

Red Palm Oil protects against the consequences of oxidative stress when supplemented with dislipidaemic diets.

Bester DJ¹, Van Rooyen J^{1*}, Du Toit EF², Esterhuyse AJ¹

¹ Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Cape Town

² Department of Biomedical Sciences, University of Stellenbosch

* Author for correspondence:

Prof J van Rooyen, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville

E-mail: vanrooyenj@cput.ac.za

1. Abstract

It is a well known fact that the modern diet contains either excess polyunsaturated fats or saturated fats, and rarely a balanced fat content. In recent research it was found that high concentrations of PUFAs in the diet may be detrimental to cardiovascular health by increasing oxidative stress. Previous studies showed that red palm oil (RPO) provided effective protection against ischaemia/reperfusion injury. In this study we developed an oxidative risk induced diet (ORD), which is rich in PUFAs and low in SFAs, and a high saturated fat diet (HFD), which is rich in SFAs and low in PUFAs. These diets were either supplemented with RPO (experimental groups) or not supplemented (control). Our aim was to investigate whether RPO could offer protection against ischaemia/reperfusion injury in these diets. Our results showed that RPO was able to protect against ischaemia/reperfusion injury in both an ORD and a HFD as indicated by an increased aortic output recovery (ORD+RPO: $83.63 \pm 1.76\%$ versus $61.40 \pm 3.76\%$; HFD+RPO: $66.17 \pm 2.85\%$ versus $50.00 \pm 1.64\%$, $P < 0.05$). This increase in reperfusion function was accompanied by an increase in cGMP concentrations during ischaemia in both RPO supplemented groups, when compared to the unsupplemented groups ($195.40 \pm 18.26\%$ versus $90.31 \pm 12.78\%$ for the ORD groups and $132.98 \pm 12.41\%$ versus $50.09 \pm 10.42\%$ for the HFD groups, $P < 0.05$). These results suggest that the NO-cGMP pathway may be stimulated by RPO during oxidative stress. This in turn would lead to elevation of cGMP during reperfusion, which is associated with protection.

2. INTRODUCTION

The fat intake in diets of the westernized world has become a matter of concern. High fat intake, especially of saturated fats and cholesterol has been identified as a potential risk factor in the development of cardiovascular disease (Elson and Quereshi, 1995). It is therefore generally recommended that saturated fats should be limited in the diet and replaced by mono-unsaturated fats and polyunsaturated fats. However, Diniz and co-workers (2004) showed that despite their beneficial effects on serum lipid concentrations, diets rich in PUFAs are deleterious to the heart by increasing cardiac susceptibility to lipid peroxidation, and therefore increasing the oxidative stress. In another study, Turpeinen and co-workers (1998) showed that diets rich in linoleic acid (LA) lead to increased oxidative stress in patients that maintained a higher than recommended intake of α -tocopherol. This increase in oxidative stress was accompanied by a decrease in excretion of nitric oxide (NO) metabolites in the urine. These authors suggest that oxidized low density lipoprotein cholesterol may have scavenged NO before it reached its target. It is also possible that the production of other reactive oxygen species (ROS) could have quenched NO (Chen *et al.*, 1999; Illarion *et al.*, 2002; Onody *et al.*, 2003). This may suggest that individuals consuming a diet rich in PUFAs may be at risk of developing cardiovascular disease, or other diseases associated with oxidative stress, such as cancer and diseases linked to DNA degradation (Turpeinen *et al.*, 1998; Chen *et al.*, 1999; Diniz *et al.*, 2004).

Palm oil is consumed worldwide as a cooking oil and as a constituent of margarines (Kheiri, 1987). Research has confirmed that RPO shortening is free of cholesterol and *trans* fatty acids. It contains beneficial micronutrient compounds such as, tocopherol, tocotrienol, lycopene, squalene, co-enzyme Q₁₀, phytosterols, oleic acid and carotenoids, of which β -carotene is the most abundant (Goh *et al.*, 1985; Sundram *et al.*, 2003).

Research has indicated that RPO is effective in protecting against oxidative stress conditions such as ischaemia/reperfusion in the heart. Serbinova and co-workers (1991) focused their research on the protective effects of the anti-oxidants contained in RPO against ischaemia/reperfusion injury. These authors found that a palm oil mixture containing both tocopherol and tocotrienol improved reperfusion functional recovery in the Langendorff perfused rat heart. They hypothesized that the free radical scavenging action of tocopherols and tocotrienols during ischaemia/reperfusion induced the protective effect. In the same study they also demonstrated that a combination of tocopherols and tocotrienols offered increased protection from ischaemia/reperfusion injury when compared with tocopherols alone. Their study also revealed that tocotrienols offered more efficient protection than tocopherol as it was preferentially consumed by ROS during the course of ischaemia and reperfusion. Esterhuyse and co-workers (2005 a and b) found that dietary supplementation with RPO shortening in rats fed a standard rat chow (control) and hypercholesterolaemic diet protected against ischaemia/reperfusion injury as reflected by improved aortic output recovery. This was associated with an increase in cGMP during ischaemia and a decrease in cAMP during ischaemia in the RPO-supplemented group versus the control group (Esterhuyse *et al.*, 2005 a and b). Their results also demonstrated that RPO-supplementation activated the mitogen protein kinase- (MAPK), protein kinase B or Akt (PKB/Akt), and inhibited caspase-3 activation (Engelbrecht *et al.*, 2005). These authors suggest that these changes may be responsible for the myocardial protection offered by RPO against ischaemia/reperfusion injury.

Esterhuyse and co-workers (2005 a and b) used a standard rat chow diet and a hypercholesterolaemic diet in their study on RPO. However, these studies did not provide information regarding the effect of RPO-supplementation of diets rich in saturated- or polyunsaturated fatty acids. The diets were also not isocaloric, and dietary RPO intake was in essence a daily supplement to a healthy diet.

In the current study isocaloric diets including an oxidative risk induced diet (which is rich in PUFAs) and a high saturated fat diet (which is rich in SFAs) was developed by our research group and supplemented with RPO to serve as experimental groups. These diets were developed to simulate individuals who increase their dietary intake of PUFAs for health purposes and also others who simply maintain a diet high in SFAs. We supplemented these diets with RPO to investigate whether RPO could protect the heart against conditions of oxidative stress. RPO-supplemented groups (experimental) were then compared to non supplemented groups (control). We referred to the diet rich in PUFAs as the oxidative risk induced diet as it would be expected to increase the oxidative stress on the body and cardiovascular system.

To our knowledge little is known about the effects of RPO-supplementation to an oxidative risk induced diet or a high saturated fat diet. Therefore, the aims of this study were: 1) to determine whether RPO-supplementation of an ORD or a HFD offered protection against ischaemia/reperfusion injury in the isolated perfused rat heart; 2) to elucidate possible mechanisms of protection; and 3) to determine whether RPO influences the changes in the myocardial total phospholipid fatty acid composition during ischaemia in these diets.

3. MATERIALS AND METHODS

3.1 Animal Care

All 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 1985).

3.2 Experimental groups

Seven-week old male Wistar rats were randomly divided into four groups. Each group received a specific diet for the following fourteen weeks.

Group 1: Oxidative risk induced diet (ORD) (control).

Group 2: Oxidative risk induced diet supplemented with RPO (ORD+RPO).

Group 3: High saturated fat diet (HFD) (control).

Group 4: High saturated fat diet supplemented with RPO (HFD+RPO).

All of the diets were isocaloric to ensure that none of the differences observed between the groups, at any time point is due to a difference in energy consumption, but rather a difference in the composition of the diets. The diets were prepared on a day-to-day basis to prevent it from spoiling. Rats in all the diet groups were given their supplements in the morning and after consumption, they were allowed ad libitum access to standard rat chow. Red Palm Oil baking fat was used in this study, and was supplied by Carotino SDN BHD (company no. 69046-T), Johor Bahru, Malaysia. The estimated composition of the diets is given in Table 1 with the assumption that rats consume an average of 25g of food per day. The RPO shortening contains no less

than 500 ppm carotene, 90% of which is present as α - and β -carotene. The tocotrienol and tocopherol content is 500 ppm of which 70% is in the form of tocotrienols (mainly as α -, β - and γ tocotrienols) (Nagendran *et al.*, 2000; Sundram *et al.*, 2003).

3.3 Working heart perfusion

At the end of the feeding program, rats weighing 450-600g were anaesthetized, by injecting 0.5 ml sodium thiopentone intraperitoneally. Hearts were rapidly excised and placed in ice-cold Krebs-Henseleit buffer. They were transferred to the standard working heart perfusion apparatus where they were perfused with a Krebs-Henseleit buffer equilibrated with 95% O₂ and 5% CO₂ at 37 °C (118.5 mM NaCl; 4.75 mM KCl; 1.2 mM MgCl₂ · 6 H₂O; 1.36 mM CaCl₂; 25.0 mM NaHCO₃; 1.2 mM KH₂PO₄; 11.0 mM glucose) at a perfusion pressure of 100 cm H₂O.

The aorta was cannulated and retrograde perfusion initiated. During this initial perfusion in the Langendorff mode, excess tissue was removed from the hearts and the opening to the left atrium was cannulated. Following a five-min stabilization period in the Langendorff mode, hearts were switched to the working mode. The temperature of both the perfusate and the air surrounding the heart was thermostatically controlled and checked at regular intervals to ensure that the temperature was maintained at 37 °C irrespective of coronary flow. A cannula, connected to a pressure transducer, was inserted through the apex of the heart into the left ventricle. Left ventricular systolic and diastolic pressure, coronary flow (CF), heart rate (HR) and aortic output (AO) were measured at 5 minute intervals during the first 25 minutes into perfusion. Hearts were then subjected to 25 minutes of total global ischaemia at a temperature of 34.5 °C, in order to assure that an approximate recovery of 50 to 60 % was achieved in ORD and HFD groups. At the end of ischaemia, hearts were reperfused in the Langendorff mode for ten minutes. In order to reduce the incidence of reperfusion arrhythmias, 2% lignocaine solution was used for the initial three minutes of reperfusion of all hearts. This was followed by a 15 minute working heart perfusion period during which cardiac function was measured. To assess fatty acid composition and other biochemical markers, hearts were freeze-clamped with Wollenberger clamps pre-cooled in liquid nitrogen.

3.4 Aortic output recovery (%)

Aortic output (AO) was measured by collecting one-minute samples of the aortic effluent. Aortic output recovery was calculated by dividing the AO after ischaemia by AO before ischaemia and expressing these values as a percentage recovery.

3.5 Biochemical analyses

The cAMP and cGMP levels were determined using radioimmunoassay kits obtained from Amersham Corp. (Amersham, UK).

3.5.1 cGMP assay

For cGMP assays, approximately 150 mg of the wet tissue was extracted in 5% trichloroacetic acid. The extracted sample was ether-washed three times for five-minute wash cycles. These samples were diluted 1:10 (V/V) and acetylated for the ¹²⁵I-labeled cGMP assay. The IC₅₀ for the cGMP assay was 25 pmol/tube (Du Toit *et al.*, 1999).

3.5.2 cAMP assay

For the cAMP assays, approximately 150 mg tissue was extracted with perchloric acid, neutralized and assayed. The IC₅₀ for this assay was 1.92 mmol/tube (Du Toit *et al.*, 1999).

3.6 Nitrate/Nitrite (Nitric Oxide)

Approximately 200 mg of wet cardiac tissue was homogenized in 0.5 ml PBS (pH 7.4) and centrifuged at 10 000 x g for 20 minutes. The supernatant was ultra-filtered using a 30 kDa molecular weight cut-off filter (Millipore) and 40 μ l of the filtrate was assayed.

Myocardial NO levels were determined using a Nitrate/Nitrite kit (Cayman Chemicals, Cayman Islands), which provides a method for measurement of total nitrate/nitrite concentration. The first step is the conversion of nitrate to nitrite utilizing nitrate reductase. The second step is the addition of the Griess Reagents, which convert nitrite into a deep purple azo compound. Photometric measurement of the absorbance due to this chromophore accurately determines nitrite concentration. This assay although being an indirect measure of NO, is able to give an accurate reflection of the difference in NO content between samples and also groups of samples.

3.7 Heart muscle total phospholipid fatty acids

Tissue samples were extracted with chloroform: methanol (2:1; v/v) according to a modified method of Folch and co-workers (1957). Neutral lipids were separated from total phospholipids by thin layer chromatography (TLC) and the total phospholipid fraction analyzed for fatty acids by gas chromatography. A fatty acid mixture was prepared from individual fatty acids (Sigma, St. Louis, MO, USA) and used as a standard (Folch *et al.*, 1957; Smuts *et al.*, 1992; Van Jaarsveld *et al.*, 2000).

3.8 Statistical methods

Values are presented as mean \pm standard error of the mean. One way ANOVA with Bonferroni's post-hoc correction was used to determine significance between the groups and for paired comparisons the Student's t test was used. *P*<0.05 was considered significant.

4. RESULTS

4.1 Functional parameters

4.1.1 Left Ventricular Diastolic Pressure and Left Ventricular Systolic Pressure

The LVSP of the ORD+RPO was significantly decreased at the 25 minute reperfusion time point when compared to the 20 minute perfusion time point (155.50 \pm 2.69 mmHg *versus* 145.91 \pm 2.65 mmHg, *P*<0.05) (Table 2).

The LVDP of the ORD+RPO was significantly decreased when compared to that of the HFD+RPO at the 20 minute perfusion time point (-14.06 \pm 1.33 mmHg *versus* -8.25 \pm 1.71 mmHg, *P*<0.05). Similarly, the LVDP of the ORD was significantly decreased when compared to that of the HFD at the 25 minute reperfusion time point (-8.08 \pm 0.70 mmHg *versus* -0.83 \pm 1.35 mmHg, *P*<0.05). Conversely, the LVDP of the HFD was significantly increased at the 25 minute reperfusion time point, when compared to the same group at the 20 minute perfusion time point (-0.83 \pm 1.35 mmHg *versus* -6.10 \pm 0.83 mmHg, *P*<0.05), signifying, that the HFD may have caused structural damage to the heart (Onody *et al.*, 2003). The LVDP of the HFD was also significantly increased when compared to the HFD+RPO at the 25 minute reperfusion time point (-0.83 \pm 1.35 mmHg *versus* -6.68 \pm 1.17 mmHg, *P*<0.05), indicating that RPO-supplementation was able to decrease this effect of the HFD diet (Table 2).

4.1.2 Aortic Output

There were no significant differences in the baseline aortic output of any of the groups (see Table 2 for baseline and reperfusion values). However, the aortic output of all the groups were significantly decreased at the 25 minute reperfusion time point, when compared to the baseline (ORD: 47.80 \pm 1.50 ml/min *versus* 28.30 \pm 2.78 ml/min; ORD+RPO: 53.50 \pm 1.45 ml/min *versus* 43.00 \pm 1.38 ml/min; HFD: 50.40 \pm 1.75 ml/min *versus* 25.20 \pm 1.35 ml/min; HFD+RPO: 53.16 \pm 3.05 ml/min *versus* 35.50 \pm 2.62 ml/min, *P*<0.05). After the ischaemic period, the aortic output of the ORD+RPO was significantly increased when compared to that of the ORD at the 25 minute reperfusion time point (43.00 \pm 1.38 ml/min *versus* 28.30 \pm 2.78 ml/min, *P*<0.05). Similarly, the aortic output of the HFD+RPO was significantly increased when compared to that of the HFD at the 25 minute reperfusion time point (35.50 \pm 2.62 ml/min *versus* 25.20 \pm 1.35 ml/min, *P*<0.05). These results may indicate that dietary RPO-supplementation is able to increase reperfusion aortic output, irrespective of the fat content of the diet.

4.1.3 Percentage Aortic Output Recovery (%)

The aortic output recovery results as shown in Figure 1, was significantly increased in the RPO supplemented groups when compared to those of the unsupplemented groups at the 25 minute reperfusion time point (ORD+RPO: 83.63 \pm 1.76 % *versus* 61.40 \pm 3.76 %; HFD+RPO: 66.17 \pm 2.85 % *versus* 50.00 \pm 1.64 %, *P*<0.05), indicating the effectiveness of RPO in preventing damage by ischaemia/reperfusion injury.

4.1.4 Heart rate and Coronary flow

The heart rate of the HFD+RPO was significantly increased when compared to that of the HFD at the 25 minute reperfusion time point (258.97 \pm 8.12 bpm *versus* 234.03 \pm 6.71 bpm, *P*<0.05) (Table 2).

There were no significant differences in coronary flow of any diet group at any time point investigated, confirming that there were no vascular effects induced in any of the diet groups (Table 2).

4.2 cGMP and cAMP (pmol/g wet weight)

The results obtained for cGMP concentrations are presented as a percentage change from the baseline values after 10 minutes ischaemia and 10 minutes reperfusion in Figure 2. The baseline values were: 11.42 \pm 2.98 pmol/g wet weight for the ORD, 8.95 \pm 1.03 pmol/g wet weight for the ORD+RPO, 10.63 \pm 1.08 pmol/g wet weight for the HFD and 10.41 \pm 0.49 pmol/g wet weight for the HFD+RPO. The ischaemic cGMP concentrations of the RPO-supplemented groups increased significantly when compared with corresponding diet groups without RPO (195.40 \pm 18.26 % *versus* 90.31 \pm 12.78 % for the ORD groups and 132.98 \pm 12.41 % *versus* 50.09 \pm 10.42 % for the HFD groups, *P*<0.05).

The baseline values obtained for myocardial cAMP concentrations were: 253.68 \pm 34.77 pmol/g wet weight for the ORD, 360.21 \pm 31.55 pmol/g wet weight for the ORD+RPO, 275.06 \pm 46.27 pmol/g wet weight for the HFD and 303.65 \pm 61.37 pmol/g wet weight for the HFD+RPO. Ischaemic and reperfusion values are presented as percentage change from the baseline in Figure 3. There was a significant increase in the % change in cAMP HFD+RPO when compared to the ORD+RPO at the 10 minutes during ischaemia time point (48.68 \pm 22.11 % *versus* -32.38 \pm 10.65 %, *P*<0.05).

4.3 Nitrate/Nitrite (Nitric Oxide)

There was a significant increase in the nitrite/nitrate concentrations of the HFD group when compared to the ORD group at the 10 minute reperfusion time point ($1.28 \pm 0.22 \mu\text{mol/L}$ versus $0.19 \pm 0.06 \mu\text{mol/L}$, $P < 0.05$). This may be due to increased oxidative stress in the ORD group which would lead to quenching of NO by ROS (Figure 4).

4.4 Myocardial Total Phospholipid Fatty Acid Content (%)

The major fatty acids (%) of myocardial total phospholipids before and after ischaemia in the four diet groups are presented in Table 3. Baseline SFA- and MUFA concentrations were similar in the four groups. These values remained unchanged after the ischaemic period for all the groups. However, the total PUFA- and n-3 PUFA content in the ORD+RPO group were significantly increased after ischaemia (PUFA content: $49.82 \pm 2.11 \%$ before ischaemia and $55.50 \pm 0.73 \%$ after ischaemia and n-3 PUFA content: $14.93 \pm 1.12 \%$ before ischaemia and $18.16 \pm 0.26 \%$ after ischaemia, respectively, $P < 0.05$). Palmitic acid was significantly decreased in the ORD+RPO group after ischaemia ($15.00 \pm 0.85 \%$ versus $12.04 \pm 0.39 \%$, $P < 0.05$) whereas arachidonic acid was significantly increased after ischaemia for the same group ($17.05 \pm 0.59 \%$ versus $18.94 \pm 0.21 \%$, $P < 0.05$). The only significant differences between the ORD and ORD+RPO groups were in the n-6:n-3 ratio after ischaemia (n-6:n-3 ratio: $2.73 \pm 0.12 \%$ versus $2.08 \pm 0.02 \%$, $P < 0.05$) and also the DHA content after ischaemia (DHA: $11.32 \pm 1.56 \%$ versus $15.83 \pm 0.18 \%$, $P < 0.05$).

In the HFD+RPO group, there was a significant decrease in linoleic acid content when compared to the HFD before ischaemia ($18.82 \pm 0.29 \%$ versus $17.62 \pm 0.51 \%$, $P < 0.05$) and also a significant decrease in palmitic acid content when compared to the HFD after ischaemia ($14.32 \pm 1.11 \%$ versus $13.12 \pm 0.54 \%$, $P < 0.05$). The eicosapentaenoic acid content of the ORD+RPO was significantly decreased when compared to the HFD+RPO before and after ischaemia (before ischaemia: $0.37 \pm 0.03 \%$ versus $0.23 \pm 0.02 \%$; after ischaemia: $0.35 \pm 0.02 \%$ versus $0.23 \pm 0.01 \%$, $P < 0.05$).

5. DISCUSSION

Our results showed that RPO-supplementation improved myocardial reperfusion functional recovery in both diets. These results support the findings of Esterhuysen *et al.* (2005 a and b) who showed that dietary RPO supplementation of a standard rat chow and a high cholesterol diet improved reperfusion aortic output recovery. The fact that RPO protected against ischaemia/reperfusion injury in a ORD and a HFD in the current study and in a high cholesterol diet (Esterhuysen *et al.*, 2005 b) provide strong evidence that may suggest that dietary RPO-supplementation protects the heart against oxidative stress in any type of diet, irrespective of the fat and/or cholesterol content of the diet.

In a study by Onody and co-workers it was found that cholesterol fed rats showed a significantly increased left ventricular diastolic pressure (LVDP), when compared to rats following a control diet. They argued that this increase in LVDP is due to abnormal distension of the heart caused by formation of peroxynitrite (ONOO⁻) in cholesterol fed rats (Onody *et al.*, 2003). The high cholesterol content in our HFD makes it tempting to speculate that the increased LVDP in the HFD may be associated with reduced compliance of the heart, as found by Onody in dyslipidaemia. In our study RPO was able to improve LVDP in the HFD, whilst it had no effect in the ORD. The HFD has high levels of cholesterol and the ORD has no cholesterol. Therefore, in the HFD peroxynitrite formation (Onody *et al.*, 2003) caused poor reperfusion recovery which could be reversed by RPO. These results confirm earlier findings by Esterhuysen and co-workers (2005 b). They found RPO protected against oxidative stress in rats fed high cholesterol diets by improving left ventricular developed pressure (LVDevP).

The nitrate/nitrite concentrations were similar in all the groups at the baseline and during ischaemia. Higher oxidative stress would lead to the production of more oxygen radicals which would in turn lead to the quenching of NO (Hare and Comerford, 1995; Maulik *et al.*, 1995; Xie and Wolin, 1996; Chen *et al.*, 1999; Onody *et al.*, 2003) by superoxide to produce peroxynitrate (ONOO⁻). This quenching of NO is associated with the production of ONOO⁻, which rapidly degenerates into to highly reactive oxidant species leading to tissue injury (Ferdinandy and Schultz, 2003). The low levels of nitrate/nitrite levels in the ORD group, compared to the HFD group, at 10 minutes into reperfusion indicate that more NO was quenched for ONOO⁻ production. This argues for increased oxidative stress in the hearts of the ORD group during ischaemia or early in reperfusion. This higher oxidative stress may be caused by the high PUFA content in the ORD group (Turpeinen *et al.*, 1998; Diniz *et al.*, 2004). Chow and co-workers (2002) attributed the protection against the adverse effects of nitrites/nitrates by vitamin E, to its ability to reduce ONOO⁻ formation. They showed that this protective effect of vitamin E is achieved by it limiting the production and availability of superoxide (O₂⁻) and NO, which forms ONOO⁻ when it reacts. In a study by Esterhuysen and co-workers (2006) it was shown that in the presence of RPO superoxide dismutase (SOD) levels was unchanged despite increased NO levels. This indicates that the NO-cGMP pathway was preferentially stimulated by RPO instead of drawing the reactions towards O₂⁻ and ONOO⁻. Therefore we speculate that the carotenoids, tocopherols, tocotrienols and

other minor compounds of RPO scavenged the O₂⁻ and ONOO⁻.

The increase in cGMP concentrations of both the RPO-supplemented groups during ischaemia is comparable to the findings of Esterhuysen and co-workers (2005 b) who used a standard rat chow diet supplemented with RPO. However, they were unable to demonstrate a significant increase in cGMP during ischaemia in cholesterol fed rats. This indicates that dietary RPO-supplementation is associated with a significant increase in cGMP concentrations during ischaemia, and that dietary cholesterol supplementation is associated with the inhibition of this effect. Our results did not show inhibition of the increase in cGMP concentrations during ischaemia by cholesterol present in the HFD. The lack of this inhibitory effect may be due to different doses of cholesterol in the different studies (2% in the study by Esterhuysen *et al.*, 2005 b, compared to 0.009% in the current study). The protective effects associated with NO and cGMP may be attributed to the suppression of cyclic adenosine monophosphate (cAMP) induced by both NO and cGMP (Chensais *et al.*, 1999; Abi-Gerges *et al.*, 2001). cAMP increases heart rate and contractile force by opening calcium ion (Ca²⁺) channels, which could ultimately lead to ventricular fibrillation (Du Toit *et al.*, 2001). cGMP has the opposite effect, by opening potassium- and sodium channels and closing Ca²⁺ channels (Csont and Ferdinandy, 2005; Schuldt *et al.*, 2005). This is achieved by stimulation of soluble guanylate cyclase which brings about reduction of Ca²⁺, partly through activation of cGMP-dependent protein kinase and termination of chain propagating lipid radical reactions caused by oxidative stress (Rubbo *et al.*, 1994). It has been suggested by Du Toit and co-workers (2001) that the cAMP to cGMP ratio may play an important role in NO induced cardioprotection. The decrease in aortic output recovery in the HFD+RPO group may be due to the opening of Ca²⁺ channels. cAMP was significantly increased in the HFD+RPO group when compared to the ORD+RPO group during ischaemia. This increase in cAMP in the HFD+RPO group may be associated with an increase in fatty acids as found by Van Rooyen and co-workers (2003). In this study we did not find an increase in myocardial cAMP concentrations during ischaemia, as is generally associated with ischaemia (Du Toit *et al.*, 2001).

In a study conducted by Van Rooyen and co-workers (1998) the authors were able to demonstrate that PUFAs replaced SFAs in erythrocyte membranes of animals supplemented with PUFAs. In the current study RPO-supplementation did not change fatty acid concentrations significantly during the supplementation phase. However during perfusions, fatty acid concentrations were changed in the ORD+RPO group from the sampling point before ischaemia to the sampling point after ischaemia. This indicates that induction of ischaemia may have triggered a change in the fatty acid profile. The ORD+RPO group had a significantly decreased PA content after ischaemia. Despite the fact that RPO contains only small amounts of alpha linolenic acid we found significant increase in n-3 fatty acid levels after ischaemia. Furthermore, mammals (including rats) are deficient of Δ12- and Δ15 desaturases and can therefore not produce n-6 and n-3 fatty acids *in vivo*. Intake of essential fatty acids (linolenic acid and α-linolenic acid) in the diet is therefore necessary for production of these fatty acids (Pereira *et al.*, 2003). As the RPO used in this study contains only 0.24 % α-linolenic acid, it is unlikely that this was used for production of n-3 fatty acids. Rat chow also contains n-3 fatty acids and can therefore be responsible for the increase in n-3 levels. However, if this was the case, we would also expect significant changes in n-3 fatty acids levels in all groups and not only in the ORD+RPO. Therefore this result needs careful consideration: We measured total phospholipid levels in the current study. Together, ischaemia and RPO, may have triggered alteration of the composition of the total phospholipids by removing the SFAs as a local protective mechanism to make the cell membrane more fluid. This would increase cell survival. Removal of SFAs from the phospholipids fraction would automatically increase the percentage of n-3 fatty acids in the same total phospholipid fraction. SFAs on the other hand will be washed out of the myocardium during early reperfusion. Our data point collection was only 10 minutes after introduction of reperfusion. Either the increase in n-3 fatty acids or the loss of SFAs in the cell membrane phospholipids may have been responsible for improved reperfusion function in RPO-supplemented hearts in our study. Increased levels of α-linolenic acid would increase DHA concentration (Table 3) in the ORD+RPO group and ultimately increase eicosanoid production which is beneficial to cardiovascular health (Abeywardena and Head, 2001).

Apart from its effects on eicosanoid production, fatty acids are also known to affect the activation of second messengers such as the mitogen activated protein kinases (Chen *et al.*, 2003). Engelbrecht *et al.*, (2005) showed that dietary RPO-supplementation is able to protect the heart against ischaemia/reperfusion injury. This protection was associated with significant increases in p38- and PKB/Akt phosphorylation, a significant decrease in JNK phosphorylation and attenuation of PARP cleavage.

6. SUMMARY AND CONCLUSIONS

Our results show that dietary RPO-supplementation protected the heart against ischaemia/reperfusion injury in both ORD and HFD fed rats.

Based on our results we propose that the improved aortic output recovery may be a result of increased myocardial concentrations of cGMP during ischaemia. We hypothesize that the antioxidant properties of a diet containing

red palm oil may contribute to the elevated cGMP which alleviated ischaemia/reperfusion injury by scavenging the superoxide.

Our results show that the SFA, palmitic acid, was removed from cell membranes during the ischaemic period. This removal of SFAs leads to the proportional increase in % PUFAs within the cell membrane. These PUFAs may induce protective mechanisms in the signal transduction pathways, and specifically affect p38 MAPK and JNK. We propose a study that supplements a standard rat chow diet with α -linolenic acid to determine fatty acids.

Our results indicate that dietary RPO-enrichment reduces ischaemia/reperfusion injury of rats fed unhealthy diets. We propose that the NO-cGMP pathway is the most likely pathway of protection and that fatty acid changes, particularly n-3 fatty acids play a complimentary role.

ACKNOWLEDGEMENTS

The authors are greatly indebted to the following people:

1. Prof A. Lochner of the Medical Physiology and Biochemistry Department of the University of Stellenbosch for the use of laboratory facilities to complete this study
2. Irma Venter of the Applied Sciences Department of the Cape Peninsula University of Technology for designing the diets used in this study
3. Carotino SDN BHD (company no. 69046-T), Johor Bahru, Malaysia for providing the red palm oil used in this study
4. The Research and Development Department of the Cape Peninsula University of Technology for financial support

REFERENCES

1. Abeywardena MY, Head RJ. Dietary polyunsaturated fatty acid and antioxidant modulation of vascular dysfunction in the spontaneously hypertensive rat. *PLEFA* 2001; 65(2): 91-97.
2. Abi-Gerges N, Fischmeister R, Mèry P-F. G protein-mediated inhibitory effect of a nitric oxide donor on the L-type Ca^{2+} current in rat ventricular myocytes. *J Physiol* 2001; 531: 117-130.
3. Chen L, Bowen PE, Berzy D, Aryee F, Stacewicz-Sapuntzakis M, Riley RE. Diet modification affects DNA oxidative damage in healthy humans. *Free Rad Biol & Med* 1999; 26: 659-703.
4. Chen H, Li D, Roberts GJ, Saldeen T, Metha JL. Eicosapentaenoic acid inhibits hypoxia-reoxygenation-induced injury by attenuating upregulation of MMP-1 in adult rat myocytes. *Cardiovasc Res* 2003; 59: 7-13.
5. Chesnais JM, Fischmeister R, Mèry P-F. Positive and negative inotropic effects of NO donors in atrial and ventricular fibres of the frog heart. *J Physiol* 1999; 518: 449-461.
6. Chow CK, Hong CB. Dietary vitamin E and selenium and toxicity of nitrite and nitrate. *Toxicol* 2002; 180: 195-207.
7. Csont T, Ferdinandy P. Cardioprotective effects of glyceryl trinitrate: beyond vascular nitrate tolerance. *Pharmacol & Therap* 2005; 105: 57-68.
8. Diniz YS, Cicogna AC, Padovani CR, Santana LS, Faine LA, Novelli ELB. Diets rich in saturated and polyunsaturated fatty acids: Metabolic shifting and cardiac health. *Nutr* 2004; 20: 230-234.
9. Du Toit EF, Muller CA, McCarthy J and Opie LH. Levosimendan: Effects of a calcium sensitizer on function and arrhythmias and cyclic nucleotide levels during ischaemia/reperfusion in the Langendorff-perfused guinea pig heart. *J Pharmacol Exp Ther* 1999; 290: 505-514.
10. Du Toit EF, Meiring J, Opie LH. Relation of cyclic nucleotide ratios to ischaemic and reperfusion injury in nitric oxide-donor treated hearts. *J Cardiovasc Pharmacol* 2001; 38: 529-538.
11. Elson CE, Quereshi AA. Coupling the cholesterol- and tumour-suppressive actions of palm oil to the impact of its minor constituents on 3-Hydroxy-3-Methylglutaryl Coenzyme A reductase activity. *PLEFA* 1995; 52: 205-208.
12. Engelbrecht AM, Esterhuysen J, Du Toit EF, Lochner A, Van Rooyen J. p38-MAPK and PKB/Akt, possible role players in red palm oil-induced protection of the isolated perfused rat heart? *J Nutr Biochem* 2006; 17(4): 265-271.
13. Esterhuysen JS, Van Rooyen J, Strijdom H, Bester D, Du Toit EF. Proposed mechanisms for red palm oil induced cardioprotection in a model of hyperlipidaemia in the rat. *PLEFA* 2006 (in press).
14. Esterhuysen AJ, Du Toit EF, Van Rooyen J. Dietary red palm oil supplementation protects against the consequences of global ischaemia in the isolated perfused rat heart. *Asian Pac J Clin Nutr* 2005; 14(4): 340-347 (a).
15. Esterhuysen AJ, du Toit EF, Benade AJS, van Rooyen J. Dietary red palm oil improves reperfusion cardiac function in the isolated perfused rat heart of animals fed a high cholesterol diet. *PLEFA* 2005; 72:153-161 (b).
16. Ferdinandy P, Schultz R. Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia-reperfusion injury and preconditioning. *Br J Pharmacol* 2003; 138: 532-543.
17. Folch J, Lees M, Sloane-Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 1957; 226: 497-509.
18. Goh SH, Choo YM, Ong ASH. Minor constituents of palm oil. *J Am Oil Chem Soc* 1985; 62: 237-240.
19. Hare JM, Comerford ML. Role of nitric oxide in the regulation of myocardial function. *Prog Lipid Res* 1995; 38: 155-166.
20. Illarion V, Murad T, Murad F. Protein nitration in cardiovascular diseases. *Pharmacol Rev* 2002; 54: 619-634.
21. Kheiri MSA. End uses of palm oil. Chapter 5 in critical Reports on applied Chemistry Vol. 15, (1987) Society of Chemical Industry. London Ed. F.D. Gunstone.
22. Maulik N, Engelman DT, Watanabe M, Engelman RM, Maulik G, Cordis GA, Das DK. Nitric oxide signalling in ischaemic heart. *Cardiovasc Res* 1995; 30: 593-601.
23. Nagendran B, Unnithan UR, Choo YM, Sundram K. Characteristics of red palm oil, a carotene- and vitamin E-enriched refined oil for food uses. *Food Nutr Bull* 2000; 21: 189-194.
24. Onody A, Csonka C, Giricz Z, Ferdinandy P. Hyperlipidaemia induced by a cholesterol-enriched diet leads to enhanced peroxynitrite formation in rat hearts. *Cardiovasc Res* 2003; 58: 663-670.
25. Pereira SL, Leonard AE, Mukerji P. Recent advances in the study of fatty acid desaturases from animals and lower eukaryotes. *PLEFA* 2003; 68: 97-106.
26. Rubbo H, Radi R, Trujillos M, Telleri R, Kaly-Anaraman B, Barnes S, Kirk M, Freeman BA. Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. *J Biol Chem* 1994; 269: 26066-26075.
27. Schuldt EZ, Bet AC, Hort MA, Ianssen C, Maraschin M, Ckless K, Ribeiro-do-Valle RM. An ethyl acetate fraction obtained from a Southern Brazilian red wine relaxes rat mesenteric arterial bed through hyperpolarization and NO-cGMP pathway. *Vasc Pharmacol* 2005 (in press)
28. Serbinova E, Kagan V, Han D, Packer L. Free radical recycling and intramembrane mobility in the antioxidant properties of alpha-tocopherol and alpha-tocotrienol. *Free Radical Biol Med* 1991; 10: 263-275.
29. Sizer FS, Whitney EN. Nutrition Concepts and controversies, Eighth edition. Woodsworth Thomson Learning, Belmont, CA. 2000; 35&402.
30. Smuts CM, Kruger M, Van Jaarsveld PJ, Fincham, Schall R, Van der Merwe KJ and Benade AJS. The influence of fish oil supplementation on plasma lipoproteins and arterial lipids in vervet monkeys with established atherosclerosis. *PLEFA* 1992; 47: 129-138.
31. Sundram K, Sambanthamurthi R, Tan YA. Palm fruit chemistry. *Asia Pac J Clin Nutr* 2003; 12(3): 355-362.
32. Turpeinen AM., Basu S., Mutanen M. A high linoleic acid diet increases oxidative stress in vivo and affects nitric oxide metabolism in humans. *PLEFA* 1998; 59(3): 229-233.
33. Van Jaarsveld PJ, Smuts CM, Tichelaar HY, Kruger M, Benade AJS. Effect of palm oil on plasma lipoprotein concentrations and plasma low-density lipoprotein composition in non-human primates. *Int J Food Sci Nutr* 2000; 51: S21-S30.
34. Van Rooyen J, Opie LH, Thomas S, Podzuweit T. Glucose protects against the development of ischaemic contracture in the isolated Langendorff perfused heart. *Cardiovasc J SA* 2003; 14(5): 274 (abstract publication).
35. Van Rooyen J, Swanevelder JC, Morgenthal JC, Benade AJS. Diet can manipulate the metabolism of EPA and GLA in erythrocyte membrane and plasma. *PLEFA* 1998; 59(1): 27-38.
36. Xie YW, Wolin MS. Role of nitric oxide and its interaction with superoxide in the suppression of cardiac muscle mitochondrial respiration. Involvement in response to hypoxia/reoxygenation. *Circulation* 1996; 94: 2580-2586.

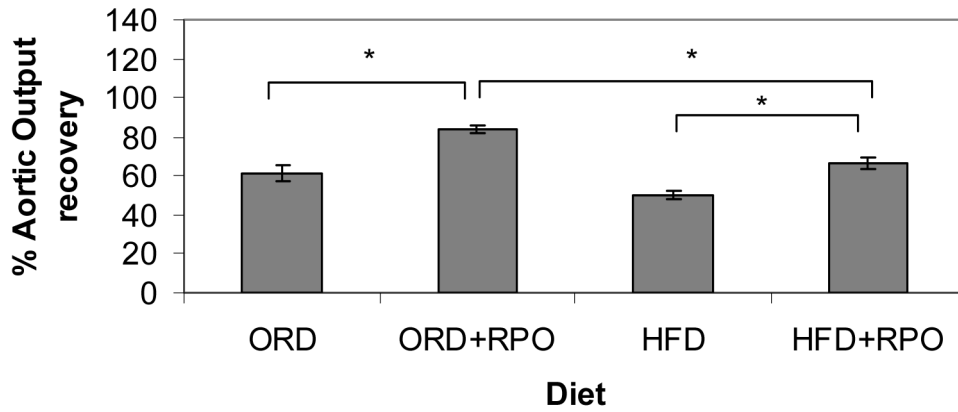


FIGURE 1. Percentage aortic output recovery of isolated perfused rat hearts in the 4 experimental groups 25 minutes into reperfusion (n=10 per group) (*P<0.05 for the group versus the indicated group) (mean ± SEM)

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

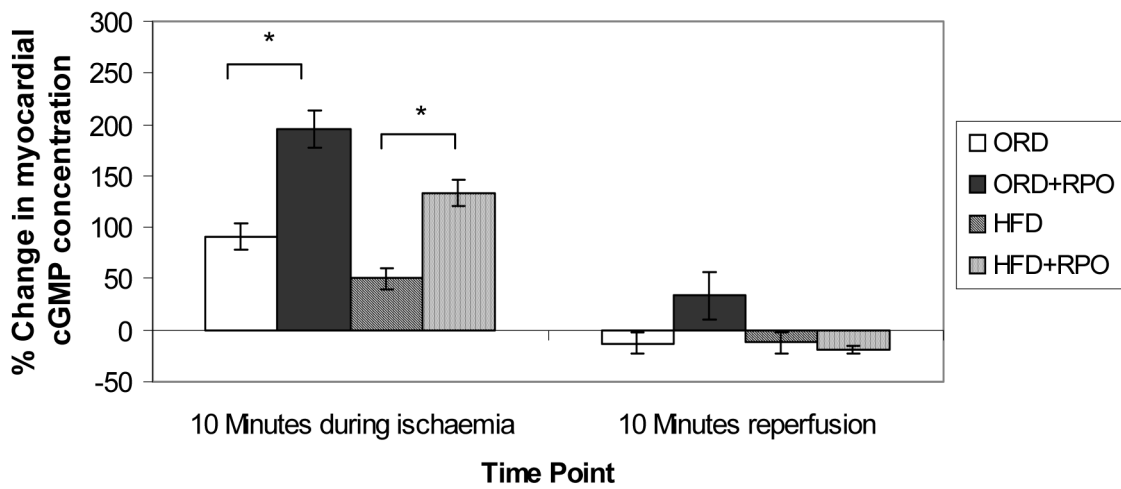


FIGURE 2. % Change in myocardial cGMP concentrations in the 4 groups at 10 minutes ischaemia and 10 minutes into reperfusion (n=5 per group per time point)(*P<0.05 for the group versus the indicated group) (mean ± SEM)

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

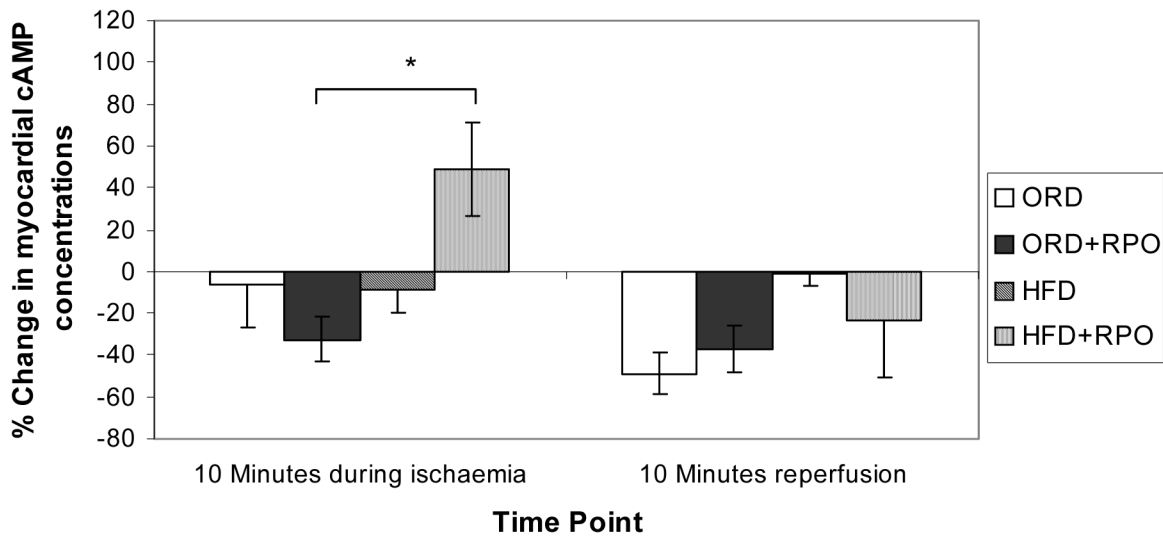


FIGURE 3. Percentage change in myocardial cAMP concentrations in the 4 experimental groups at 10 minutes ischemia and 10 minutes into reperfusion (n=5 per group per time point) (*P<0.05 for the group versus the indicated group) (mean ± SEM)

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

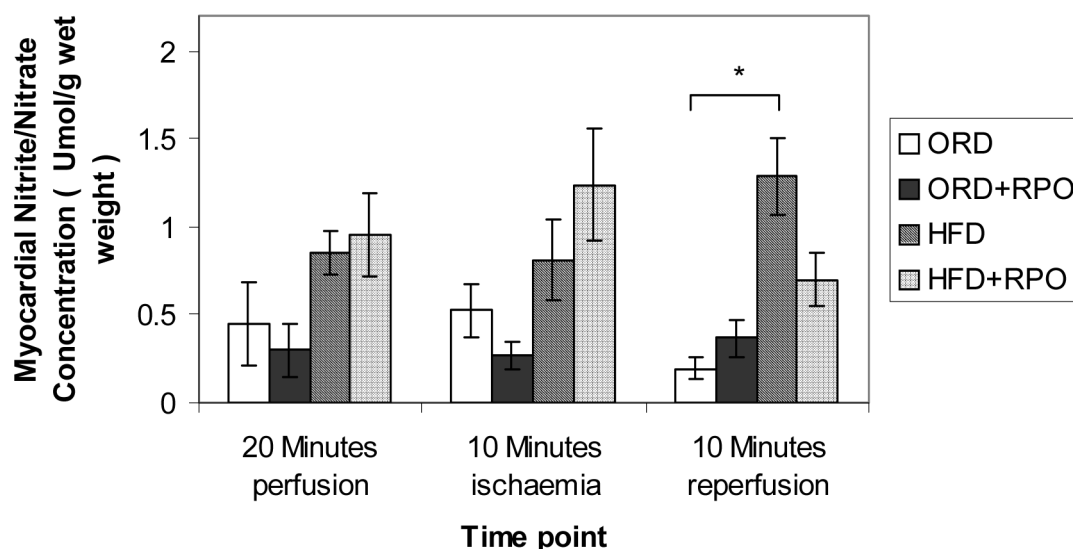


FIGURE 4. Myocardial NO concentrations of rats fed different supplemented diets for a period of fourteen weeks. (n=5 per group per time point)(*P<0.05 for the group versus the indicated group) (mean ± SEM)

ORD: Oxidative risk induced diet

HFD: High saturated fat diet

ORD+RPO: ORD with RPO-supplementation

HFD+RPO: HFD with RPO-supplementation

TABLE 1: Summary of total energy content (kJ) and macronutrient content (%) of rat diets as compared to recommendations made by the World Health Organization

	ORD	ORD+RPO	HFD	HFD+RPO	WHO
Total energy	240.50 kJ	242.30 kJ	230.70 kJ	232.60 kJ	-
Total protein	15.50 %	15.40 %	13.60 %	13.50 %	10-15 %
Total carbohydrate	61.90 %	61.40 %	58.60 %	58.10 %	55-75 %
Total fat	22.60 %	23.20 %	27.80 %	28.40 %	15-30 %
SFA	4.10 %	3.30 %	10.40 %	9.60 %	<10 %
PUFA	12.60 %	14.10 %	7.70 %	9.30 %	3-7 %
MUFA	4.90 %	4.50 %	6.80 %	6.40 %	>10 %
Refined sugar	10.50 %	10.50 %	11.00 %	10.90 %	0-10 %
Cholesterol	-	-	2.20 mg/d* (513.33 mg/d/70kg)	2.20 mg/d* (513.33 mg/d/70kg)	0-300mg/d#

* These values are the actual amounts of cholesterol fed to the rats.

This value is recommended for human daily intake by the WHO.

ORD: Oxidative risk induced diet

HFD: High saturated fat diet

ORD+RPO: ORD with RPO-supplementation

HFD+RPO: HFD with RPO-supplementation

MEDICAL TECHNOLOGY SA

TABLE 2: Functional parameters measured in perfused rat hearts at 20 minute perfusion and 25 minutes reperfusion time points (n=6 per group per time point)

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

a: P<0.05 for the group versus its RPO supplemented group of the same diet

b: P<0.05 for the group versus the group of the other diet without RPO-supplementation

c: P<0.05 for the group versus the RPO supplemented group of the other diet

d: P<0.05 for the group before ischaemia versus the same group after ischaemia

ORD: Oxidative risk induced diet

ORD+RPO: ORD with RPO-supplementation

HR: Heart rate

LVDP: Left ventricular diastolic pressure

AO: Aortic output

HFD: High saturated fat diet

HFD+RPO: HFD with RPO-supplementation

CF: Coronary flow

LVSP: Left ventricular systolic pressure

bpm: Beats per minute

MEDICAL TECHNOLOGY SA

TABLE 3: Major fatty acids (%) of myocardial total phospholipids in the 4 experimental groups before and after ischaemia (n=5 per group per time point) (mean \pm SEM)

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

SFA=Saturated fatty acids

MUFA=Monounsaturated fatty acids

PUFA=Polyunsaturated fatty acids

Total n-6= (n-6) Polyunsaturated fatty acids

Total n-3= (n-3) Polyunsaturated fatty acids

a: P<0.05 for the group versus its RPO supplemented group of the same diet

b: P<0.05 for the group versus the control group of the other diet

c: P<0.05 for the group versus the RPO supplemented group of the other diet

d: P<0.05 for the group before ischaemia versus the same group after ischaemia

ORD: Oxidative risk induced diet

HFD: High saturated fat diet

ORD+RPO: ORD with RPO-supplementation

HFD+RPO: HFD with RPO-supplementation