while the antioxidant activity of rooibos has also been linked to its potential anti-inflammatory and DNA protective effects in a rat colitis model [33]. RPO (fat soluble) and rooibos (water soluble) contain different types of antioxidants which reside and exert their biological effects in different cellular compartments [12, 13, 24, 40]. Therefore, it is tempting to speculate that supplementation with a combination of these two natural food compounds can enhance the spectrum of available dietary antioxidants in different cellular compartments and hence offer a better protection against certain pathological conditions such as inflammation.

Accumulating scientific evidence shows that inflammation is the underlying pathological cause for most chronic diseases, including cardiovascular diseases, cancer and rheumatoid arthritis [41, 42, 43, 44, 45]. Ischaemic heart disease is the commonest form of cardiovascular disease leading to increased morbidity and mortality [46]. The majority of heart attacks and strokes are caused by rupturing of the atherosclerotic plaque in the arterial wall and the tendency of clot formation, which results from plaque rupture [46, 42]. It is now a scientifically accepted fact that inflammation in the lining of the artery is the initiating trigger in the pathogenesis of atherosclerosis [42]. Therefore, preventive and therapeutic interventions which are aimed at combating or alleviating inflammation could aid in reducing the health and economic burden associated with cardiovascular diseases and other diseases where inflammation is known to play a critical role.

Administration of lypopolysaccharide (LPS) to animals is widely used to study responses to *in vivo*-induced acute systemic inflammation [48, 49]. The inflammatory response forms part of the host innate immune response, which represents the first line of defence against invading pathogens or to injury [50]. Cytokine production forms an important part of the initial response to microbial agents and they are also important pathophysiological mediators of cardiovascular pathologies such as artherosclerosis and systemic sepsis-induced cardiac dysfunction [51, 52]. The isolated rat heart model and the LPS-induced inflammatory model were used to determine if rooibos and RPO supplementation could protect against the negative effect of LPS-induced inflammation on baseline cardiac function.

2.0 Materials and Methods

Animals 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). The rats had free access to water or rooibos and rat chow. They were individually caged in an experimental animal facility at a constant room temperature of 27°C and exposed to a twelve-hour artificial day-night cycle. The ethical clearance for this study was granted by the Faculty of Health and Wellness Science's Research Ethics Committee of the Cape Peninsula University of Technology: Ethics Certificate no (CPUT/HW-REC 2010/A004).

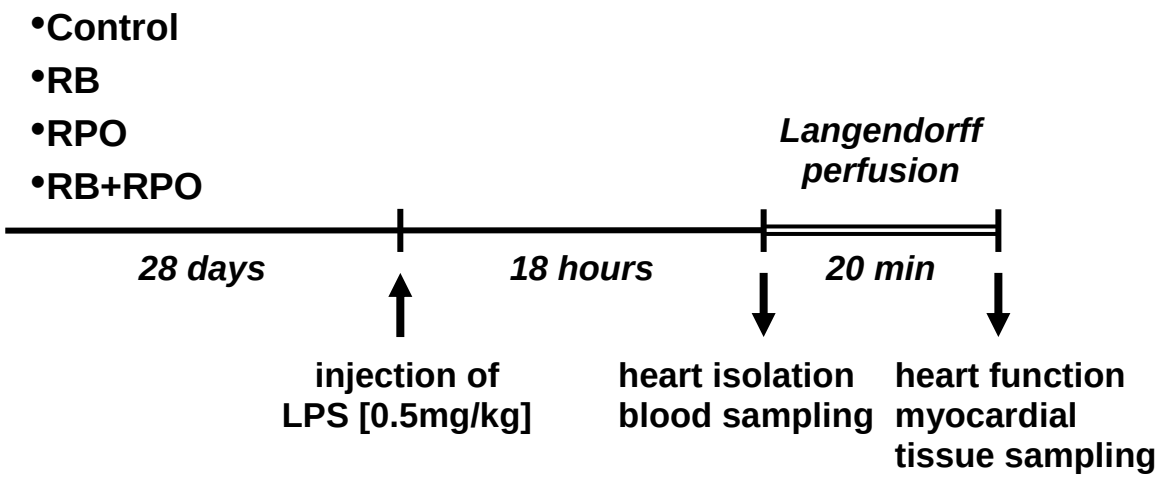

Figure 1: This figure illustrates the experimental groups, feeding period, the perfusion protocol and the time points where cardiac function was measure and biochemical samples collected.

RB- rooibos

RPO- red palm oil

RB+RPO – rooibos + red palm oil

LPS - lypolysccharide

2.1 Experimental model

Male Wistar rats weighing 150-200 g were randomly divided into 8 groups and supplemented with fermented/traditional rooibos, red palm oil (RPO) or a combination of the two for 28 days. The eight groups were further subdivided into two groups, either receiving 1) No-LPS or 2) LPS injection. Group 1 which is the NO-LPS group consisted of the control group receiving standard rat chow and water, rooibos group receiving standard rat chow and rooibos, RPO group receiving standard rat chow supplemented with RPO 0.2 mL (7 g/kg diet) daily and water. The red palm oil concentrate was supplied by Carotino SND BHD (Company no. 69046-T) Malaysia. The composition of RPO consumed by the rats is shown in (Table 1). The rooibos+RPO group received combination of rooibos and RPO (without LPS treatment). Group 2 which is the LPS group consisted of the control group receiving standard rat chow and water, rooibos group receiving standard rat chow and rooibos, RPO group receiving standard rat chow supplemented with RPO 0.2 mL (equivalent to 7 g/kg diet) daily and rooibos+RPO group receiving the combination of rooibos and RPO (with LPS treatment). Superior grade fermented rooibos was provided by Rooibos Ltd(Clanwilliam, South Africa). The rooibos aqueous extract was prepared by the addition of 100 mL of freshly boiled water to 10 g of tea leaves, filtered and stored at -40°C, and diluted 5 times, a concentration customarily used for tea consumption purposes, before being given to the rats [53]. Phenolic content, antioxidant capacity and flavonoids composition of the rooibos are shown in (Table 2). The animals were given 100 mL of the freshly diluted rooibos every second day. The rooibos and water consumption was monitored throughout the feeding period and there were no statistical differences observed in either rooibos or water consumption among the experimental groups (data not shown). At the end of the feeding period (28 days), 18 hours prior to sacrificing, animals in the LPS group were injected (intraperitoneal) with lipopolysaccharide (*Escherichia coli* serotype) to induce inflammation. The LPS was dissolved in sterile filtered phosphate buffered saline (PBS) to obtain 0.5 mg/kg body weight in 0.1 ml [48]. The animals in the NO-LPS were injected (intraperitoneal) with 0.1 ml of PBS (Fig 1).

At the end of the feeding period and inflammation injection protocol, rats were fasted for 16 hours before sacrificed and anaesthetized with an intraperitoneal injection of 2 mg/kg intraval sodium (sodium pentobarbital). Blood was collected from the abdominal aorta (approximately 5-8 ml) and placed into plain tubes for cytokine analysis. Serum was then separated immediately by centrifuging at 5000 g for 5 min at 4˚C, the samples were then stored at -80˚C till analysis were performed. Hearts were rapidly excised and placed in ice-cold Krebs-Henseleit buffer and transferred to the Langendorff perfusion system. Hearts were 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) and a perfusion pressure of 100cmH_2O was maintained throughout the protocol. Hearts were mounted to the Langendorff system and perfused for 15 minutes. Coronary flow, heart rate, LVDevP, RPP, ±dp/dt max derivatives, EDLVP were documented at baseline phase. LVDevP was measured with the aid of a balloon made from transparent sandwich wrap film inserted into the left ventricle through the opening of the left atrium. The balloon was connected to a power lab system (AD Instruments Pty Ltd., Castle Hill, Australia). After insertion, the balloon was inflated to 2mmHg, and the contraction force of the heart against the balloon caused water displacement that was converted to pressure. The systolic and diastolic pressures as well as the heart rate and minimum and maximum derivatives were documented on the computer. At the end of the perfusion protocol hearts were removed from the system and stored at -80º C till biochemical analysis were performed.

Table 1: The composition of RPO consumed by the rats

Parameters	Specifications	Typical
Fatty acids %	0.1 max	0.058
Moisture and impurities, %	0.1 max	0.03
Iodine Value	48-53	51.2
Slip melting point, c	33-37	36.4
Carotenes, ppm	400 min	420
Tocopherols and Tocotrienols, ppm	400 min	860
Nutritional information		
Amt/serving	Qty per 14 g	Qty per 100 g
Energy	518 kJ	3700 kJ
Protein	0.0 g	0.0 g
Fat, total	14 g	100 g
saturated	7.0 g	50.0 g
Trans	0.0 g	0.0 g
polyunsaturated	1.5 g	11.0 g
monounsaturated	5.5 g	39.0 g
Cholesterol	0.0 g	0.0 g
Carbohydrates	0.0 g	0.0 g
sugars	0.0 g	0.0 g
Sodium	0.0 g	0.0 mg
Carotenes as Vitamin A activity	640 ug	4600 ug
Vitamin E	2.5 mg	18.0 mg
Tocopherols	1.7 mg	12.0 mg
Tocotrienols	4.8 mg	34.0 mg

Certificate of analysis prescribed by Carotino 2010.
www.carotino.com

Table 2: Phenolic content, antioxidant capacity and flavonovoids composition of the 2% rooibos tea consumed by the rats.

Soluble solids(mg/mL)	**Total phenolic content** (mg gallic acid equivs/mg soluble solids)	**Flavonol content** (mg quecetin equivs/mg soluble solids)	**Flavanol content** (mg catechin equivs/mg soluble solids)	**FRAP** (µmol AAE/mL)	**TEAC** (µmol TE/ml)	**ORAC** (µmol TE/mL)
2.743±0.26	**0.303±0.006**	**0.159±0.004**	**0.058±0.002**	**4.90±0.35**	**5.22±0.22**	**14.72±1.57**

Phenolic compounds	**Concentration**	**% of Soluble solids**
Aspalathin	**28.32±1.65**	**1.03±0.06**
Orientin	**16.94±1.67**	**0.62±0.06**
Isoorentin	**23.64±2.34**	**0.86±0.09**
Vitexin	**6.06±0.061**	**0.22±0.02**
Isovitexin	**6.50±0.68**	**0.24±0.02**
Hypersoside/rutin	**14.55±1.30**	**0.53±0.05**
Quercetin	**0.89±0.11**	**0.03±0.003**
Luteolin	**0.22±0.03**	**0.01±0.001**
Chrrysoeriol	**0.23±0.02**	**0.01±0.001**

Soluble solids (mg/mL) 2.74 ± 0.26

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2.2 Immunoassay for plasma and myocardial cytokine analysis

Analyses of samples were performed on undiluted myocardial tissue homogenates which were originally prepared in phosphate buffer at a dilution of 1:4. In order to analyse the myocardial cytokines, hearts from all the 8 groups were freeze-clamped with Wollenberger tongs clamp pre-cooled in liquid nitrogen. The heart samples were then grinded into powder and 100mg of heart tissue powder was diluted into 500µl of phosphate buffer. The mixture was homogenized by ultrasonic homogenizer at maximum power (2x20 sec), and the homogenate was centrifuged at 4˚c for 20 minutes at 5000g. The supernatant was collected and stored at -80˚c till analysis were carried out. Protein tissue content was determined using Bradford technique [54]. Plasma and myocardial IL-1 beta, IL-6 and IL-10 levels were measured using the Bio-Plex bead array system (Bio Rad Laboratories, USA). Assays were carried out in 96-well filter plates, while the rat cytokine kits, (Cat #: RCYTO-80K) were obtained from Millipore (USA). Samples were evaluated in duplicate. All levels of analytes in quality control reagents included in the kits were within the expected references ranges.

2.3 Data analysis

Results were expressed as mean ± standard error of the mean (SEM). Differences between the NO-LPS control group and the LPS control group were determined using an unpaired Student's *t*-test. To compare differences in multiple groups, ANOVA plus FisherLSD with a post hoc test was used. $P < 0.05$ was considered to be statistically significant.

3.0 Results

3.1 Plasma cytokine levels

3.1.1 IL-1*β* Fig 2A

The plasma pro-inflammatory cytokine IL-1β was significantly increased ($^{\#}p<0.05$) in LPS control (positive controls) rats (367.52±60 pg/ml) when compared to the NO-LPS control (negative controls) rats (63.71±10 pg/ml) (Fig 2 A). No differences were observed in plasma IL-1β of the rooibos and RPO-supplemented LPS-treated rats compared to the LPS control (Fig 2A).

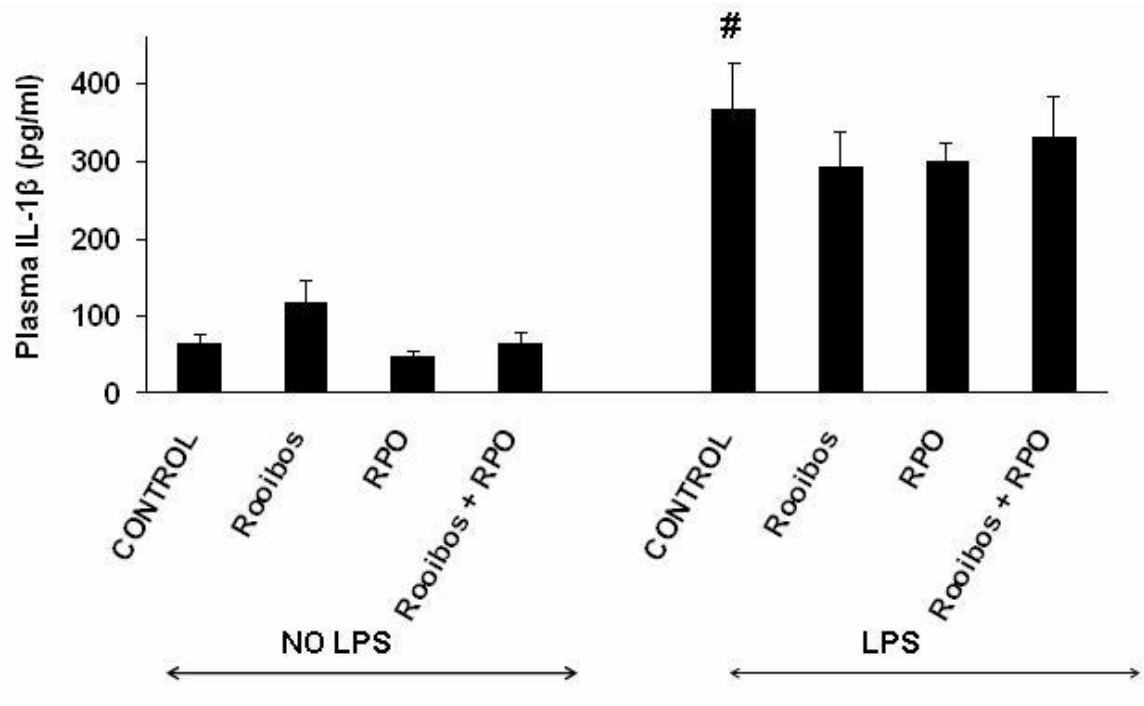

Figure 2A: Effects of inflammation, rooibos and RPO on plasma IL-1β. Results are expressed as means ± SEM, n=4-8/group, #p<0.05 for LPS control vs NO-LPS control. Abbreviations: RPO = Red palm oil.

3.1.2 IL-6 Fig 2B

The level of plasma pro-inflammatory cytokine, IL-6 in the positive control rats was not significantly different from the levels in the negative control animals. There were also no differences observed in plasma IL-6 of LPS-induced rooibos and RPO-supplemented rats compared to the positive control (Fig 2B).

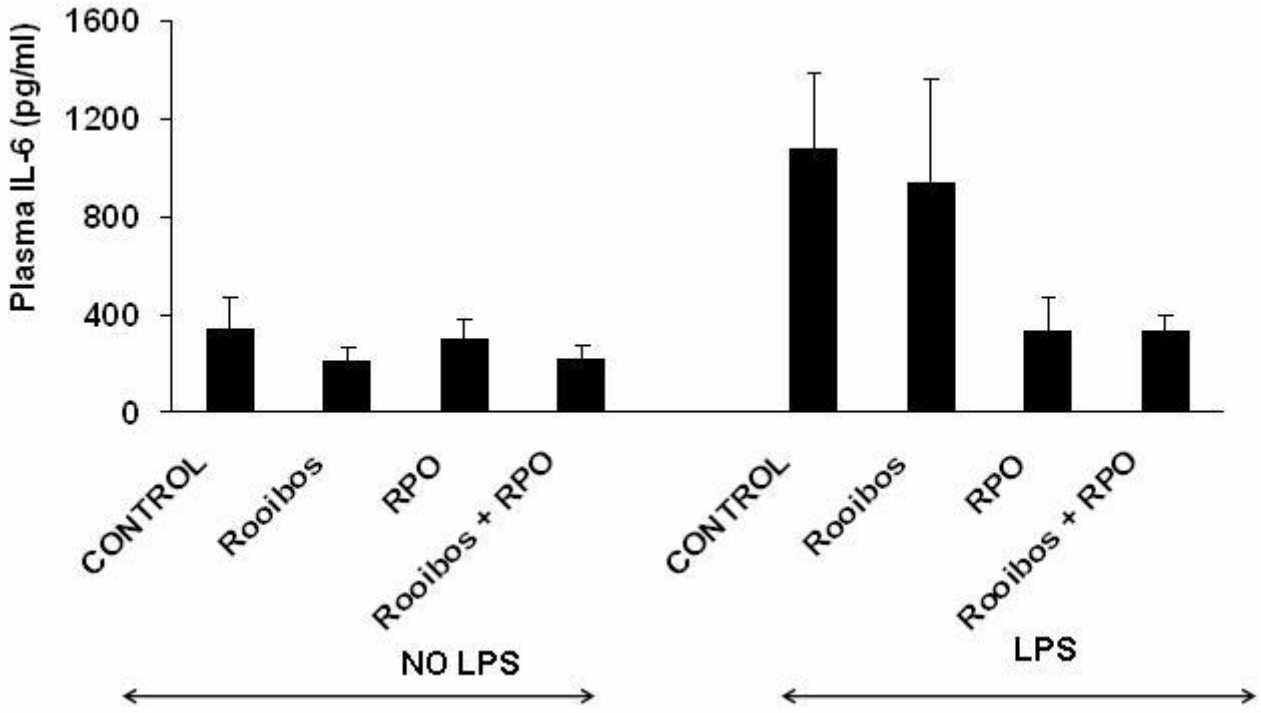

Figure 2B: Effects of inflammation, rooibos and RPO on plasma IL-6 Results are expressed as means ± SEM, n=6-8/group. No differences observed between the groups. Abbreviations: RPO = Red palm oil.

3.1.3 IL-10 Fig 2C

The plasma anti-inflammatory cytokine, IL-10 was significantly (*p<0.05) increased in LPS-induced rats consuming rooibos (4082.19±180 pg/ml) compared to the positive control (1462.63±372 pg/ml). A similar pattern of results were also observed for LPS-induced rats supplemented with RPO, where the plasma IL-10 level was significantly (*p<0.05) increased (2375.28±264 pg/ml) compared to the positive control (1462.63±372 pg/ml). There were no differences observed in plasma IL-10 levels between the negative control and the positive control and there was also no differences observed in plasma IL-10 level of the LPS-induced rats consuming the combination of rooibos and RPO compared to the positive control (Fig 2C).

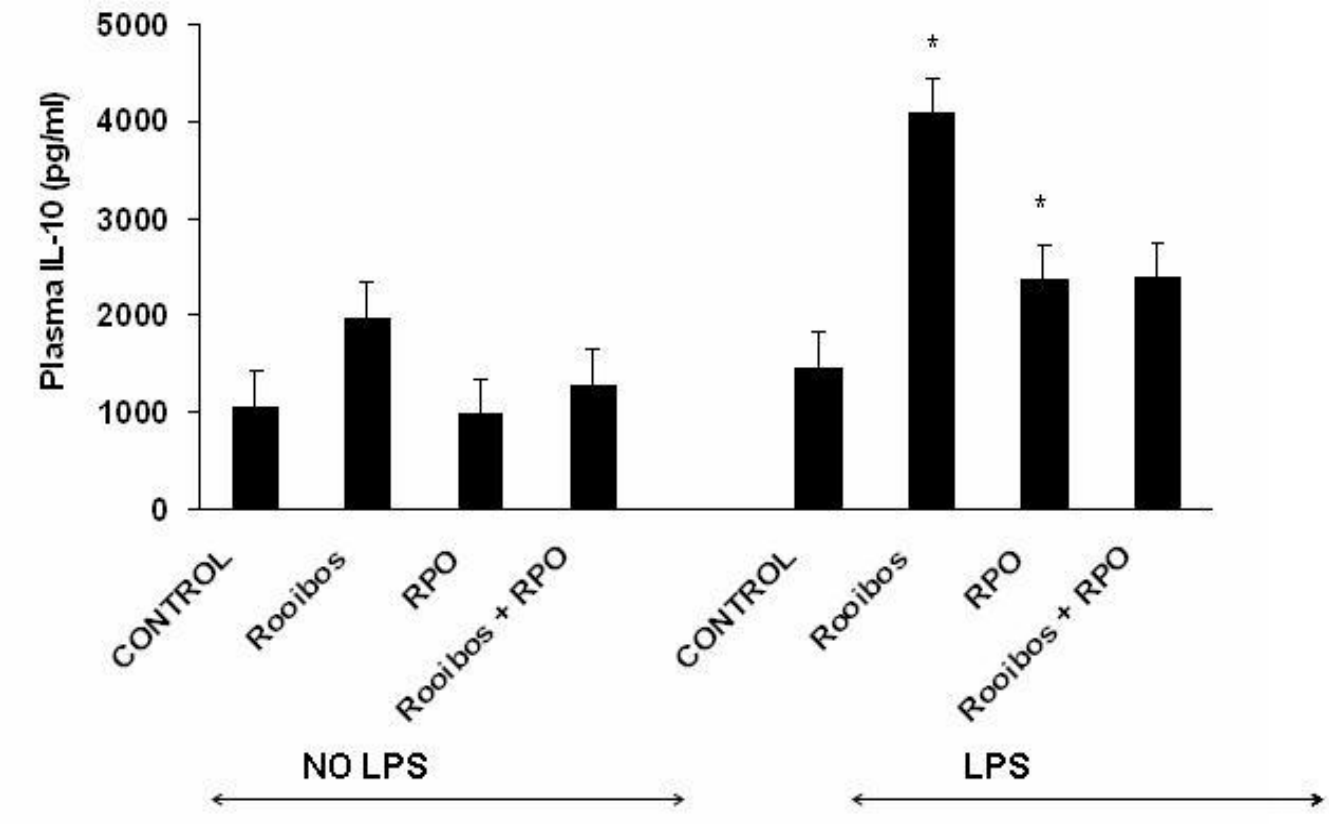

Figure 2C: Effects of inflammation, rooibos and RPO on plasma IL-10. Results are expressed as means ± SEM, n=5-7/group, *p<0.05 significantly different from the positive control. Abbreviations: RPO = Red palm oil.

3.2 Myocardial cytokine levels

3.2.1 IL-1*β* Fig 3A

When considering the myocardial IL-1β levels, there were no differences observed between the NO-LPS control and the LPS control animals. The level of myocardial IL-1β was significantly (*p<0.05) increased in LPS-induced rats consuming the rooibos (172.36±23 pg/ml) when compared to the LPS control (73.29±14 pg/ml) rats (Fig 3A).

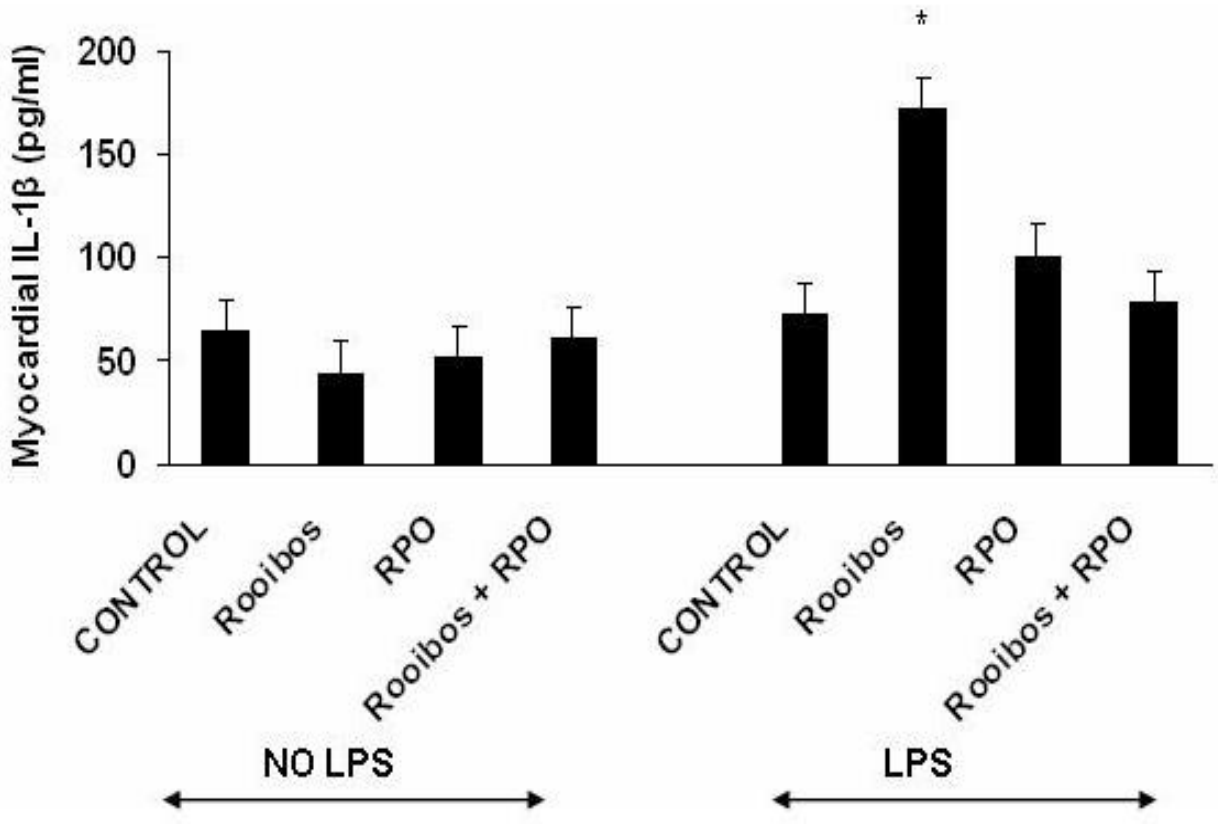

Figure 3A: Effects of inflammation, rooibos and RPO on myocardial IL-1β. Results are expressed as means ± SEM, n=5-6/group *p<0.05 significantly different from the positive control. Abbreviations: RPO = Red palm oil.

3.2.2 IL-6 Fig 3B

Myocardial IL-6 was significantly (#p<0.05) lower in the positive control (121.53±23 pg/ml) compared to the negative control (233.85±38 pg/ml) rats. The level of myocardial IL-6 was significantly (*p<0.05) increased in LPS-treated rats consuming rooibos (235.58±38 pg/ml) compared to the positive control (121.53±23 pg/ml). The LPS-induced rats which consumed the combination of rooibos and RPO also showed a significant (*p<0.05) increase in myocardial IL-6 (283.50±29 pg/ml) compared to the LPS control (121.53±23 pg/ml), (Fig 3B).

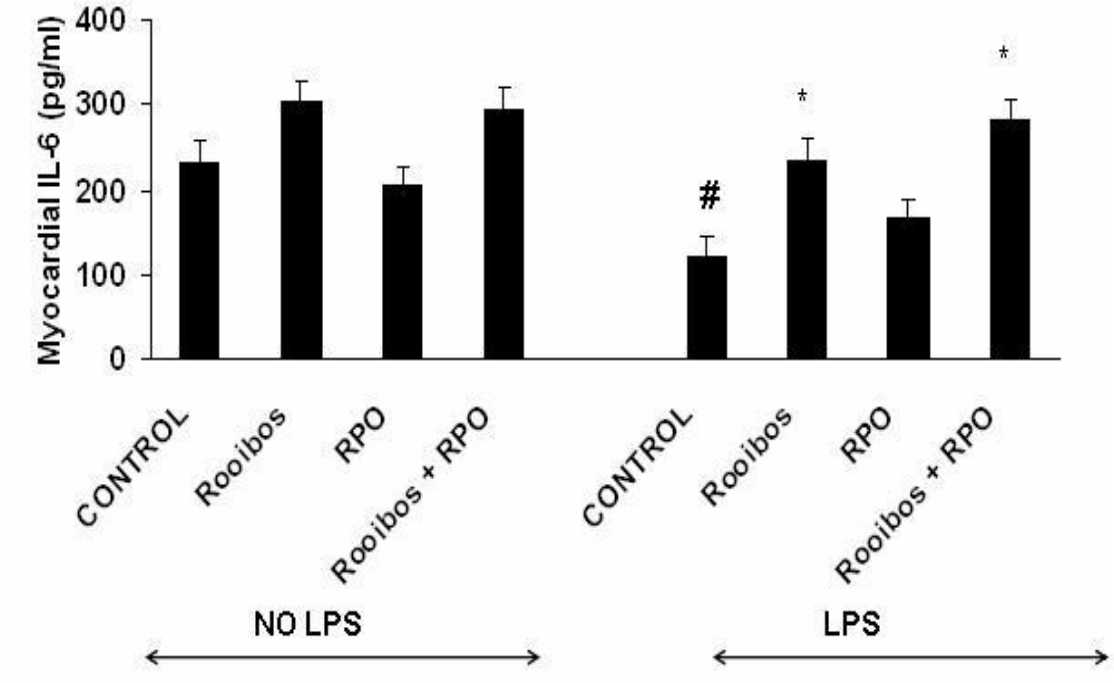

Figure 3B: Effects of inflammation, rooibos and RPO on myocardial IL-1 6. Results are expressed as means ± SEM, n=5-6/group *p<0.05 significantly different from the LPS control. #p<0.05 for LPS control vs NO-LPS control. Abbreviations: RPO = Red palm oil.

3.2.3 IL-10 Fig 3C

When considering the myocardial IL-10 levels, significantly ($^{\#}p<0.05$) increased levels of myocardial IL-10 were measured in the negative control (915.60±71 pg/ml) compared to the positive control (468±60 pg/ml). While the combination of rooibos and RPO significantly ($^{\#}p<0.05$) increased myocardial IL-10 levels (739.09±48pg/m) compared to positive control. The LPS-induced rats consuming either rooibos or RPO alone did not show significant differences in induction of myocardial IL-10 compared to the positive control (Fig 3C).

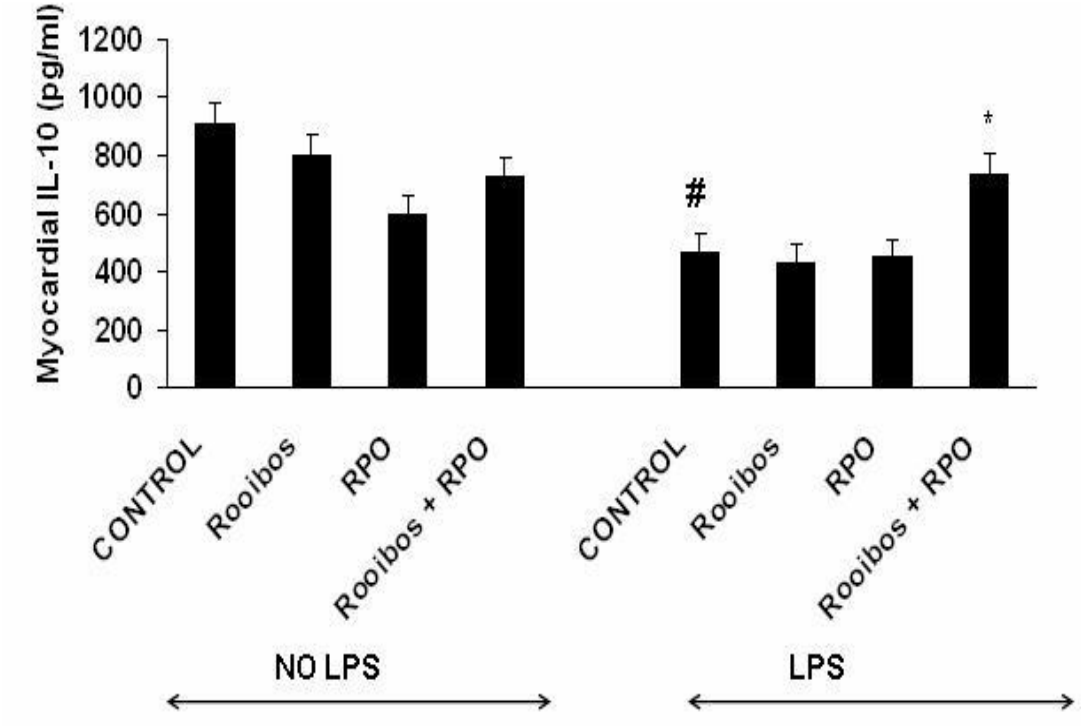

Figure 3C: Effects of inflammation, rooibos and RPO on myocardial IL-10. Results are expressed as means ± SEM, n=5-7/group, *p<0.05 significantly different from the LPS control, #p<0.05 for LPS control vs NO-LPS control. Abbreviations: RPO = Red palm oil

Table 3: Effects of inflammation, rooibos and RPO on baseline cardiac function in the NO-LPS group and the LPS group. Body weights and heart weights are also shown. Non significant differences were observed between the groups. Results are expressed as SEM, n=5-7. Abbreviations: CF- Coronary flow, HR- Heart rate, LVDevP- Left ventricular developed pressure, RPP- Rate pressure product, +dp/dt max-maximum derivative of Left ventricular time, -dp/dt-minimum derivative of Left ventricular pressure max, EDLVP- End diastolic left ventricular pressure, HW- Heart weigt, BW- Body weight.

	NO-LPS				LPS			
	Control	**Rooibos**	**RPO**	**RB+RPO**	**Control**	**Rooibos**	**RPO**	**RB+RPO**
CF (ml/mi)	13.92 ±1.00	13.84 ±1.00	14.6 ±1.00	15.2 ±1.00	14.30 ±0.60	13.70 ±0.70	14.60 ±0.70	15.10 ±0.20
HR bpm	294.33 ±6.00	277.87 ±13.00	279.69 ±15.00	296.313 ±8.00	293.14 ±14.00	302.39 ±12.00	296.86 ±10.00	300.00 ±13.00
LVDevP (mmHg)	92.804 ±6.00	86.40 ±6.00	86.47 ±5.00	97.00 ±3.00	106.25 ±4.30	89.60 ±2.40	95.500 ±4.50	100.90 ±4.410
RPP (Bpm*mmHg)	27593.83 ±1814.00	23706.98 ±660.00	24383.99 ±2503.00	28319.93 ±764.00	30133.51 ±1394.00	24805.68 ±1366.00	24701.52 ±1551.00	26176.72 ±1212.00
dp/dt (+) mmHg/sec	2822.951 ±149.00	2829.72 ±84.00	2661.67 ±198.00	2664.75 ±112.00	3065.48 ±103.00	2888.66 ±129.00	2885.71 ±80.00	2814.45 ±113.00
dp/dt (-) (mmHg/sec)	1933.91 ±70.00	1906.73 ±82.00	1918.94 ±103.00	1996.27 ±94.00	2140.53 ±90.00	1969.86 ±69.00	2024.86 ±120.00	1952.45 ±51.00
EDLVP (mmHg)	10.256 ±0.92	13.424 ±2.20	11.558 ±0.72	12.29 ±1.34	13.74 ±2.36	12.93 ±1.33	12.22 ±2.24	11.78 ±1.16
HW (g)	1.17±0.09	1.16±0.05	1.11±0.041	1.09±0.03	1.32±1.0	1.11±0.0	2.82±1.4	1.28±0.1
BW (g)	333.70±4.53	343.90±10.02	346.60±6.80	348.20±3.40	350.20±7.5	352.40±7.4	334.20±8.8	340.40±90

4.0 Discussion

The aim of the current study was to induce inflammation *in vivo* and to establish if dietary supplementation with rooibos and RPO would reverse or suppress the effects of inflammation. We have shown that administration of LPS induced systemic inflammation as evidenced by elevated levels of IL-1*β*, the inflammation marker, in the plasma of LPS-treated animals compared to non-treated animals. The increase in plasma IL-1*β* levels of the LPS-induced rats consuming either rooibos or RPO alone were associated with elevated levels of plasma anti-inflammatory cytokine. The results indicate a potential anti-inflammatory property of rooibos and RPO at systemic level when supplemented individually. However, the combination of rooibos and RPO significantly enhanced endogenous myocardial IL-10 level in LPS-induced rats, arguing for potential protection against inflammation on organ level.

4.1 Effects of inflammation, rooibos and RPO on IL-1*β*.

The increased levels of plasma IL-1*β* in the LPS-induced rats indicate that there was induction of inflammation in response to the presence of the endotoxin (LPS), (Fig 2 A). IL-1*β* is one of the initial pro-inflammatory cytokines to be released in response to the invading microbial pathogens (specifically the lipopolysaccharide, an endotoxin embedded within the bacterial membrane) and it plays a crucial role in the induction of inflammation [55]. Therefore, increased levels of circulating IL-1*β* are indicative of a systemic inflammatory response [56, 57). The response to LPS is initiated upon the recognition of LPS by the LPS-binding protein, following the binding of LPS to its binding protein a series of multiple complex signalling pathways is initiated. This will ultimately result in activation of the Toll-like Receptor (TLR) 4 through various adaptor proteins leading to NFkB activation and eventual induction of inflammatory cytokines [58, 59, 60, 61]. LPS triggers the release of inflammatory cytokines from various cells of the immune system, the released cytokines leads to an acute inflammatory response directed towards the invading pathogen [62, 63, 64]. The finding of elevated plasma levels of IL-1*β* therefore confirms that inflammation was induced in the current model. Our results are in agreement with previous reports by Ohsaki and co-workers (2006) [48] which used a similar dose of LPS and showed increased IL-6 mRNA levels indicating that inflammation was induced. Our results also show that supplementation of either rooibos or red palm oil, together and separate, could not prevent an increase in plasma IL-1*β*.

However, in the myocardium, our results show that consumption of rooibos in the LPS-induced rats was associated with increased myocardial levels of IL-1*β* compared to the positive control, while RPO and rooibos+RPO did not affect the induction of myocardial IL-1*β* (Fig 3A). Myocardial and endothelial cells have the capacity to respond to LPS via activation of the TLRs leading to induction of inflammatory cytokines [64]. The role of cytokines in inflammation is complex and is determined by various factors such as the magnitude of cytokine induction, the presence of receptors to cytokines and also by the presence of antagonist mediators such as anti-inflammatory cytokines. The increased myocardial levels of IL-1*β* in the LPS-induced rats consuming rooibos may represent a normal cellular response to the presence of the endotoxin [65], especially because the observed increases in myocardial levels of IL-1*β* in LPS-induced rats consuming rooibos were not associated with alterations cardiac function.

4.2 Effects of inflammation, rooibos and RPO on IL-6.

Dietary intervention with rooibos, RPO or their combination did not have any effect on plasma IL-6 levels in LPS-treated animals and their non-treated counterparts (Fig 2B).

There was differential modulation of myocardial IL-6 by the dietary supplements in the LPS-induced supplemented rats. Consumption of rooibos and the combination of rooibos and RPO in LPS-induced rats resulted in increased myocardial IL-6 compared to the positive control (Fig 3B). Even though IL-6 is classically characterized as a pro-inflammation cytokine, it has been shown to have both pro-inflammatory and anti-inflammatory features [66, 67]. IL-6 can evoke an anti-inflammatory environment by inducing the production of anti-inflammatory cytokines, such as IL-10 and IL-1ra in humans [67]. Our results show that the increase in myocardial IL-6 in the LPS-induced rats consuming rooibos and rooibos+RPO was associated with enhanced up-regulation of myocardial IL-10. Therefore the elevation of both IL-6 and IL-10 indicates that in this instance, IL-6 might be acting as an anti-inflammatory cytokine leading to enhancement of IL-10 production. There is evidence showing that in some instances acute elevation of IL-6 may be beneficial, especially following exercise in humans [67]. Xing and co-workers (1998) [68] showed that endogenous IL-6 plays an anti-inflammatory role in both local and systemic acute inflammatory responses in mice. This mechanism acts by controlling the level of pro-inflammatory, but not anti-inflammatory, cytokines [69, 70]. Others have also shown that blockade of IL-6 in patients with rheumatoid arthritis led to enhanced cholesterol and plasma glucose levels, indicating a role for IL-6 in modulation of glucose and lipid metabolism [71, 72]. Results in the current study would therefore indicate that endogenous IL-6 rather protected than

harmed the heart against induction of LPS, especially in the local organ region as is presented in Fig 3B. The results further show that dietary intervention can influence the levels of IL-6 in cardiac tissue. There is also a difference between systemic and local response to IL-6 levels with LPS induction in the presence of dietary supplements such as red palm oil and rooibos. This needs to be further investigated and clarified.

4.3 Effect of inflammation, rooibos and RPO on IL-10.

The current results report that plasma IL-10 levels were significantly elevated in the two LPS-treated groups consuming either rooibos or RPO when compared to the LPS control. However, the LPS-induced rats consuming the combination of rooibos and RPO did not show any effect on plasma IL-10 levels indicating that there was no additional benefit on plasma IL-10 levels when rooibos and RPO were given in combination (Fig 2C). The results indicate that dietary supplementation with rooibos and RPO up-regulated the production of IL-10 in response to the presence of inflammation. IL-10 is a potent ant-inflammatory cytokine whose role is to counteract the effects of pro-inflammatory mediators in various forms of shock and inflammation [73]. Inflammatory cells in the circulation are activated in response to invasion of LPS and the initial induction of inflammatory cytokine in response to LPS is aimed at clearing local effect of the invading pathogen [74]. However, the body has also evolved regulatory systems to maintain the balance between the levels of pro-inflammatory mediators and anti-inflammatory mediators in order to sustain cellular homeostasis and immune system integrity. We have shown that rooibos and RPO, when supplemented individually, enhanced production of IL-10 in the blood, suggesting a potential anti-inflammatory effect at systemic level. Therefore the concomitant release of IL-1 β and IL-10 in plasma of LPS-induced rats consuming rooibos and RPO indicate that dietary intervention with rooibos and RPO modulated the inflammatory response in the model of inflammation by enhancing systemic production of the anti-inflammatory cytokine. Rooibos is rich in various polyphenolic compounds, some unique to the plant as well, of which flavonoids are the most predominant [75]. Various polyphenolic molecules have been shown to exhibit anti-inflammatory activity [76, 77]. Polyphenols have a wide range of biological effects which include antioxidant and anti-inflammatory effects [78, 79, 80]. In the current study we have shown that rooibos consumption was associated with increased levels of plasma IL-10. This is in line with previous studies where polyphenols were associated with enhanced production of IL-10 and suppression of IL-1 beta [78].

Just as polyphenols form a vital part of a healthy diet, vitamins are also equally essential for human health. RPO is rich in various forms of vitamin E and carotenoids which function as cellular antioxidants [81, 13]. Inflammation and oxidative stress are closely related and are usually common features underlying etiological and pathological mechanisms for most chronic diseases including cardiovascular diseases [82, 83]. Both tocotrienols and tocopherol are potent antioxidants and have also been shown to possess potential anti-inflammatory properties [19, 22].

Dietary supplentation with the combination of rooibos and RPO resulted in increased myocardial levels of IL-10 in the LPS-induced rats compared to the positive control while, when supplemented individually, rooibos and RPO in the presence of LPS, did not have any effect on myocardial IL-10 levels (Fig 3C). To our knowledge this is the first evidence showing that the combination of rooibos and RPO resulted in up-regulation of myocardial IL-10 levels. Elevated myocardial levels of IL-10 have been linked to cardio-protection [84]. The importance of IL-10 in maintaining myocardial integrity has also been shown by studies which showed that genetic deletion of IL-10 was associated with enhanced inflammation and increased myocardial infarction and necrosis [85].

4.4 Effects of inflammation, RB and RPO on baseline cardiac function in normal and LPS treated hearts and body weights and heart masses are shown on Table 3.

LPS treatment and dietary supplementation with rooibos and RPO did not have an effect on baseline cardiac functional parameters. The increases in plasma IL 1β levels and in myocardial tissue of the LPS-induced rooibos group were not associated with ventricular dysfunction or reduction in coronary flow (Table 3). This is contrary to reports that IL-1β has a negative inotropic effect and that it also leads to endothelial dysfunction [86, 51]. The reason for this could be that the dose of LPS that we used in this study was sufficient to induce inflammation but not high enough to induce cardiac dysfunction. In previous studies where ventricular dysfunction was reported, higher doses of LPS were used [87, 88]. Lew *et al.,* 2013 [89] also reported that sub-lethal dose of LPS had minimal effect on cardiac function. Another plausible reason could be that low doses or sub-lethal doses of LPS have been shown to have a pre-conditioning effect [90, 91, 92].

5.0 Conclusion

In this study we have shown that LPS induction caused inflammation. Evidence presents, for the first time, that the dietary combination of rooibos and RPO significantly enhanced the up-regulation of endogenous myocardial anti-inflammatory IL-10 levels, a phenomenon shown to have great potential in cardio-protection. This study also showed that IL-6 in this model acted more like an anti-inflammatory rather than pro-inflammatory cytokine. It was also evident from the results that there is a difference in inflammatory response to LPS injection between the myocardium and the systemic circulation. Therefore, the results argue that the combination of these two natural food substances exhibit potential anti-inflammatory properties worth investigating further.

6.0 Conflict of interest

The authors declare that there is no conflict of interest

7.0 Acknowledgements

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Chapter 5

Red palm oil improves myocardial ischaemic/reperfusion tolerance in a model of induced inflammation.

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Abstract

Red palm oil (RPO) is rich in various bioactive compounds including fatty acids, carotenoids, tocotrienols, tocopherols, sterols and squalene. Even though there is convincing evidence of the cardio-protective effects of RPO, the efficacy of RPO as a cardio-protective agent against ischaemia-reperfusion injury has not yet been investigated in a model of *in vivo*-induce inflammation. Therefore, the aim of this study was to establish if dietary RPO will improve functional recovery and reduce infarct size in LPS-treated hearts. Animals were supplemented with 0.2 ml of RPO (7g/kg diet) daily for 28 days. Potential mechanisms of protection were also elucidated. Male Wistar rats weighing 150-200 g were divided into 4 groups receiving standard rat chow and water for 28 days, 18 hours prior to sacrificing animals were injected with either LPS or PBS (intraperitoneal). Hearts were rapidly removed and mounted on the Langendorff system for determination of cardiac function and infarct size. Myocardial IL-1*β* was increased, while IL-10 was reduced in LPS-treated hearts compared to the non-treated hearts, indicating that inflammation was induced. RPO supplementation improved functional recovery and reduced infarct size in control and LPS-treated hearts. RPO-induced cardio-protection was associated with increased myocardial protein expression of SOD1, SOD2, GPX1 and increased p38 phosphorylation during reperfusion. LPS treatment increased myocardial protein expression of NFkB p65 which was reversed by RPO. RPO offered protection with or without LPS-treatment. Reduction of myocardial NFkB protein expression, increased p38 phosphorylation and elevated mitochondrial antioxidant enzymes (SOD2 and GPX1) as well as cytosolic SOD (SOD1) are proposed as potential mechanisms underlying the RPO-induced cardio-protection in this model of *in vivo*-induced inflammation.

1.0 Introduction

Cardiovascular diseases, particularly ischaemic heart diseases, poses significant a health problem across all economic strata of societies worldwide (Callow, 2006; Gaziano et al., 2010). Myocardial infarction is the common complication of ischaemic heart disease. Even though timely reperfusion of the myocardium after myocardial infarction is the prerequisite to salvage the viable myocardial cells, it has been shown to contribute to lethal injury following prolonged periods of ischaemia (Przyklenk, 1997; Cannon et al., 2000). The pathogenesis of ischaemia-reperfusion injury is complex and involves multiple pathological mechanisms including oxidative stress, intracellular calcium overload, endothelial dysfunction, metabolic alterations and inflammation (Jordan et al., 1999; Di Napoli et al., 2002; Kaminski et al., 2002; Sabine et al., 2009; Pashkow, 2011). Manifestations of ischaemia reperfusion injury include myocardial stunning, reperfusion arrhythmias and the no-reflow phenomenon (Eltzschig and Collard 2004). Ischaemia-reperfusion is associated with an increased release of oxygen free radicals and inflammatory mediators, including cytokines, which can lead to endothelial dysfunction and myocardial cell death (Jordan et al., 1999). It has been shown that targeting reperfusion injury with anti-inflammatory agents and antioxidants may be beneficial in reducing reperfusion injury (Panes et al., 1999, Tsujita et al., 2004). Cytokines play a key role in mediating myocardial response to injury following myocardial infarction, in acute reperfusion injury and also in cardiac repair (Nian et al., 2004). The inflammatory response may also contribute to adverse remodelling of the ventricle by triggering degradation of the extracellular matrix (Steffens et al., 2009).

Dietary components have the capacity to affect cellular processes by modulating gene and protein expression which can lead to changes in metabolic pathways and homeostatic regulation, with the potential to affect health and disease (Afman and Muller 2006). RPO is a natural oil obtained from the fruits of the oil palm tree, *Elaeis guineensis* (Family *Arecaceae*). It is rich in various bioactive compounds, including fatty acids, carotenoids, tocotrienols, tocopherols, sterols and squalene (Nagendran et al., 2000). It has been shown to protect the heart from the consequences of ischaemia-reperfusion injury (Engelbrecht et al., 2006; Bester et al., 2010). Furthermore studies have shown that RPO, or some of its components, were associated with important modulation of signalling events early in reperfusion (Das et al., 2008; Engelbrecht et al 2009; Katengua-Thamahane et al., 2012). This has important therapeutic implications since treatment early in reperfusion has been identified as a very important window for therapeutic intervention (Ovize et al., 2010).

Cardio-protective interventions applied at the onset of reperfusion have been shown to improve functional recovery and reduced infarct size after myocardial infarction (Piper, 1998, Ovize et al., 2010). This indicated that addition of adjunct reperfusion therapies at the onset of reperfusion have the potential to salvage more myocardial cells over and above that which can be salvaged by reperfusion alone. It has recently been shown that dietary RPO supplementation has the potential to improve myocardial ischaemic tolerance in experimentally-induced pathological conditions such as hyperlipidemia and hypertension (Szucs et al., 2011; Bačová et al., 2012). Infarct size is an important determinant of prognosis after acute myocardial infarction (Gaudron et al., 2001; Ertl and Frantz, 2005). Reduction in infarct size by dietary RPO supplementation has been reported previously. In this regard Bester and co-workers (2010) showed that RPO reduced infarct size in healthy rat hearts and Szucs et al., (2011) reported that dietary RPO supplementation reduced infarct size in a model of hyperlipidemia.

Attenuation of reperfusion injury and reduction in infarct size greatly determine the clinical outcome in patients with ischaemic heart disease (Bolli et al., 2004). Several studies have investigated and reported the cardio-protective effect of dietary RPO supplementation (Engelbrecht et al., 2006; Bester et al., 2010; Engelbrecht et al 2009; Wergeland et al., 2011; Szucs et al., 2011). However, this is the first study to our knowledge to investigate the cardio-protective effects of dietary RPO supplementation in a model of inflammation. Inflammation is an established risk factor for cardiovascular diseases and it has been implicated as an important phathophysiological mechanism underlying most cardiovascular conditions including ischaemic heart disease (Albert et al., 2002). Ridker and colleagues (1997) reported that the efficacy of cardio-protective interventions in reducing the risk of a first myocardial attack appeared to correlate with the level of baseline inflammation in apparently healthy men. This may suggest that the presence of inflammation could potentially modify the ability of the myocardium to respond to the ischaemic insult and/or alter the way that it respond to cardio-protective interventions.

Antioxidant enzymes represent the first line of defense against conditions of acute oxidative stress such as ischaemia-reperfusion injury and inflammation (Bolli and Marbán, 1999; Christofidou-Solomidou et al., 2006; Ratnam et al., 2006). Hence, in the current study the myocardial protein levels of SOD1, SOD2 and GPX1 were measured to establish if modulation of myocardial antioxidants could constitute a potential protective mechanism underlying RPO-induced cardio-protection. LPS administration is associated with recruitment of the innate immunity including the inflammatory response, with ultimate induction of cytokines (Nian, 2004). Activation of the MAPK and the JAK-STAT pathway are upstream to the NFkB signaling

pathways, which upon activation can lead to induction of cytokines (Nian, 2004). NFkB is an important redox sensitive transcription factor involved in regulating transcription of various genes that play key roles in regulating the immune system and also in the modulation of myocardial survival and apoptosis (Catz and Johnson, 2001; Mustapha et al., 2000). Activation of NFkB can be beneficial or detrimental depending on the cell type and stimulus involved (Luo et al., 2005). In general, NFkB activation plays an important role in regulating the genes that help the organism to adapt to environmental and cellular stress in acute conditions. while if activated for prolonged periods of time it can become detrimental (Mustapha et al., 2000; Misra et al., 2003; Hamid et al., 2011; Hayden and Ghosh 2012). Oxidative stress plays a crucial role in mediating ischaemia-reperfusion injury especially during the early phase of reperfusion (Griendling et al., 1997). The oxidative burst that is associated with the early moments of reperfusion can alter signaling vents including the activity of NFkB which is mainly regulated by the redox status of the cell (Li and Karin 1999, Li et al., 1999). p38 MAPK signaling plays an important role in response to ischaemia-reperfusion and in the regulation of inflammatory responses (Schulze et al., 2002; Clerk and Sugden 2006). The role of p38 in the setting of ischaemia and reperfusion is controversial with some authors showing protection and other reporting the negative effects (Wang et al., 1998; Mocanu et al., 2000).

In the current study inflammation was induced with LPS following the protocol already described by Ohsaki et al. (2006). Induction of inflammation was followed by ischaemia-reperfusion to investigate the cardio-protective effects of dietary RPO in a clinically relevant condition such as inflammation. Therefore, the aim of the current study was to establish if dietary RPO will improve functional recovery and reduce infarct size in LPS pre-treated hearts. The levels of myocardial IL-1 beta and IL-10 were measured, firstly to establish if there was any inflammation induced and also to determine if dietary RPO modulated the inflammatory response after ischaemia-reperfusion injury in LPS treated hearts. The roles of the redox sensitive transcription factor NFkB and the p38 MAPK signalling were investigated as possible mechanisms of protection and selected myocardial antioxidant enzymes were also determined to investigate their involvement.

2.0 Materials and Methods

Animals 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). Animals were caged individually and housed in an animal house at a constant temperature of 27°C and they were exposed to a twelve-hour artificial day-night cycle. The rats had free access to water and food. The ethical clearance for this study was granted by the Health and Applied Sciences Research Ethics Committee of the Cape Peninsula University of Technology, (NHREC: REC-2304408-014).

2.1 Experimental model

Male Wistar rats weighing 150-200 g were randomly divided into 8 groups which were then subdivided into 2 groups. The one group received standard rat chow and water for 28 days without LPS injection (NO-LPS group) and the other group received rat chow and water for 28 day plus LPS injection (LPS group) . The animals in the NO-LPS group consisted of a negative control receiving standard rat chow and water while the other group received standard rat chow and water plus 0.2 ml RPO/day (equivalent to 7g/kg diet). At end of the feeding period, 18 hours prior to sacrificing, these animals were injected (intraperitoneal) with 0.1 ml of PBS (pH 7.2). The animals in the LPS group received the same feeding protocol but at the end of the feeding time, 18 hours prior to sacrificing they were injected (intraperitoneal) with a lipopolysaccharide (*Escherichia coli* serotype, 0111:B4) dissolved in sterile filtered phosphate buffered saline (PBS) to obtain 0.5 mg/kg body weight in 0.1 ml (LPS group) (Ohsaki et al., 2006) Fig 1. The composition of RPO that the rats consumed is shown in Table 1. At the end of the feeding period, rats were anaesthetized with an intraperitoneal injection of 2mg/kg intraval sodium (sodium pentobarbital). Hearts were then rapidly excised and placed in ice-cold Krebs-Henseleit buffer and transferred to the Langendorff perfusion apparatus. Hearts were 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) and a perfusion pressure of 100cmH_2O was maintained throughout the protocol. After mounting to the Langendorff system, hearts were stabilised for 10 minutes after which they were subjected to 15 minutes perfusion period and baseline measurement were then documented. At the end of perfusion time, hearts were subjected to 30 minutes of global ischaemia followed by 120 minutes of reperfusion (Fig 1). Left ventricular developed pressure (LVDevP) was measured

with the aid of a balloon made from transparent sandwich wrap film inserted into the left ventricle through the opening of the left atrium. The balloon was connected to a power lab system (AD Instruments Pty Ltd., Castle Hill, Australia). After insertion, the balloon was inflated to 2mmHg, and the contraction force of the heart against the balloon caused water displacement that was converted to pressure. LVDevP, minimum and maximum of left ventricular pressure derivatives, heart rate as well as coronary flow were measured at baseline and at 10 minutes reperfusion.

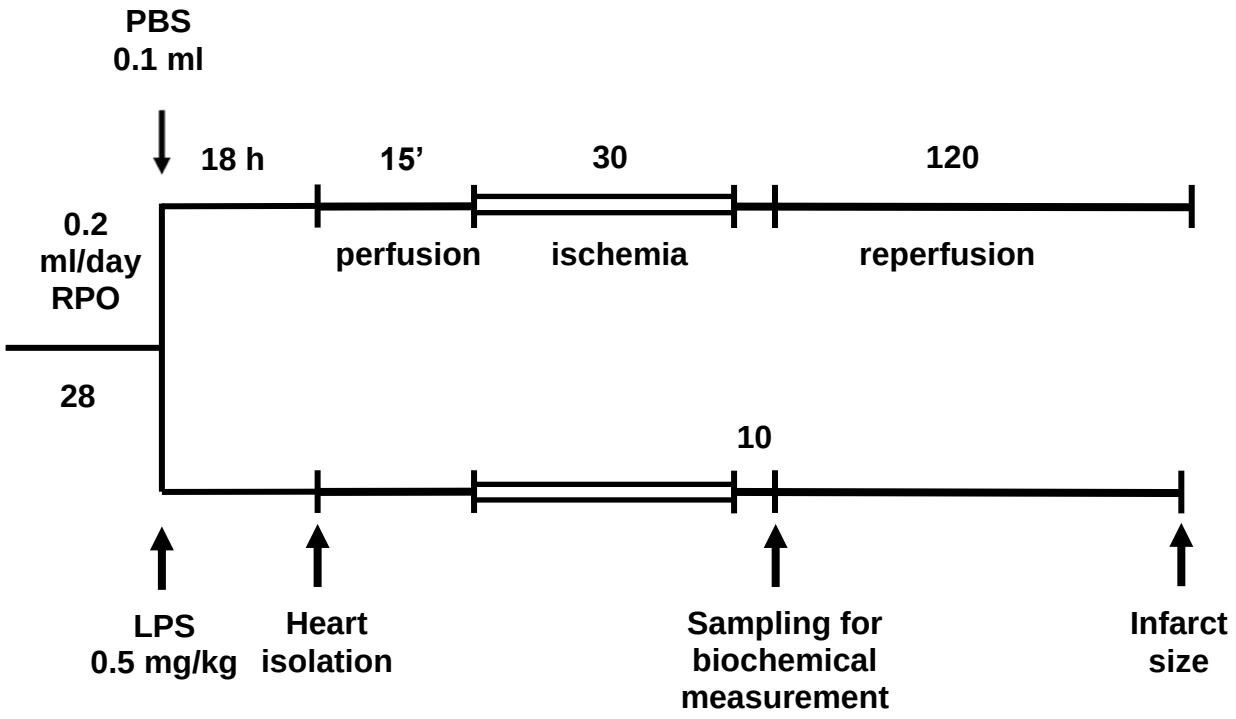


Figure 1: This figure illustrates the experimental protocol. Four experimental groups (NO-LPS control, LPS control, NO-LPS RPO and LPS RPO) were feed for 28 days. At the end of the feeding period, 18 hours prior to sacrificing animals were injected with either PBS or LPS intraperitoneally. Hearts were then excised and mounted on a Langendorrf perfusion system. Hearts were perfused for 15 minutes and then subjected to 30 minutes global ischaemia after which reperfusion was reinitiated for 120 minutes. At the end of the perfusion protocol hearts were removed from the system and frozen for infarct size determination. Mechanical function was measured both at baseline phase and at 10 minutes reperfusion. Biochemical samples were collected at 10 minutes of reperfusion.

Table 1: The composition of RPO consumed by the rats

Parameters	Specifications	Typical
Fatty acids %	0.1 max	0.058
Moisture and impurities, %	0.1 max	0.03
Iodine Value	48-53	51.2
Slip melting point, c	33-37	36.4
Carotenes, ppm	400 min	420
Tocopherols and Tocotrienols, ppm	400 min	860
Nutritional information		
Amt/serving	Qty per 14 g	Qty per 100 g
Energy	518 kJ	3700 kJ
Protein	0.0 g	0.0 g
Fat, total	14 g	100 g
saturated	7.0 g	50.0 g
Trans	0.0 g	0.0 g
polyunsaturated	1.5 g	11.0 g
monounsaturated	5.5 g	39.0 g
Cholesterol	0.0 g	0.0 g
Carbohydrates	0.0 g	0.0 g
sugars	0.0 g	0.0 g
Sodium	0.0 g	0.0 mg
Carotenes as Vitamin A activity	640 ug	4600 ug
Vitamin E	2.5 mg	18.0 mg
Tocopherols	1.7 mg	12.0 mg
Tocotrienols	4.8 mg	34.0 mg

Certificate of analysis prescribed by Carotino 2010.
www.carotino.com

2.2 Infarct size determination

At the end of the perfusion protocol hearts were removed from the system and frozen at -20°C over night for infarct size determination. The frozen hearts were taken out of the -20°C freezer and cut into approximately 2mm thick cross-sectional slices. The slices were placed into a 10% formalin solution for 10 minutes and then transferred in phosphate buffer (pH 7.4). The thin heart slices placed between the two glass sheets, scanned and analysed for infarct size using planimentry software (Infarctsize^{TM} 1.0 Pharmahungary, Szeged, Hungary). Infarct size was expressed as a percentage of the area at risk, in global ischaemia the whole heart is the area at risk.

2.3 Western blot analysis

Myocardial SOD1, SOD2, GPX1, Total and phosphorylated p38, NFkB protein expression levels were determined using western blot protein analysis. Cardiac proteins 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) 1, dithiothreitol (DTT) 1, aprotinin 10 μg/ml. The tissue lysates were diluted in Laemmli sample buffer, boiled for 5 min and equal amounts of protein concentration were loaded per lane and subjected to PAGE-SDS gel electrophoresis (Bio-RAD Mini Protein Tetra cell 552BR). 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). Membranes were then incubated with the primary antibodies overnight, after which they were subsequently washed with large volumes of TBST (5x3 minutes), and incubated with the secondary antibody conjugated with alkaline-phosphotase for one hour with continuous shaking at room temperature.

2.4 Immunoassay for cytokine analysis

Determination of cytokines levels in the myocardial tissue samples was performed using the Bio-Plex bead array system (Bio Rad Laboratories, USA). Assays were carried out in 96-well filter plates. The cytokine kits were obtained from Millipore (USA). Analyses of samples were

performed using undiluted myocardial tissue homogenates, originally prepared in phosphate buffer at a dilution of 1:4. Samples were evaluated in duplicate. All analyte levels in quality control reagents included in the kits were within the expected references ranges.

2.5 Data analysis

Results are expressed as mean ± standard error of the mean (SEM). Differences between the groups were determined using an unpaired Student's *t*-test and to compare differences in multiple groups, a Two-way ANOVA with a Bonferoni and a post hoc test was used. $P < 0.05$ was considered to be statistically significant.

3.0 Results

3.1 Effects of dietary RPO on myocardial IL-1 β and IL-10 at 10 minutes reperfusion.

Administration of LPS resulted in significant increase of myocardial IL-1 β in the LPS treated hearts, both in non-supplemented rats and in RPO supplemented rats compared to their NO-LPS counterparts: NO-LPS control vs LPS control (57.03±6.0 vs 78.65±9.6 pg/ml, 0.01) and for supplemented hearts, NO-LPS RPO vs LPS RPO (55.70±6.5 vs 86.6±13.2 pg/ml, $^{\#}p<0.01$) Fig 2 A. When considering the myocardial IL-10 levels, a significant decrease in IL-10 was observed in LPS-induced rats compared to their non-treated counterparts: NO-LPS control vs LPS control (849.2±85.9 vs 431.4±47.4 pg/ml, $^{\#}p<0.01$) and for supplemented hearts, NO-LPS RPO vs LPS RPO (768.7±134.4 vs 455.7±36.4 pg/ml, $^{\#}p<0.01$) Fig 2 B.

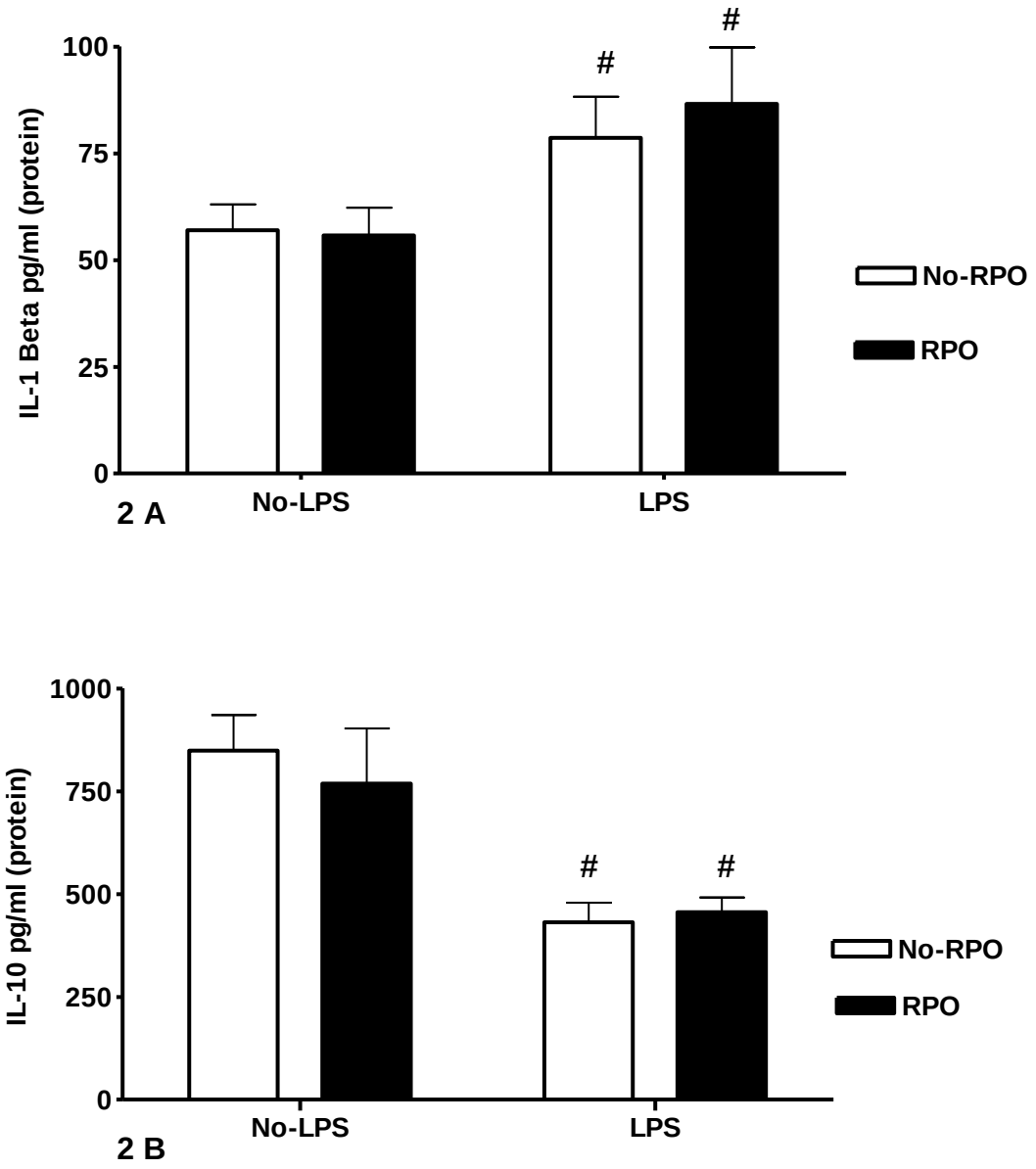


Figure 2: Effects of dietary RPO on myocardial IL-1 beta and IL-10 at 10 minutes reperfusion. Results are expressed as means ± SEM, n=6-7/group. Two-way ANOVA with a post hoc was used to compare the groups. $^{\#}p<0.05$ for LPS treated groups significantly different from their NO-LPS counterparts.

3.2 Effects of dietary RPO on LVDevP %recovery at 10 minutes of reperfusion

Dietary RPO supplementation significantly improved LVDevP recovery with or without LPS treatment. The effect of dietary RPO in improving functional recovery was more pronounced in NO-LPS RPO hearts compared to their control counterparts. NO-LPS control vs NO-LPS RPO: (34.09±6.3% vs 64.17±4.7%, **p<0.01). RPO supplementation improved functional recovery to a lesser degree in the RPO LPS-induced rats compared to the LPS (31.51±2.7% vs 55.18±7.8%, *p<0.05), Fig 3. Inflammation (LPS) did not have a negative effect on post-ischaemic functional recovery, whether in the presence of dietary RPO supplementation or without the dietary intervention.

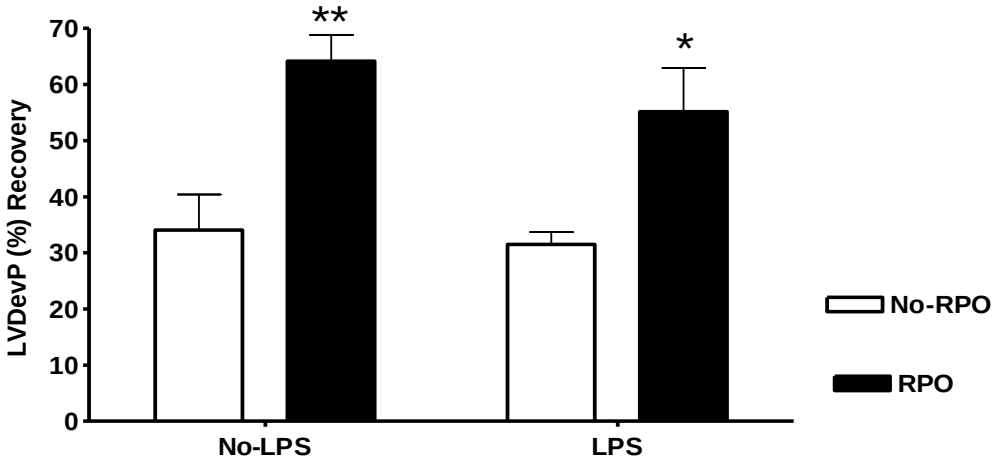


Figure 3: Effects of dietary RPO on LVDevP %recovery at 10 minutes of reperfusion. Results are expressed as means ± SEM, n=5-6/group. Two-way ANOVA with a post hoc was used to compare the groups. NO-LPS control group was compared to the NO-LPS RPO group, **p<0.01 for NO-LPS control group significantly different from NO-LPS RPO group, *p<0.05 for LPS control group significantly different from LPS RPO group.

3.3 Effects of dietary RPO on infarct size after 2 hours of reperfusion

RPO supplementation significantly reduced infarct size with or without LPS treatment. The infarct size reduction was more pronounced in the healthy rats consuming RPO vs NO-RPO control: (16.04±1.3% vs 31.90±1.0%, ***p<0.001). RPO still reduced the infarct size in LPS RPO hearts compared to the LPS control but the reduction was to a lesser degree, (28.86±2.1% vs 20.89±2.2%,**p<0.01), Fig 4. No differences were observed between the NO-LPS control group vs LPS control and between NO-LPS RPO vs LPS RPO showing that LPS did not have any effect on infarct size reduction.

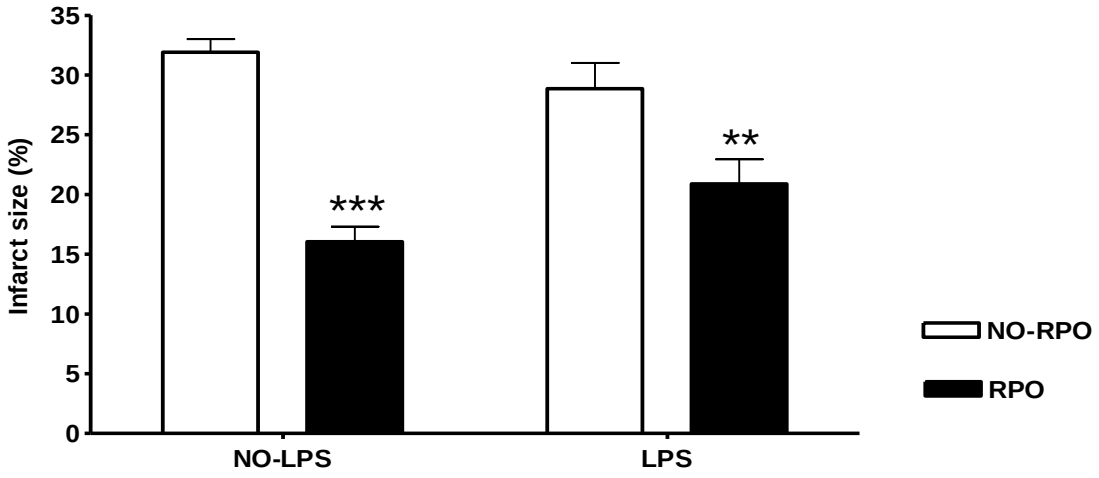


Figure 4: Effects of dietary RPO on infarct size after 2 hours of reperfusion. Results are expressed as means ± SEM, n=5-6/group. Two-way ANOVA with a post hoc was used to compare the groups. NO-LPS control group was compared to the NO-LPS RPO group, ***p<0.001 for NO-LPS control group significantly different from NO-LPS RPO group, **p<0.01 and also for LPS control group significantly different from LPS RPO group.

3.4 Effects of dietary RPO and LPS on baseline cardiac function

Dietary supplementation with RPO did not alter baseline LVDevP, but administration of LPS significantly increased baseline LVDevP (**p<0.01) when considering NO LPS control vs LPS control: (91.4±4.8 vs 99.9±2.5 mmHg). A similar pattern of results were observed for NO-LPS RPO vs LPS-RPO **p<0.01 (92.7±3.3 vs 104.7 mmHg). There were no differences observed in body weights and heart weights across all experimental groups, (Table 2).

Table 2: Effects of dietary RPO and LPS on baseline cardiac function and post-ischaemic LVDevP (absolute values). Body weights and heart weights are also shown. BW- body weight, HW- heart weight, CF- coronary flow, HR- heart rate, LVDevP- left ventricular developed pressure.p<0.01 for LPS treatment vs NO-LPS treatment.**

Groups	BW	HW	CF	HR	LVDevP	LVDevP (post-ischaemic-Absolute values)
NO-LPS cont	366.8±4.43	1.26±0.09	14.08±0.54	301.20±5.92	91.4±4.41	35.8±5.97
LPS-cont	342.4±11.50	1.2±0.11	14.28±0.62	300.40±14.39	99.9±2.45**	31.0±1.88
NO-LPS RPO	338.4±12.19	1.12±0.07	13.48±0.49	287.70±10.32	92.7±3.31	59.5±4.67
LPS-RPO	345.6±10.14	1.18±0.07	13.52±0.23	286.50±9.12	104.7±3.04**	58.1±8.95

****p<0.01 significantly different from their NO-LPS control group**

Abbriviations

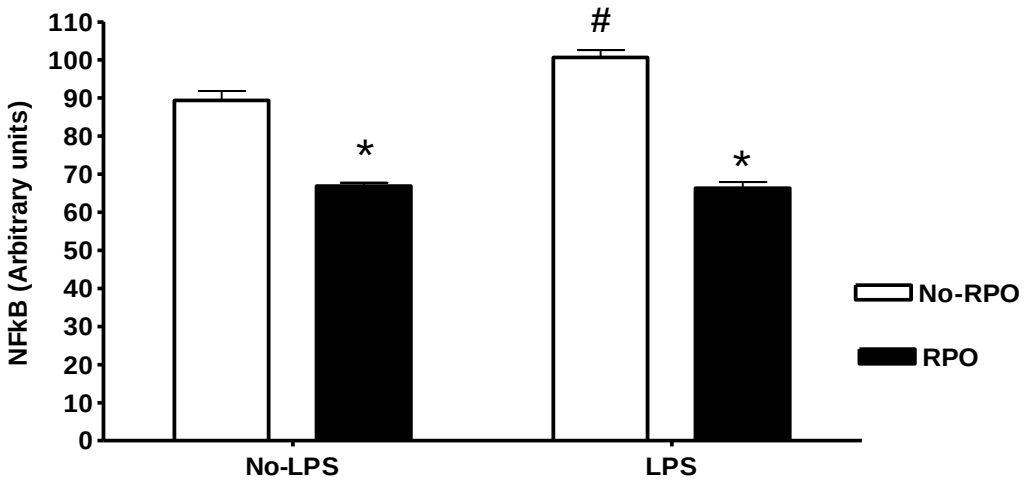
BW- Body weight
HW- Height weight
CF- Coronary flow
HR- Heart rate
LVDevP- Left ventricular developed pressure
LPS- Lypopolysaccharide

3.5 Effects of dietary RPO on cytosolic NFkB p65 protein levels at 10 minutes

The myocardial cytosolic NFkB p65 protein expression was significantly reduced in LPS-induced rats consuming RPO compared to the LPS controls, (66.32±1.6 vs 100.70±1.9 arbitrary units, *p<0.01). A similar pattern of results was also observed in the healthy rats consuming RPO when compared to the NO-LPS control, (66.85±0.9 vs 89.39±2.9 arbitrary units, *p<0.01). On the other hand, cytosolic NFkB p65 levels were significantly increased in the LPS control vs NO-LPS control, (100.70±1.9 arbitrary units vs 89.39±2.9 arbitrary units, #p<0.05), Fig 5 A.

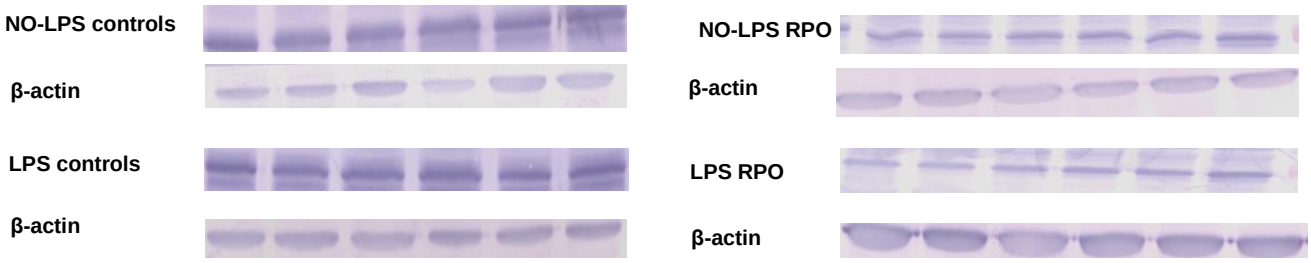
3.6 Effects of dietary RPO on phosphorylation of p38 at 10 minutes of reperfusion

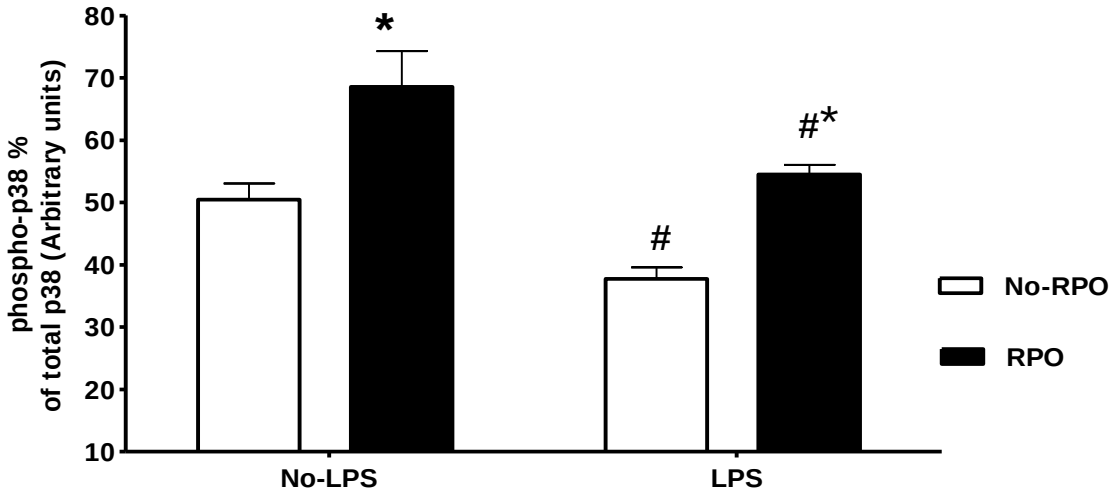
Dietary RPO supplementation significantly increased phosphorylation of p38 MAPK in the NO-LPS RPO hearts compared to the NO-LPS control hearts: (68.60±5.7 vs 50.46±2.6 arbitrary units, *p<0.05). This increase in p38 phosphorylation was maintained in the LPS-induced rats consuming RPO compared to the LPS control hearts, (54.50±1.5 vs 37.75±1.9 arbitrary units, *p<0.05). LPS-induction significantly reduced p38 phosphorylation in the LPS control compared to the NO-LPS control: (37.75±1.9 vs 50.46±2.6 arbitrary units, #p<0.05). A reduction in p38 phosphorylation was also observed when LPS RPO was compared to NO-LPS RPO: (54.50±1.5 vs 68.60±5.7 arbitrary units, #p<0.05), Fig 5 B.



5 A

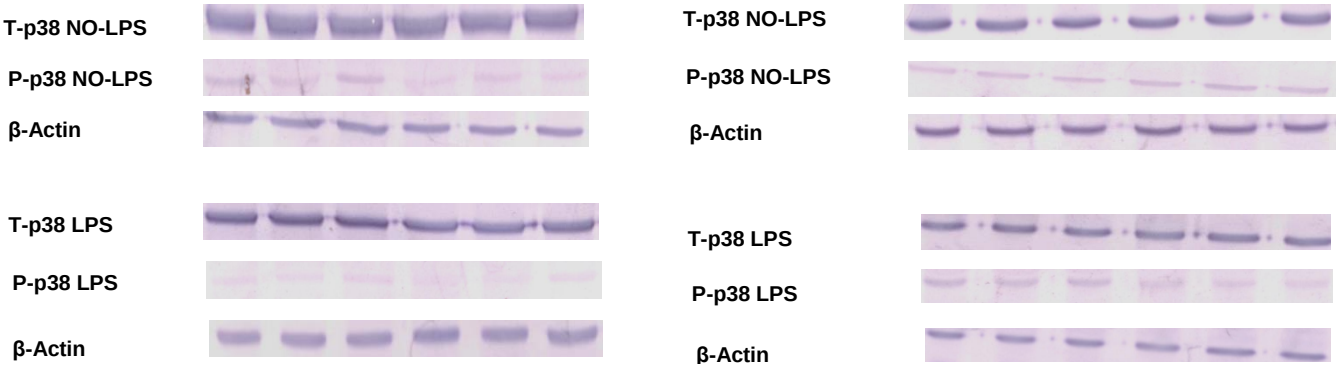
Figure 5: A: Effects of dietary RPO on cytosolic NFkB p65 protein levels at 10 minutes of reperfusion. Results are expressed as means ± SEM, n=6-7/group. Two-way ANOVA with a post hoc was used to compare the groups. NO-LPS control group was compared to the NO-LPS RPO group, *p<0.05 for NO-LPS control group significantly different from NO-LPS RPO group and also for LPS control group significantly different from LPS RPO group. #p<0.05 for LPS control group significantly different from the NO-LPS control.





5 B

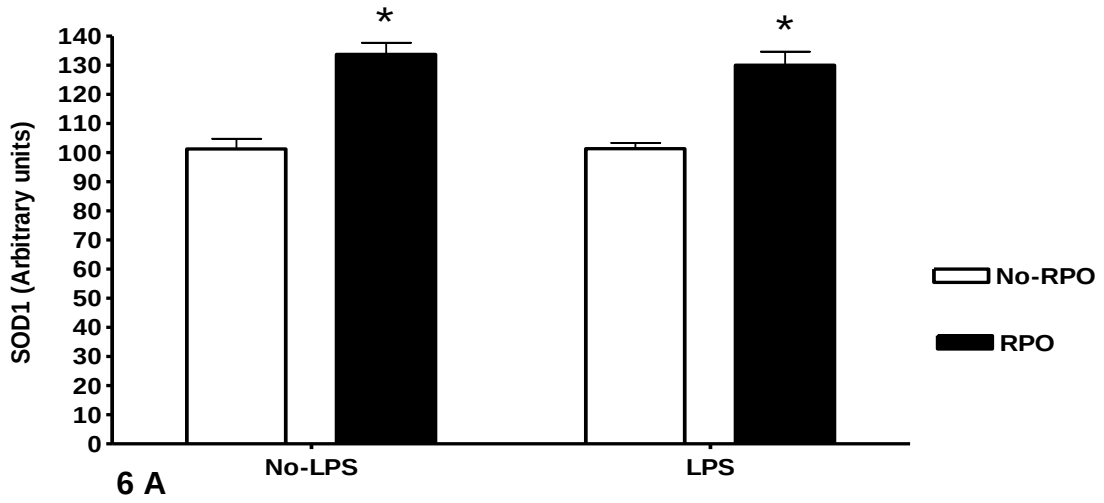
Fig 5 B: Effects of dietary RPO on phosphorylation of p38 at 10 of reperfusion. Results are expressed as means ± SEM, n=6-7/group. Two-way ANOVA with a post hoc was used to compare the groups. NO-LPS control group was compared to the NO-LPS RPO group, *p<0.05 for NO-LPS control group significantly different from NO-LPS RPO group and also for LPS control group significantly different from LPS RPO. #p<0.05 for LPS control significantly different from the NO-LPS control and for RPO LPS vs NO-LPS RPO.

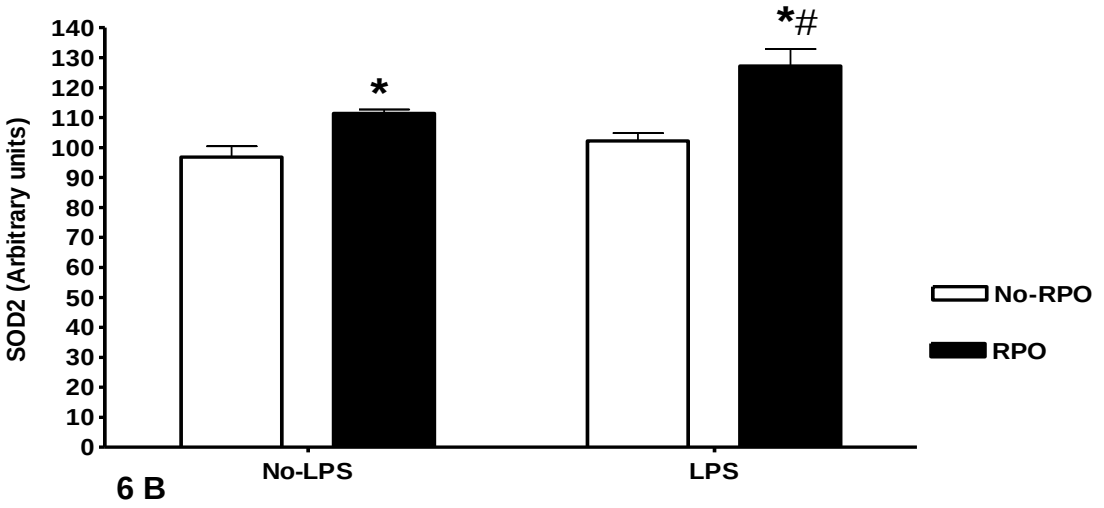


T-p38 = Total p38,
P-p38 = phosphorylated p38

3.7 Effects of dietary RPO supplementation on protein expression levels of SOD1, SOD2 and GPX1 at 10 minutes reperfusion.

Dietary RPO supplementation significantly increased SOD1 protein expression levels with or without LPS treatment, NO-LPS control vs NO-LPS RPO: (133.80±4.0 vs 101.30±3.5 arbitrary units, *p<0.05). Similarly an increase in the two LPS treated groups, RPO vs control was observed, (130.00±4.7 vs101.40±1.9 arbitrary units, *p<0.05), Fig 6 A. LPS induction alone did not have effect on SOD 1 protein expression as there were no differences observed between the NO-LPS control and the LPS control, likewise there were no significant differences observed between NO-LPS RPO and LPS RPO. When considering myocardial SOD2 proteins expression, a significant increase in SOD2 protein level was observed in healthy rats consuming RPO compared to the NO-LPS control hearts, (111.40±1.3 vs 96.86±3.6 arbitrary units, *p<0.05). A similar pattern of results were observed in LPS-induced rats consuming RPO compared to their control counter parts, (127.20±5.7 vs 102.20±2.6 arbitrary units, *p<0.05). An increase in SOD2 protein levels was observed in the LPS RPO vs NO-LPS RPO (127.20±5.7 vs 111.40±1.3 arbitrary units, $_{\#}$p<0.05), Fig 6 B. When considering the myocardial GPX1 protein expression, RPO significantly increased GPX1 protein expression with or without the LPS treatment, a significant increase in GPX1 was observed in healthy rats consuming RPO compared to the NO-LPS control rats consuming water (85.60±2.8 vs 73.67±1.2 arbitrary units,*p<0.05). Likewise, there was a significant increase in the expression of GPX1 in LPS-induced rats consuming RPO compared to the LPS control, (89.62±1.3 vs 65.12±1.2 arbitrary units, *p<0.05). GPX1 was significantly reduced in the LPS control compared to the NO-LPS control (73.67±1.2 Arbitrary Units vs 65.12±1.2 arbitrary units, $^{\#}$p<0.05), Fig 6 C.





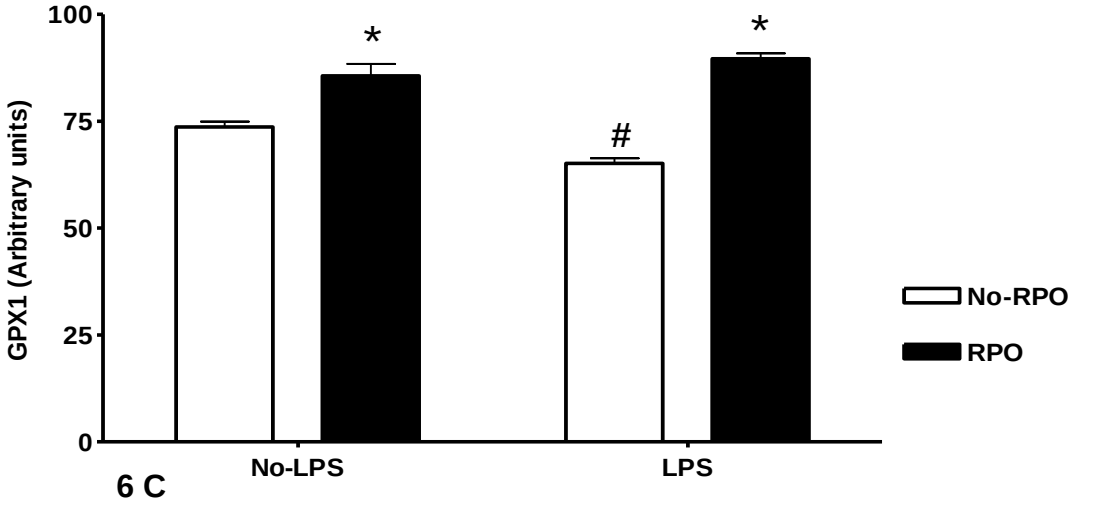
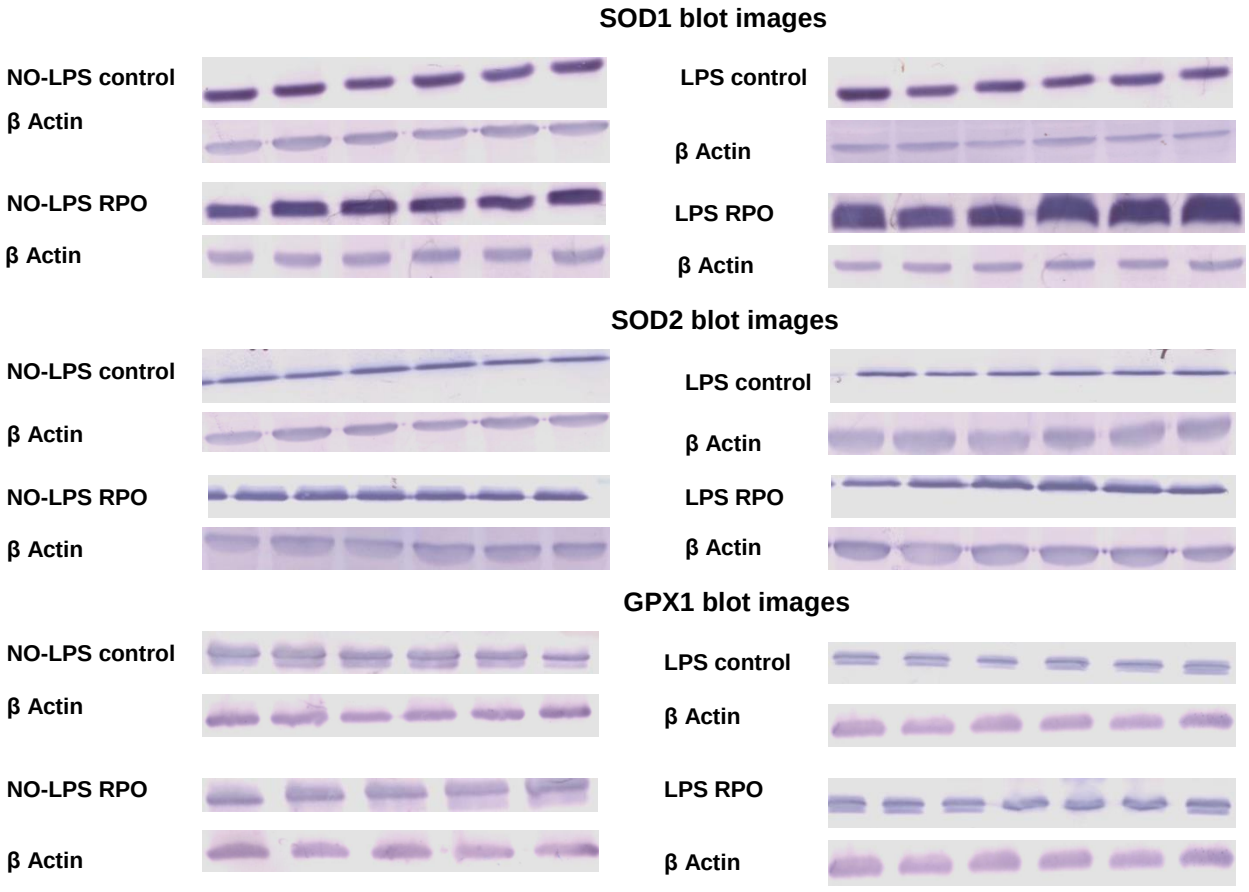


Figure 6: Effect of RPO on antioxidant enzymes

Fig 6 A: Effects of dietary RPO supplementation on SOD1 protein expression levels at 10 minutes reperfusion. Results are expressed as means ± SEM, n=6-7/group. Two-way ANOVA with a post hoc was used to compare the groups. NO-LPS control group was compared to the NO-LPS RPO group, *p<0.05 for NO-LPS control group significantly different from NO-LPS RPO group and also for LPS control group significantly different from LPS RPO.

Fig 6 B: Effects of dietary RPO supplementation on SOD2 protein expression levels at 10 minutes reperfusion. Results are expressed as means ± SEM, n=6-7/group. Two-way ANOVA with a post hoc was used to compare the groups. NO-LPS control group was compared to the NO-LPS RPO group, *p<0.05 for NO-LPS control group significantly different from NO-LPS RPO group and also for LPS control group significantly different from LPS RPO. #p<0.05 for LPS RPO significantly different from NO-LPS.

Fig 6 C: Effects of dietary RPO supplementation on GPX1 protein expression levels at 10 minutes reperfusion. Results are expressed as means ± SEM, n=6-7/group. Two-way ANOVA with a post hoc was used to compare the groups. NO-LPS control group was compared to the NO-LPS RPO group, *p<0.05 for NO-LPS control group significantly different from NO-LPS RPO group and also for LPS control group significantly different from LPS RPO. #p<0.05 for LPS control significantly different from NO-LPS control.



Blot images for antioxidant enzymes

4.0 Discussion

The aim of the current study was to establish if RPO offered protection in LPS-treated hearts by improving functional recovery and reducing infarct size. RPO improved mechanical functional recovery without LPS treatment as well as in the presence of LPS. The improvement in functional recovery was coupled with a reduction in infarct size. However, the cardio-protective effect of RPO was more pronounced in the absence of LPS compared to the LPS treated group, showing that myocardial ischaemic tolerance of the LPS-treated hearts was reduced compared to their NO-LPS counterparts, (Fig 3 and 4) as shown by the p values of the respective control vs their treated counter parts. The improved functional recovery and infarct size reduction in both LPS treated and NO-LPS rats consuming RPO were associated with augmentation of myocardial SOD1, SOD2 and GPX1 during reperfusion. LPS treatment increased myocardial expression of NFkB p65, which was reversed by RPO supplementation. RPO-induced cardio-protection was also associated with increased phosphorylation of p38 MAPK both in the absence and presence of LPS treatment.

4.1 Effects of dietary RPO and LPS on myocardial IL-1 beta and IL-10 at during reperfusion.

Inflammatory response and subsequent cytokine release constitutes an integral component of the body's normal response to myocardial injury following myocardial infarction (Nian et al., 2004). Acute induction of cytokines can confer cellular survival mechanisms or it can be detrimental, depending on the magnitude of cytokines released and the degree of inflammatory response elicited after myocardial infarction (Nian et al., 2004). Our results show that LPS resulted in increased myocardial levels of IL-1 beta and decreased levels of IL-10 with or without RPO dietary intervention, showing that RPO did not have an effect on the modulation of these cytokines during reperfusion. Pro-inflammatory cytokines are not constitutively expressed in the normal heart (Kapadia et al., 1995; Kapadia et al., 1997). Therefore up-regulation of myocardial IL-1 beta indicates that there was recruitment of the inflammatory response in the myocardium (Mann, 2003). The increased levels of IL-1 beta in the myocardium of LPS-treated hearts compared to their NO-LPS counterparts indicate that LPS caused an exacerbation of the myocardial inflammatory response in the LPS-treated hearts.

4.2 Effects of dietary RPO supplementation and LPS on baseline LVDevP

Administration of LPS caused increased cardiac function at baseline as evidenced by increased LVDevP in LPS-induced hearts compared to their non-treated hearts, suggesting a protective effect by LPS in the absence on ischaemic event. However, upon subjection to ischaemia the preconditioning effect of LPS was lost in non-supplemented hearts, while, LPS-exposed hearts of rats consuming RPO, maintained protection even after subjection to an ischaemic insult. The cardiomyocytes are among some of the different cell types in the body which express TLR 4 and therefore, they are potential targets for LPS (Boyd et al., 2006). The Toll-like receptor 4 (TLR4) are important pathogen recognition receptors that play an integral part in mediating cellular response to the antigenic component of the gram-negative bacterial cell wall (LPS). TLR 4 has also been shown to be triggered in response to endogenous substances released during ischaemia-reperfusion injury (Fitzgerald et al., 2001). The binding of LPS to the TLR 4 induce recruitment of various adaptor molecules to the receptor molecules which then initiate activation of complex signal transduction pathways. The activated signalling pathways then result in phosphorylation of down stream kinases that ultimately lead to activation of the NFkB pathway and increased expression of inflammatory cytokines (Liew et al., 2005, Boyd et al., 2006, O'Neill 2006). LPS has previously been shown to have a preconditioning effect in different cell types as well as in the myocardial cells (Wang et al., 2002; Lastres-Becker and Molina-Holgado 2006; Rosenzweig et al., 2007; Yao et al., 2011). Yao and co-workers (2009) reported that pretreatment of mesechymal stem cells with LPS, before transplantation into the ischaemic myocardium resulted in enhanced survival of the engrafted cells as well as improved cardiac function after experimental myocardial infarction.

4.3 Effects of dietary RPO supplementation and LPS on %LVDevP recovery and infarct size.

RPO protected the hearts from ischaemia-reperfusion in a model of LPS-induced inflammatory model as evidenced by improved %LVDevP recovery at 10 minutes of reperfusion and reduced infarct size after 2 hours of reperfusion. The improvement in functional recovery was observed in both LPS treated hearts and their non-treated counterparts. However the cardio-protect effect of RPO was more pronounced in NO-LPS hearts compared to their treated counterparts indicating that the susceptibility of the myocardium to ischaemic insult in LPS treated hearts was increased compared to the NO-LPS hearts. The first minutes at the onset of reperfusion are associated with a huge burst of reactive oxygen species production, a phenomenon which is believed to

play an important role in the pathogenesis of myocardial stunning (Bolli and Marbán, 1999). The induction of myocardial antioxidants observed in the current study may have been at least in part responsible for the observed improvement in functional recovery during the first 10 minutes of reperfusion. One of the most serious and devastating manifestations of ischaemia-reperfusion injury following acute myocardial infarction is reperfusion-arrhythmias (Zheng et al., 2001; Bolli et al., 2004). Bačová and colleagues (2012) have recently shown that dietary RPO supplementation significantly reduced lethal reperfusion induced-arrhythmias during the first five minutes of reperfusion in spontaneously hypertensive rats. The reduction of reperfusion-induced arrhythmia in their study was associated with improved post-ischaemic function and up-regulation of connexin 43.

Bester and co-workers (2010) previously reported that RPO reduced infarct size after exposure to 30 minutes of ischaemia and this reduction in myocardial infarction in RPO supplemented hearts was associated with attenuation of LDH in the coronary effluent indicating an anti-necrosis effect. Further more Engelbrecht and colleagues (2009) demonstrated that RPO-induced cardio-protection in the isolated perfused rat heart was associated with reduced markers of apoptotic cell death during reperfusion. It has previously been shown that dietary RPO supplementation was associated with modulation of oxidative stress during pre-ischaemic phase and this was associated with reduced infarct size after subjection to ischaemia-reperfusion in cholesterol supplemented rats (Szucs et al., 2011). The authors from this study showed that dietary RPO supplementation was associated with reduced MMP2 activation which is an indication of reduced oxidative stress. Dietary RPO supplementation has been previously linked to infarct size reduction in health hearts and in rats fed a high cholesterol diet. In this regard Bester and co-workers showed that RPO reduced infarct size in healthy rats. On the other hand Szucs et al. (2011) reported that dietary RPO supplementation reduced infarct size in rat fed a high cholesterol diet. In the current study we have shown that the presence in an inflammatory condition did not abolish the infracting limiting effect of dietary RPO supplementation.

4.4 Effects of dietary RPO and LPS on cytosolic NFkB p65 protein levels during reperfusion.

Dietary RPO supplementation was associated with a reduction in myocardial NFkB protein expression of NFkB. The presence of LPS did not affect the modulation of NFkB by RPO as the reduction in cytosolic NFkB levels was observed in the supplemented groups with or without LPS treatment. Reduction of myocardial mRNA and protein expressions have been linked to improved homodynamic function and inhibition of cardiac structural changes in rats with chronic heart failure (Huang et al., 2013). Regulation of the activity of NFkB is complex and is mainly controlled by the upstream signalling pathways such as the IKK and the MAPK signalling pathways. However, the cellular levels of NFkB has also been shown to play an important role via the mechanism of autoregulation (Scott et al., 1993). NFkB is a ubiquitously expressed redox sensitive transcription whose activation is triggered by various cellular stress stimuli including cytokines, oxidative stress, ischaemia and reperfusion (Li et al., 1999; Mustapha et al., 2000; Kis et al., 2003). In resting cells without the activating stimuli, NFkB remains sequestered in the cytoplasm bound to its inhibitor proteins. The binding of NFkB to its inhibitor causes it to be retained in the cytoplasm and thus preventing its activation and subsequent nuclear translocation (Valen, 2004). We have also demonstrated that cytosolic reduction in NFkB was associated with increased p38 phosphorylation an incidence which has been shown to lead to activation of NFkB (Bassi et al., 2008). In the presence of activating stimuli the inhibitor of NFkB becomes phosphorylated at specific regulatory serine residues leading to subsequent degradation by proteasome (Ghosh et al., 1998; Chen et al., 1999; Valen et al., 2001). The release of NFkB from its inhibitor protein causes a structural conformation which permits translocation of NFkB to the nucleus where it can induce transcription of its target genes (Ghosh et al., 1998; Chen et al., 1999; Valen et al., 2001; Gordon et al., 2011). NFkB has been shown to regulate cardiac myocyte survival through inhibition of apoptotic cell in ventricular myocytes (Mustapha et al., 2000). In another study Misra and colleagues (2003) reported that trans-genetic mice expressing a cardiac specific inhibitor of NFkB alpha displayed a greater infarct size and increased apoptosis. One of the ways by which NFkB is believed to regulate cell survival is through induction of anti-apoptotic protein genes such as Bcl2 and SOD2 (Catz and Johnson 2001: Shungo et al., 2009). Our results show that reduced cytosolic NFkB levels in RPO supplemented hearts was associated with increased SOD2 protein levels, an important mitochondrial antioxidant enzymes whose transcriptional genes are regulated by NFkB (Shungo et al., 2009).

4.5 Effects of dietary RPO and LPS on phosphorylation of p38 MAPK during reperfusion.

p38 MAPK is a mitogen-activated protein kinase which has been shown to play an important role in response to ischaemia-reperfusion injury (Weinbrenner et al., 1997; Mocanu et al., 2000; Schulz et al., 2002; Bassi et al., 2008). Other biological functions of p38 include regulation of cell death and inflammation (Baines and Molkentin 2005; Cleark and Sugden, 2006). Dietary RPO supplementation increased phosphorylation of p38 in both the LPS treated hearts and the NO-LPS hearts, in both groups the increase in p38 was associated with improved mechanical function as well as a reduction in infarct size. This is contrary to several previous reports indicating that increased activation of p38 during ischaemia-reperfusion aggravated myocardial injury (Meldrum et al., 1998, Barancik et al., 2000, Pu Liao et al., 2002). However there is also credible evidence showing that activation of p38 during ischaemia-reperfusion conferred cardio-protection (Maulik et al., 1998, Mocanu et al., 2000, Schulz et al., 2002). A study by Engelbrecht and colleagues (2006) demonstrated that dietary RPO supplementation to healthy rats resulted in increased phosphorylation of p38 and this augmentation in p38 phosphorylation was linked to an improved functional recovery. Available scientific data shows that activation of p38 can be both protective and detrimental depending of the circumstances. These seemly contradicting effects of p38 have been attributed to the presence of different p38 isoforms present in the heart (Wang et al., 1998). Evidence presented by Wang and co-workers (1998) demonstrated that the two p38 isoforms expressed in the heart exhibited divergent functions, p38 α was implicated in apoptosis while p38 β was linked to anti-apoptotic effects. Another important factor which has been shown to determine the outcome of p38 activation in the heart is the timing of activation or inhibition. In this regard Mocanu and co-workers (2000) clearly demonstrated that inhibition of p38 phosphorylation was associated with abrogation of ischaemic preconditioning in rat heart but the timing of administration of the inhibitor was critical to this effect.

The antibody that we used to determine the levels of phosphorylation of p38 in the current study was not isoform specific, therefore, it did not discriminate phosphorylation between the different isoforms of p38 expressed in the heart. However, it has previously been shown that dietary RPO supplementation exhibited potential anti-apoptotic effects during reperfusion (Engelbrecht et al., 2009) and evidence by Wang et al. (1998) identified p38 β as the p38 isoform with anti-apoptotic effect. Therefore, it can be argued that p38 β is the most likely isoform whose activity is modulated by dietary RPO supplementation.

4.6 Effects of dietary RPO and LPS o several n protein expression levels of SOD1, SOD2 and GPX1 during reperfusion.

Myocardial antioxidant enzymes play a central role in protecting the heart against the deleterious effects of ischaemia-reperfusion injury as they represent the first line of defense against acute insults due to oxidative stress (Christofidou-Solomidou et al., 2006). Dietary RPO supplementation was associated with increased protein expression of SOD1, SOD2 and GPX1 during reperfusion. One of the ways by which organisms protect themselves against oxidative stress is through enhanced production of endogenous antioxidant defence systems (Poljsak, 2011). Reperfusion of an ischaemic myocardium results in an increase in production of ROS, generating a condition of oxidative stress with potential depletion of myocardial antioxidants. It has been shown that the mitochondria are the major generators and the main targets of ROS during oxidative stress conditions (Rana et al., 2012). SOD2 and GPX1 are antioxidant enzymes which are mainly located in the mitochondria. In the current study we have shown that dietary RPO up-regulated the expression of these two antioxidant enzymes. Enhanced SOD2 protein expression with simultaneous increases in GPX1 represents a plausible mechanism by which RPO may improve the redox status of the mitochondria during ischaemia-reperfusion injury and ultimately protecting the myocardium from associated oxidative damage. To effectively cope with excessive oxidative stress imposed on the mitochondria during ischaemia-reperfusion, an increase in SOD2 should be accompanied by increases in either GPX and/or catalase in order to prevent excessive buildup of hydrogen peroxide (Ratnam et al., 2006). Mitochondrial oxidative stress leads to induction of the opening of the mitochondrial permeability transition pores and disruption of the mitochondrial membrane potential thereby triggering cells to undergo apoptosis or necrosis (Rana et al., 2012).

5.0 Conclusion

Inflammation was induced in LPS-treated hearts as evidenced by increased myocardial IL-1 β levels in LPS-treated hearts compared to non-treated hearts. Furthermore, the results of the current study demonstrate that dietary RPO supplementation offered cardiac protection as demonstrated by improved functional recovery and reduced infarct size in normal and LPS-treated hearts. For the first time we report evidence showing that dietary RPO improves myocardial tolerance against ischaemia-reperfusion injury in a model of *in vivo*-induced inflammation. Increased expression of myocardial NFkB was associated with poorer functional recovery and larger infarcts in LPS-treated hearts, while RPO-induced cardio-protection was

associated with decreased expression of NFkB and increased p38 phosphorylation during the first 10 minutes of reperfusion. Therefore, NFkB and p38 could at least in part be responsible for RPO-induced cardio-protection observed in this model. The mitochondria are important sources of ROS in inflammation and ischaemia-reperfusion and the induced ROS production can lead to depletion of endogenous antioxidants. Therefore, increased expression of mitochondrial antioxidant enzymes (SOD2 and GPX1) in RPO-supplemented hearts, argues for an enhanced defence against oxidative stress, specifically the oxidative stress that might be imposed on the mitochondria.

6.0 Conflict of Interest

The authors declare that there is no conflict of interest.

7.0 Acknowledgements

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Chapter 6: General Discussion

1.0 Introduction

Cardiovascular disease (CVD) is the major cause of death in industrialized countries and rapidly becoming a significant cause of morbidity and mortality in middle and low-income countries (Mayosi et al., 2005; Adeyi et al., 2007; WHO 2009e; Gaziano et al., 2010). The rising incidence of CVD in African countries has huge economic implications, especially because most of these countries are also faced with the burden of infectious diseases (Mayosi et al., 2005). Therefore, radical reforms in the health systems of these countries should be done, including those which emphasize the importance of research aimed at preventive and new therapeutic approaches in combating the surge of CVD. Sound understanding and appreciation of the role of cellular and molecular mechanisms underlying the aetiology and pathogenesis of CHD is of paramount importance if the battle against the ever increasing incidence of CVD is to be won in the long the term. Dietary interventions with food stuffs that are rich in antioxidants and also targeting of other modifiable CVD risk factors have the potential to attenuate and modulate some of the important pathogenic factors such as inflammation and oxidative stress (Carty et al., 2000; Carpenter et al., 2003; Duda et al., 2009; Moertl et al., 2011; Nodari et al., 2011). However, basic scientific research which addresses the role and mechanism of action of the bioactive compounds should be given priority so that their effectiveness as preventive and therapeutic agents in attenuating morbidity and mortality associated with CVD can be establised. In 2011, South African Health Minister Aaron Motsoaledi remarked that “South Africa’s healthcare model needs to shift from being hospi-centric to more preventative if the country is to win the fight against the burden of diseases” when addressing the first Global Ministerial Conference on healthy lifestyles and non-communicable disease in Moscow (www.mg.co.za/article/2011-04-29-motsoaledi-cals-for-more-emphasis-on-disease-prevention).

Non-phamacological strategies, including modification of dietary patterns, have been shown to be effective in reducing the risk of major cardiovascular events such as hypertension, stroke and CHD (Carretero and Oparil 2000; Staessen et al., 2001; Mehler et al., 2003; Bakris 2007; Jenkins et al., 2005; Estruch et al.,2013). RPO and rooibos are two natural plant extracts which have been shown to exhibit potential cardioprotective properties and recent evidence from experimental studies suggests that RPO may have anti-hypertensive and anti-arrhythmic properties. However, the cellular and molecular mechanisms underlying the anti-hypertensive and anti-arrhythmic effect of RPO are still to be elucidated.

This thesis reports results from three studies where different experimental models were employed to investigate the cardio-protective effects of dietary RPO and/ or rooibos, alone or in combination. In the first study the Langendorrf perfusion system and the model of genetically hypertensive rats (SHR) were used to investigate the effect of red palm oil on cardiac function in genetically hypertensive rats. Nitric oxide and antioxidant defence enzymes were investigated as potential mechanisms of protection. Evidence obtained from this study demonstrated that dietary RPO supplementation conferred cardio-protection in SHR as evidenced by improved reperfusion left ventricular pressure, maximum velocity of pressure rise (+dp/dt) max and fall (-dp/dt) max compared to their controls (i.e non-supplemented SHR). An increase in coronary flow was also observed in SHR supplemented with RPO compared to the non-supplemented SHR group. Taken together the functional results in this study indicated that RPO supplementation improved ventricular function and coronary circulation in hypertensive rats.

In the second study the Langendorff system and an acute model of LPS-induced inflammation were employed to establish if RPO and rooibos, when supplemented alone or in combination, will reverse the negative effects of LPS on cardiac function at baseline. The effect of dietary intervention on modulation of pro-inflammatory and anti-inflammatory cytokines in plasma and myocardial tissue was also investigated. The results of this study showed that LPS caused an increase in IL-1 β in the plasma of treated animals compared to non-treated animals. The elevation of IL-1 β levels in plasma of the LPS-induced rats consuming either RPO or rooibos alone were paralleled with increased levels of the anti-inflammatory cytokine, IL-10. Dietary intervention with RPO, rooibos or their combination, did not affect plasma IL-6 in both LPS-treated and non-treated rats. However, in the myocardium, rooibos consumption and the combination of RPO and rooibos were associated with increased levels of IL-6, while RPO alone did not have any effect on myocardial IL-6 levels. Neither LPS treatment nor dietary intervention with either RPO, rooibos or their combination had effect on baseline cardiac functional parameters.

The third study was designed to investigate whether dietary RPO improves myocardial ischaemic/reperfusion tolerance in a model of LPS-induced inflammation. Possible underlying mechanisms of protection which include NFkB, p38 MAPK and selected antioxidant enzymes were also investigated. Treatment of animals with LPS caused increased myocardial IL-1 β levels in LPS-treated animals compared to their control counterparts while myocardial IL-10 was reduced in treated animals compared to NO-LPS hearts. Dietary RPO supplementation resulted in improved myocardial tolerance to ischaemia-reperfusion injury in healthy hearts and hearts of LPS-treated animals as evidenced by improved functional recovery and reduction in infarct size.

RPO-induced cardio-protection was associated with increased myocardial protein expression of SOD1, SOD2, GPX1 and increased p38 phosphorylation during reperfusion. LPS treatment increased myocardial protein expression of NFkB p65 which was reversed by RPO.

1.1 Effect of red palm oil on cardiac function in genetically hypertensive rats: role of NO and antioxidant defence enzymes

The SHR model is one of the best experimental models to study human essential hypertension, because the development and progression of hypertension in SHR follows a similar pattern in many respect as the disease process in humans. The SHR model is also a widely used model to test the efficacy of new anti-hypertension medications (Doggrell and Brown 1998; Sarikonda et al., 2009). Therefore, the results obtained in this study have potential clinical implications. The aim of the first study was to establish whether red palm oil could protect the heart in a hypertensive model via the NO-cGMP pathway and the anti-oxidant enzyme system. The improved functional recovery in SHR hearts was associated with increased aortic NOS activity in both normotensive and hypertensive rats compared to their control counterparts. Bacova and co-workers (2012) used the same model of SHR and showed that RPO significantly reduced blood pressure in SHR compared to the non-supplemented counterparts. This created an opportunity to do a follow up study to investigative the involvement of the NO-cGMP pathway in SHR, as there is circumstantial evidince implicating cGMP in RPO-induced cardioprotection in hyperlipidemic rats (Esterhuyse et al., 2006). NO plays a pivotal role in regulating cardiovascular homeostasis, specifically the control of blood pressure. The increased NO activity observed in the current study can be linked to the RPO blood pressure-lowering effect previously reported by Bacova and co-workers (2012). RPO resulted in elevated coronary flow after reperfusion in both genotypes compared to the non-supplemented counterparts. However, this is specifically important in hypertensive rats as hypertension is associated with coronary insufficiency which usually results from associated long term complications (Houghton et al., 1990; Rosendorff et al., 2007). Moreover endothelial dysfunction plays a critical role in the pathogesis of hypertension, thus, the increased coronary flow during reperfusion together with increased aortic NOS activity strongly aurgues for the role of RPO in improving endothelial function in hypertensive rats.

The RPO-induced cardioprotection in SHR was also associated with an increase in protein expression of myocardial SOD2. SOD2 is an important mitochondrial antioxidant enzyme

involved in detoxification of superoxide anions resulting in hydrogen peroxide and oxygen (Dikalova et al., 2010). Myocardial cells are highly specialized aerobic cells with a large number of mitochondria. The large amount of ROS produced by the myocardial mitochondria during conditions of oxidative stress renders the heart more prone to oxidative damage. Therefore, increased myocardial SOD2 protein may lead to reduced oxidative stress. It has been shown that overexpression of SOD2 can lead to reduction of mitochondrial ROS production and restoration of NO bioavailibility in hypertension (Dikalova et al., 2010). Evidence presented in this study demonstrates that RPO resulted in increased NOS activity in aortic tissue which cause elevated NO production in the aorta, and thus leading to maintenance of endothelial function. Oxidative stress and endothelial dysfunction are common features in hypertension. Therefore, it could be argued that NO-cGMP pathway and antioxidant defense systems acted synergistically to restore cardiovascular function in SHR. Studies reporting on the anti-hypertensive effect of RPO are scarce and to the best of our knowledge this is the first study.

It has recently been shown that supplementation of dietary RPO to animals resulted in significant reduction in hypertension in both normotensive and hypertensive rats (Bacova et al., 2012, Boon et al., 2013). However, the cellular and molecular mechanisms underlying the anti-hypertensive properties of RPO are poorly understood. This is the first evidence to the best of our knowledge to provide mechanistic evidence linking the beneficial effect of RPO in SHR, to augmentation of myocardial SOD2 and improved increased activity of nitric oxide synthase.

1.2 The combination of red palm oil and rooibos show anti-inflammatory effects in rats

Dietary supplementation with either RPO, rooibos or their combination did not have an effect on baseline cardiac function, these results are not surprising, as previous studies from our laboratory have shown that dietary intervention with either RPO or rooibos did not alter function at baseline, but their beneficial effect on functional recovery was observed during reperfusion phase (Esterhuyse et al., 2006; Engelbretch et al., 2009; Pantsi et al., 2011). In the current study elevated plasma IL-1β levels were not associated with ventricular dysfunction or reduced coronary flow as previously reported in other studies (Rietschel and Brade 1992; Zeuke et al., 2002). Lew *et al.* (2013) also reported that sub-lethal dose of LPS had minimal effect on cardiac function even in the presence of significant myocardial structural changes.

IL-1β is one of the initial pro-inflammatory cytokines to be released in response to the invading microbial pathogens, and it plays a critical role in the induction of acute inflammation (Kadokami et al., 2001). Thus, the presence of elevated plasma IL-1β in this study confirms that inflammation was induced in our model. Cells that express pattern recognition receptors for LPS, including the cardiomyocytes, are activated upon exposure to the activating stimulus. Once activated, a complex signal transduction pathway is triggered which ultimately lead to the release of inflammatory cytokines. The released cytokines and other inflammatory mediators play a crucial role in orchestrating the inflammatory response (Sweet and Hume 1996; Feng et al., 2008; Chao, 2009). It is worth noting that inflammation is a complex process and does not only involve cytokines but there are other inflammatory mediators that are also recruited in a coordinated manner by the immune system to respond to tissue injury. These include recruitment and activation of leukocytes, antibodies and the complement system (Medzhitov 2010). The results indicate a potential anti-inflammatory property of rooibos and RPO at systemic level when supplemented individually.

IL-10 is a potent ant-inflammatory cytokine whose main role is to down-regulate production of inflammatory cytokines, and as such plays an important role in mechanisms that regulate inflammatory resolution (Rennick et al., 1997; Johnidis et al., 2008). IL-10 is one of the important anti-inflammatory cytokines that plays a critical role in determining the net effect of cytokines during the inflammatory response (Heumann et al., 1992; Fichtner-Feigh et al., 2008). Therefore, the increased production of IL-10 in rats supplemented with RPO and rooibos argues for the role of RPO and rooibos in modulating the inflammatory response in this model. Evidence suggest that IL-6 has both pro-inflammatory and anti-inflammatory characteristics (Steensberg et al., 2003) and in some circumstances IL-6 has been shown to evoke an inflammatory response by inducing production of anti-inflammatory cytokines such as IL-10 (Steensberg et al., 2003). The simultaneous increase of IL-6 and IL-10 in hearts from rats that consumed a combination of RPO and rooibos, suggests that IL-6 might be acting as an anti-inflammatory cytokine leading to the enhancement of IL-10 production. RPO and rooibos did not have any effect on IL-10 levels when supplemented individually. However, the combination of rooibos and RPO enhanced endogenous production of myocardial IL-10 in LPS-treated rats. This argues for an enhanced anti-inflammatory effect with the combination of RPO and rooibos at organ level. This was not observed with either RPO or rooibos alone.

1.3 Red palm oil improves myocardial ischaemic/reperfusion tolerance in a model of induced inflammation.

Inflammation is an important confounding factor in cardiovascular associated morbidities and mortalities therefore in this study, induction of inflammation was followed by an ischaemia-reperfusion injury model to to establish the cardio-protective effects of dietary RPO in a clinically relevant condition such as inflammation. The results of this study show that RPO offered cardioprotection against ischaemia-reperfusion injury in a model of LPS-induced inflammation, as evidenced by improved LVDevP pressure recovery and reduced necrotic cell death (infarct size). RPO offered cardio-protection in LPS-treated hearts as well as in healthy hearts. We observed no differences in function and infarct size between the normal controls and the LPS-control hearts. This is in agreement with previous reports where pre-treatment of isolated hearts with low dose of LPS resulted in preservation of left ventricular function after ischaemia-reperfusion injury (Yao et al., 2011).The cardioprotective effects of RPO in inflammation-induced hearts as well as in normal hearts ware associated with reduced expression of myocardial NFkB and increased phosphorylation of p38 during reperfusion. However poorer functional recovery and bigger infarcts in LPS-treated hearts were associated with increased myocardial expression of NFkB and reduced p38 phosphorylation. Therefore, NFkB and p38 could at least in part be responsible for RPO-induced cardio-protection observed in this model.

Augmentation of myocardial SOD1, SOD2 and GPX1 by RPO in inflammation-induced hearts and healthy hearts represent a potential mechanism of protection. Ischaemia-reperfusion injury and inflammation are conditions that are both associated with increased production of ROS with potential depletion of myocardial endogenous antioxidants. One way by which organisms protect themselves against oxidative stress is through enhanced production of endogenous antioxidant defence systems (Polisak, 2011). Overexpression of SOD2 has been shown to attenuate mitochondrial ROS generation as well as oxidative damage and reduced cell death (Motoori et al., 2001). In another study, SOD2 overexpression resulted in protection of mitochondrial respiratory function and blockade of apoptosis in ischaemia-reperfusion injury (Suzuki et al., 2002). Partial deficiency of SOD2 in heterozygous SOD2 knockout mice resulted in impaired contractile function after 30 minutes of ischaemia suggesting the importance of SOD2 in determining the myocardial tolerance to oxidative damage (Gregory et al., 2002). Increased expression of myocardial antioxidant enzymes during reperfusion argues for an enhanced defense against oxidative damage and a potential role of dietary RPO supplementation in different pathologies where oxidative stress is known to play an important role. SOD2 and GPX1

are primarily mitochondrial antioxidant enzymes. Therefore, their upregulation is particularly important for protecting the mitochondria against the oxidative stress that is associated with inflammation and ischaemia-reperfusion injury. Protection of the mitochondria from oxidative damage is important in ultimately protecting the myocardium from oxidative damage and cell death. Mitochondrial oxidative stress leads to induction of the opening of the mitochondrial transition pores and disruption of the mitochondrial membrane potential thereby triggering cells to undergo apoptosis or necrosis (Kokoszka et al., 2001, Rana et al., 2012).

2.0 Conclusion

Using a model of genetically hypertensive rats (SHR) we have shown that RPO improved functional recovery in SHR, which was associated with increased aortic NOS activity and increased myocardial protein expression of SOD2. Endothelial dysfunction and depletion of endogenous antioxidant defense systems, which may lead to oxidative stress, are common features in hypertension. Therefore, we propose that the NO-cGMP pathway and antioxidant defense systems could have acted synergistically, leading to restoration of cardiac and vascular function in SHR.

Inflammation is an important pathogenic factor in cardiovascular pathologies. We present evidence showing potential anti-inflammatory effect of dietary RPO and rooibos at systemic and at myocardial levels. Most importantly the results show that combination of RPO and rooibos enhanced the up-regulation of endogenous myocardial anti-inflammatory IL-10 levels, a phenomenon shown to have great potential in cardio-protection. This study also showed that IL-6 in this model, acted more like an anti-inflammatory rather than pro-inflammatory cytokine. Therefore, the results obtained in this study opens new research opportunity for further investigations into the anti-inflammatory and cardio-protective effects of combined RPO and rooibos supplementation.

In the third study we present evidence showing that dietary RPO improves myocardial tolerance against ischaemia-reperfusion injury in a model of in *vivo*-induced inflammation. Increased expression of myocardial NFkB was associated with poorer functional recovery and larger infarcts in LPS-treated hearts, while RPO-induced cardio-protection was associated with decreased expression of NFkB and increased p38 phosphorylation during the first 10 minutes of reperfusion. Therefore, NFkB and p38 could at least, in part, be responsible for RPO-induced cardio-protection observed in this model. Increased expression of mitochondrial antioxidant enzymes (SOD2 and GPX1) as well as increased cytosolic SOD1 in RPO-supplemented hearts, argues for an enhanced defence against oxidative stress. The results from the three studies show that RPO and rooibos provide cardioprotection in healthy rats and in differect pathological models employed. This has great clinical potential as it suggests that these dietary interventions are effective as preventive and as therapeutic agents.

3.0 Recommendations

Future studies should be designed to investigate the anti-inflammatory effects of RPO and rooibos in models of chronic inflammation such as diabetic and metabolic syndrome, as they are significant causes of cardiovascular disease-associated morbidity and mortality. Based on the evidence provided in this study and over the years from other RPO studies, we suggest that it is very important to now also start evaluating the effectiveness of RPO and rooibos as cardio-protective agents, in clinical intervention studies as they have been shown to be effective in protecting the animal heart against oxidative challenges. With a new product, such as rooibos, more basic research studies are needed to elucidate the molecular and cellular mechanisms underlying rooibos.

References chapter 6

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Research Outputs

Publications

Emma Thamahane-Katengua, Anna-Mart Engelbrecht, Adriaan J. Esterhuyse and Jacques Van Rooyen (2012). Inhibition of Akt Attenuates RPO-Induced Cardioprotection. *Cardiology Research and Practice* 1-9.

Olawale R. Ajuwon, **Emma Katengua-Thamahane**, Jacques Van Rooyen, Oluwafemi O. Oguntibeju, and Jeanine L. Marnewick (2013). Protective Effects of Rooibos (Aspalathus linearis) and/or Red Palm Oil (Elaeis guineensis) Supplementation on tert-Butyl Hydroperoxide-Induced Oxidative Hepatotoxicity in Wistar Rats. *Evidence-Based Complementary and Alternative Medicine*. (2013): 1-19.

Oguntibeju, **ET Katengua**, AJ Esterhuyse & ET Truter. Modulations of erythrocyte antioxidant enzymes levels by red palm oil in male Wistar rats.

Submitted Manuscripts

Emma Katengua-Thamahane, Barbara Bačová Iveta Bernatova, Csilla Viczenczová, Vladimir Knezl, Narcis Tribulová, Jacques Van Rooyen (2013). Effect of red palm oil on cardiac function in genetically hypertensive rats: role of NO and antioxidant defence enzymes (*Lipids and Health*).

Emma Katengua-Thamahane, Marnewick J.L, Ajuwon O.R, Chegou Novel N, Szucs G, Ferdinandy P, Csont T, Csonka C, Van Rooyen J (2013). The combination of Red Palm Oil and Rooibos show anti-inflammatory effects in rats. (*Plant Foods for Human Nutrition*)

Emma Katengua-Thamahane, Ajuwon O.R, Marnewick J.L, Szucs G, Ferdinandy P,Csont T, Csonka C, Van Rooyen J (2013). Red palm oil improves myocardial ischaemic/reperfusion tolerance in a model of induced inflammation. (*Acta physilogica*)

Abstracts

ETM Katengua-Thamahane, A Engelbrecht, AJ Esterhuyse, J van Rooyen
ORAL PRESENTATION: A Role for Akt in the Protective Effect of Antioxidant-rich Oil Against Ischaemia/reperfusion Injury. (South African Congress for pharmacology and toxicology.

Olawale R Ajuwon, **Emma Katengua-Thamahane**, Jacques Van Rooyen, Oluwafemi O. Protective Effects of Rooibos (Aspalathus linearis) and/or Red Palm Oil (Elaeis guineensis) Supplementation on tert-Butyl Hydroperoxide-Induced Oxidative Hepatotoxicity in Wistar Rats