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

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Abstract

Dietary red palm oil (RPO) supplementation offers protection against ischaemia/reperfusion injury. Several pathways have been suggested to convey this protection. Recently it has been shown that RPO supplementation increases glutathione peroxidase (GPX) activity in the myocardium, but the mechanism behind this increase in GPX activity remains unknown. Antioxidant activity is known to play a role in pro-survival kinase signaling. The involvement of extracellular signal regulated kinases (ERK) early in reperfusion gave different results in previous studies, depending upon the diet to which RPO was supplemented. The aims of this study were to investigate the effects of dietary RPO supplementation on ERK 1/2 phosphorylation and GPX1, GPX3 and GPX4 transcription. Male Wistar rats were randomly divided into two groups. 1) SRC control group fed a standard rat chow diet (SRC) for 6 weeks and 2) RPO experimental group fed a standard rat chow diet supplemented with 2 ml of RPO olein per day. After the feeding period, rats were sacrificed and hearts perfused on a working heart perfusion apparatus. Cardiac function was measured before and after ischaemia in order to determine aortic output recovery. Hearts were also freeze clamped at 20 min perfusion, 10 min reperfusion and 25 min reperfusion, in order to determine the level of ERK and phosphorylated ERK by Western blotting. Regulation of glutathione peroxidase mRNA was determined before ischaemia using real-time polymerase chain reaction (RT-PCR). RPO supplementation did not produce significant changes in GPX1 or GPX3 mRNA expression when compared to the SRC control group. The mRNA expression of GPX4 was significantly higher in the RPO supplemented group when compared to controls. ERK44 phosphorylation was significantly higher in the RPO supplemented group when compared to the control group at 20 min perfusion. Our results confirmed improved aortic output recovery in the RPO group, as reported in previous studies after ischaemia/reperfusion injury. The minor changes found in ERK phosphorylation in this study may suggest that RPO has little effect on this pathway. However, the increase at baseline should be investigated further. Our findings also suggest that RPO may increase glutathione peroxidase activity, through up-regulation of the mRNA levels of GPX4.

Key words: Antioxidant activity, extracellular signal regulated kinases, phosphorylation, improved aortic output.

Introduction

Recent studies have shown that dietary red palm oil (RPO) supplementation is able to offer protection against ischaemia/reperfusion injury ¹⁻³. Several pathways of protection have been suggested for this protection. RPO is an oil, containing several antioxidants ⁴. The most abundant antioxidants in RPO are carotenoids and vitamin E. At least 500 ppm carotenoids is contained in RPO, of which the majority is in the form of α - and β -carotene ^{5,6}. RPO also contains approximately 500 ppm vitamin E of which the majority is in the form of tocotrienols.

Since RPO contains high levels of antioxidants, it would be of value to know whether it influences the antioxidant enzymes *in vivo*. Little is known about the effects of dietary supplementation of RPO on the regulation of antioxidant enzymes such as glutathione peroxidase. RPO may increase glutathione peroxidase (GPX) activity in rat hearts ⁷, but the mechanism for this increase in GPX activity is still unknown. Increased GPX activity would lead to decreased oxidative stress, as all isoenzymes of GPX reduce hydrogen peroxide and alkyl hydroperoxides ⁸. GPX 1 reduces only soluble hydroperoxides and is expressed intracellularly. GPX 3 may reduce hydroperoxides from complex lipids. GPX 4 can

reduce hydroperoxides which are integrated in cellular membranes, as well as hydroperoxide groups of complex lipids, lipoproteins, cholesterol esters and thymine ⁸.

The effect of RPO on the reperfusion induced salvage kinase (RISK) pathway has been a key feature in recent studies ⁹⁻¹¹. The PKB/Akt branch of this pathway was shown to be upregulated during reperfusion in normal rats receiving RPO supplementation ⁹. It is well known that ERK induces anti-apoptotic effects through phosphorylation of p90 ribosomal S6 kinase (P90RSK) and Bcl-2-associated death promotor (BAD) ¹²⁻¹⁵. ERK is also known to be involved in cardiac hypertrophy and heart failure ^{14,16}. However, it has not yet been established whether ERK plays a protective or causative role in heart failure. Pro-survival effects of ERK may also be dependant upon more variables than the Akt pathway. Badrian *et al.* ¹⁸ suggested that the pro-survival effects of ERK phosphorylation are dependant on the insult and also the length of time of ERK activation. It may therefore be necessary to investigate the effects of dietary RPO supplementation on ERK activation in greater depth.

We hypothesize that GPX activity may be increased in RPO

supplemented rat hearts through genetic regulation and thereby reduce ischaemia/reperfusion injury in these hearts.

Our aims were therefore to investigate: 1) the effect of dietary RPO supplementation on GPX 1, GPX 3 and GPX 4 gene transcription and 2) to determine the involvement of ERK in RPO mediated protection against ischaemia/reperfusion injury.

Materials and Methods

All rats received humane animal care in accordance with the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH publication 8523, revised 1985). Ethical approval for this study was obtained from the ethics committee of the faculty of Health and Wellness of the Cape Peninsula University of Technology (HAS-REC 26/01/2007).

Experimental design: Forty male Wistar rats (weighing approximately 180 g) were randomly divided into two diet groups. These groups were placed on the following diets for a five week period: Control group 1, standard rat chow diet (SRC) (n = 20); Experimental group, SRC plus 2 ml red palm oil (RPO) per day (n = 20).

Rats were individually caged in order to ensure that they consume similar amounts of supplements, and were allowed *ad libitum* access to rat chow and water, after supplements were consumed. Eight rats in each group was perfused for functional data, while the hearts of the remaining rats were freeze clamped at either 20 min perfusion or 10 min reperfusion for biochemical analysis.

Working heart perfusion: At the end of the feeding period rats were sacrificed by intra-peritoneal injection with Euthenase (sodium pentobarbitol). Hearts were rapidly (in less than 30 s) excised and mounted on the working heart perfusion apparatus. Once the aorta was cannulated retrograde perfusion was initiated and sustained for the 10 min stabilisation period. After the stabilisation period, the heart was switched to the working heart mode for 20 min, before being subjected to 20 min of normothermic global ischaemia and 25 min reperfusion, of which the first 10 min was retrograde perfusion and the last 15 min working heart perfusion.

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

Western blot: Heart tissue was homogenized by adding homogenization buffer and PMSF to the sample. For phosphoprotein determination a NaF, Na₃VO₄ solution was added to the sample after which samples were sonicated three times for 10 s and then centrifuged at 5000 rpm for 10 min. Protein concentration was determined through the Bradford method².

Samples were diluted with Laemmli sample buffer and boiled, after which 40 µg of protein was separated by SDS-PAGE electrophoresis. After electrophoresis the proteins were transferred to PVDF membranes. Nonspecific binding was blocked by overnight incubation in 5% fat free milk in TBST. Membranes were then incubated with primary antibodies that recognize Erk p42/44 (Thr²⁰²/Thr²⁰⁴) and PKB (Thr³⁰⁸). Membranes were subsequently washed and incubated with secondary antibody.

After thorough washing with TBST, membranes were covered with ECL and exposed to autoradiography films which were densitometrically analyzed.

Quantitative real-time polymerase chain reaction: Total RNA was isolated from snap frozen left ventricular tissue by homogenisation in Trizol (1ml/100 mg tissue) using a bead mill homogenizer (Precellys 24, Bertin Technologies). Insoluble material was removed from the homogenate by centrifugation, and the supernatant with 200 µl chloroform was transferred to a phase lock gel (Eppendorf, Hamburg, Germany) for separation of total RNA from genomic DNA. After DNase treatment and ammonium acetate precipitation, total RNA was purified using RNeasy Mini Kit (Qiagen), and the concentration measured on a NanoDrop Spectrophotometer (ND 1000). First strand cDNA was synthesised from 1µg total RNA primed with 4 µl of oligo(dT) using qScript cDNA SuperMix (Quanta Biosences) in a total volume of 20 µl and subjected to PCR; 2.5 µl cDNA synthesis template was mixed with 5µl PerfeCta SYBR Green 2x FastMix (Quanta Biosences), 0.5 µM forward and reverse primers (Gpx1, Gpx 3 and Gpx 4; qPCR primers assays from SA Biosences) and RNase free water to a total volume of 10 µl. The qPCR was performed on a Roche Light Cycler 480. The level of each Gpx mRNA was analysed and expressed relative to the housekeeping gene GAPDH using the Light Cycler_R 480 SW software (f'CCAAGGTCATCCATGA CAACTT, 'AGGGGCCATCCACAG TCTT; Invitrogen).

Statistical methods: All values are presented as mean plus or minus standard error of the mean. Significance between groups was determined with Student's t-test. *P* was considered significant if it was less than 0.05.

Results

Animal weight: There was no significant difference between the body weights of any of the diet groups after the feeding period (Fig. 1).

Coronary effluent: Coronary effluent of the RPO group was significantly increased when compared to the SRC group after ischaemia (17.62±0.73 ml/min versus 14.14±1.13 ml/min) (Fig. 2). There was no difference in coronary effluent at baseline.

Aortic output recovery: Aortic output recovery of the RPO supplemented group was significantly higher compared to the SRC group (47.16±5.46% versus 13.44±6.34%) (Fig. 3).

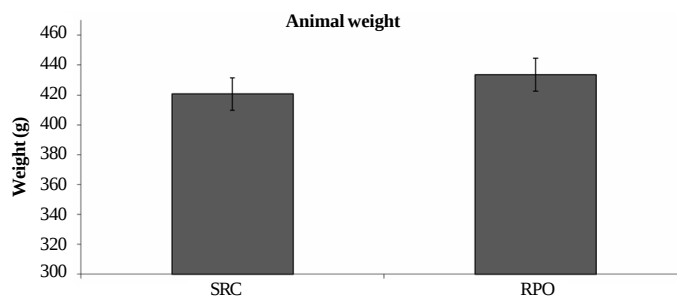


Figure 1. Animal weight (g) at the time of sacrifice.

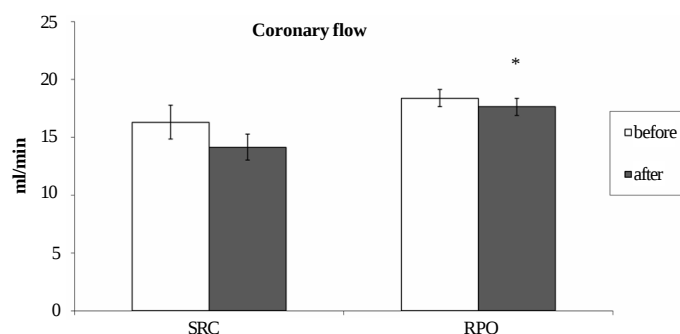


Figure 2. Coronary flow of hearts before and after ischaemia (* $P < 0.05$ for the group versus the control after ischaemia).

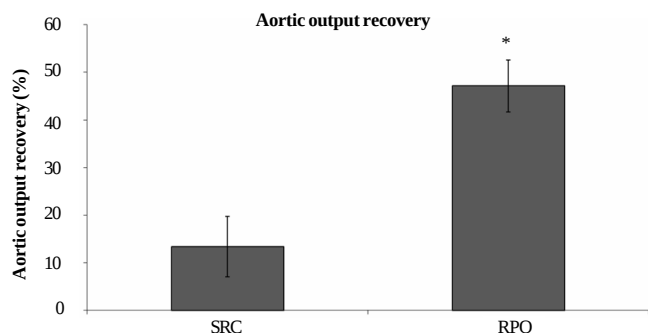


Figure 3. Aortic output recovery of hearts after ischaemia (* $P < 0.05$ for the group versus the control group).

Phosphorylation status of ERK 42/44: No significant differences in ERK 42 phosphorylation between the diet groups or time points were observed (Fig. 4). ERK 44 phosphorylation was significantly increased in RPO supplemented hearts compared to the SRC group, at the 20 min perfusion time point ($80.39 \pm 0.59\%$ versus $76.36 \pm 1.18\%$ ($P = 0.03$)) (Fig. 5). This may suggest that dietary RPO supplementation may have a cardio-protective at baseline, as ERK is associated with increased cytoprotection. ERK 44 phosphorylation was significantly decreased in the RPO supplemented group between the 20 min perfusion time point and the 10 min reperfusion time point ($80.39 \pm 0.59\%$ versus $72.05 \pm 1.55\%$). The 25 min reperfusion time point was also significantly lower than the 10 min reperfusion time point in the RPO supplemented group ($72.05 \pm 1.55\%$ versus $63.92 \pm 3.18\%$).

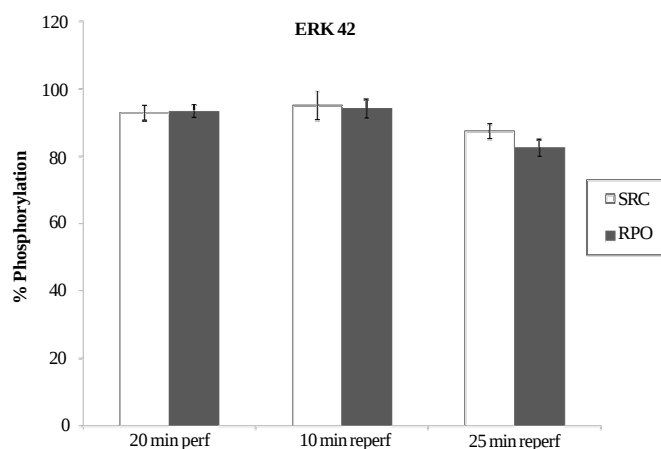


Figure 4. Percentage phosphorylation of ERK42 in cardiac tissue of each group at 20 min perfusion, 10 min reperfusion and 25 min reperfusion.

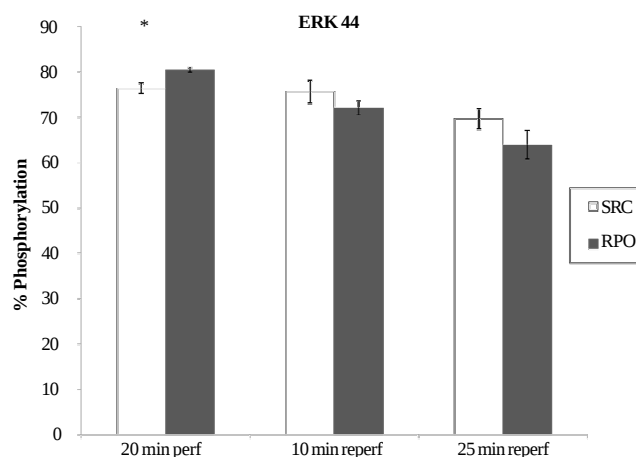


Figure 5. Percentage phosphorylation of ERK44 in cardiac tissue of each group at 20 min perfusion, 10 min reperfusion and 25 min reperfusion. (* $P < 0.05$ for the group versus the control before ischaemia).

Glutathione peroxidase transcription: GPX 4 expression was significantly higher in the RPO group compared to the SRC (1.09 ± 0.23 arbitrary units versus 0.44 ± 0.89 arbitrary units) (Fig. 6). GPX1 and GPX3 expression showed no significant differences between the diet groups (Figs. 7 and 8).

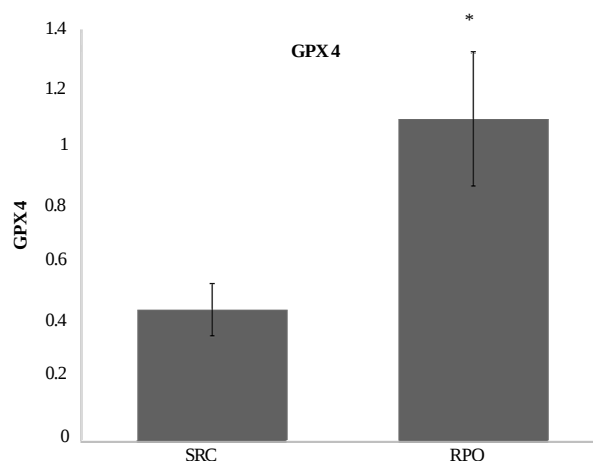


Figure 6. Expression of GPX4 mRNA in cardiac tissue of each group. (* $P < 0.05$ for the group versus the control group).

* $P = 0.05$

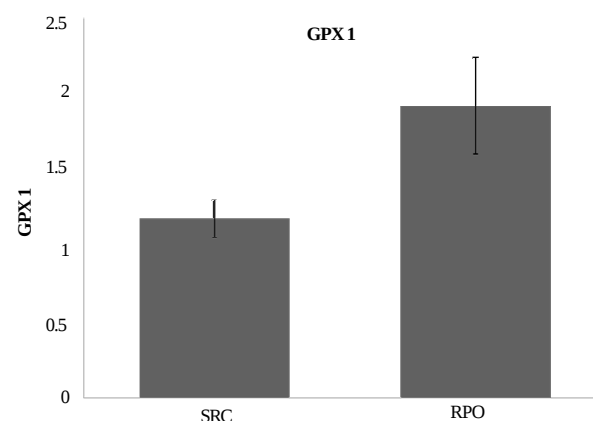


Figure 7. Expression of GPX1 mRNA in cardiac tissue of each group.

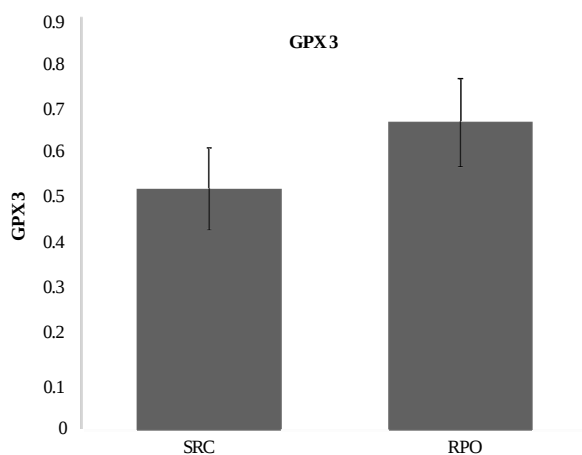


Figure 8. Expression of GPX3 mRNA in cardiac tissue of each group.

Discussion

The results indicate that dietary RPO supplementation was able to increase functional recovery of hearts after ischaemia/reperfusion compared to hearts of SRC fed controls. This confirms results from previous studies which also found improved functional recovery in RPO supplemented hearts¹⁻³. The significant increase in coronary flow after ischaemia in the RPO group, when compared to the SRC group, may indicate that red palm oil was able to reduce the vascular effects of ischaemia.

Narang *et al.*⁸ found that dietary RPO supplementation was able to increase GPX and other antioxidant enzyme activity in myocardial tissue. They suggested that this increase was followed by reduced oxidative stress in an ischaemia/reperfusion model. We have demonstrated that dietary RPO supplementation increases the expression of GPX 4 mRNA. Our results therefore suggest that increased GPX activity found by Narang *et al.*⁸ is due to upregulation of the GPX 4 gene by RPO supplementation. This may be due to the increased amount of antioxidants provided in the diet by RPO supplementation, which should lead to improvement of intracellular oxidative stress status¹⁸. It may therefore be advisable to measure the ratio of glutathione to reduced glutathione in the myocardium in future studies, in order to obtain a better understanding of the intracellular oxidative stress. In our study we did not perform transcription analysis in samples after ischaemia. In previous studies we showed that RPO's mechanism of protection was maximized after ischaemia. It would therefore be advisable to analyse GPX transcription after ischaemia. However, Borch *et al.*²⁰ reported increased GPX activity in the failing human heart with no significant change in mRNA or protein expression. They suggested that this may take place through post-translational alterations, such as tyrosine phosphorylation of the protein.

Results regarding ERK phosphorylation are in agreement with those of Engelbrecht *et al.*¹⁰. There seems to be no significant effects of RPO supplementation on ERK phosphorylation early in reperfusion. The fact that ERK 42 phosphorylation showed decreased levels of phosphorylation at 25 min reperfusion, when compared to the SFO supplemented hearts, supports the suggestion that ERK phosphorylation does not play a role in RPO mediated protection. The increased ERK 44 phosphorylation in hearts of dietary RPO supplemented rats before ischaemia is lost during ischaemia, and is therefore probably not involved in

protection against ischaemia/reperfusion injury. Similar studies in cholesterol fed rats may, however, have yielded different results¹². Our results together with those of Engelbrecht and *et al.*^{10,11} show that RPO supplementation to SRC fed rats induced upregulation of mainly the protein kinase B/Akt (PKB/Akt) branch of the reperfusion induced salvage kinase (RISK) pathway. However, further investigation of ERK phosphorylation before ischaemia may be needed in order to draw a conclusion.

Conclusions

Our results are in agreement with previous studies, showing that dietary RPO supplementation improves functional recovery at reperfusion in the isolated perfused rat heart. Furthermore, RPO supplementation was able to increase genetic transcription of GPX 4, but not GPX 1 and 3. Finally, it is inconclusive whether phosphorylation of ERK 1/2 plays any significant role in RPO mediated protection against ischaemia/reperfusion injury.

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