Dietary red palm oil supplementation protects against the consequences of global ischemia in the isolated perfused rat heart

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Activation of the NO-cGMP pathway is associated with myocardial protection against ischemia. During ischemia, function of this pathway is disturbed. Little is known about the effects of supplements such as Red Palm Oil (RPO) on the myocardial NO- cGMP- signalling pathway. RPO consists of saturated (SFAs), monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids and is an antioxidant rich in natural B-carotene and vitamin E (tocopherols and tocotrienols). This study determined whether dietary RPO-supplementation protects against the consequences of ischemia and identified a possible mechanism for this protection. Long-Evans rats were fed a control diet or control diet plus 7g RPO per kg diet for six weeks. Hearts were excised and mounted on a working heart perfusion apparatus. Cardiac function was measured before and after hearts were subjected to 25 minutes of global ischemia. Left ventricular systolic (LVSP) and diastolic pressure (LVDP), coronary flow (CF), heart rate (HR) and aortic output (AO) were measured. To assess NO-cGMP pathway activity, hearts subjected to the same conditions, were freeze-clamped and analysed for tissue cAMP and cGMP levels using a RIA method. Furthermore, composition of myocardial phospholipid fatty acids by gaschromatography and blood samples were collected for serum lipid determinations. The percentage aortic output recovery of hearts supplemented with RPO was 72.9 ± 3.43 % vs 55.4 ± 2.48 % for controls (P < 0.05). Ten minutes into ischemia the cGMP levels of the RPO-supplementation group were significantly higher than the control group (26.5 ± 2.78 pmol/g vs 10.1 ± 1.78 pmol/g. Total myocardial PUFA content in hearts supplemented with RPO increased from 54.45 ± 1.11% before ischemia to 59.03 ± 0.30 % after ischemia (P<0.05). Results demonstrated that RPO-supplementation protected hearts against the consequences of ischemia/reperfusion injury. These findings suggest that dietary RPO protects via the NO-cGMP pathway and/or changes in PUFA composition during ischemia/reperfusion.

Key Words:  red palm oil, ischemia, reperfusion, aortic output recovery, cGMP, phospholipid fatty acids

Introduction

Red Palm oil (RPO) and its liquid fraction, palm olein, are consumed worldwide as cooking oils and as constituents of margarines. These oils are also incorporated into fat blends used in the manufacture of a variety of food products and in home food preparation. It plays a useful role in meeting energy and essential fatty acid needs in many regions of the world. RPO contains a mixture of SFAs (48%), MUFAs (42%) and PUFAs (10%). Several clinical trials have evaluated palm oil’s effects on blood lipids and lipoproteins. These studies suggest that palm oil and palm olein diets do not raise serum total cholesterol (TC) and LDL cholesterol levels to the extent expected from its fatty acid composition.

Although many animal feeding studies have shown that fish-oil diets rich in n-3 PUFAs prevent ischemia-induced cardiac arrhythmias, only a few reports have been published on the protective effects of RPO-supplementation against ischemia/reperfusion injury. Palm oil and palm olein products are also naturally occurring sources of the antioxidant vitamin E constituents, tocopherols and tocotrienols. These natural antioxidants act as scavengers of damaging oxygen free radicals. Two studies published in the New England Journal of Medicine show that both men and women who supplement their diet with at least 100 IU of vitamin E per day for at least two years have a 37-41% drop in the risk of heart disease. Vitamin E is believed to be the major lipid-peroxidation chain-breaking antioxidant found in blood plasma and membranes. The insight into the mechanism of heart injury has suggested that administration of antioxidants may lessen oxidative damage of the heart. It has been shown that palm oil vitamin E mixture containing both alpha-tocopherol and alpha-tocotrienol was more efficient in the protection of the isolated Langendorff heart against ischemia/reperfusion injury than tocopherol alone as measured by its mechanical recovery.
Palm oil vitamin E completely suppressed LDH enzyme leakage from ischemic hearts prevented the decrease in ATP and creatine phosphate levels and inhibited the formation of endogenous lipid peroxidation products.\textsuperscript{13} Nitric Oxide (NO) is an important regulator of both cardiac and vascular function and tissue reperfusion.\textsuperscript{14} Myocardial NO formation is increased during ischemia and reperfusion, offering protection against ischemia/reperfusion injury.\textsuperscript{15,17} However, when NO plummets during reperfusion, due to rapid quenching by superoxide, the vascular protective regulatory properties become dysfunctional. This may lead to exacerbation of tissue injury. The role of reactive oxygen species, including superoxide radical ($\text{O}_2^-$), hydrogen peroxide ($\text{H}_2\text{O}_2$) and hydroxyl radical (OH) have long been implicated in the pathogenesis of ischemia/reperfusion injury. These radicals are predominant both during ischemia and at the time of reperfusion and can react with nucleic acids, proteins and lipids, resulting in damage to the cell membrane or intracellular organelles.\textsuperscript{18} Peroxynitrite (ONOO\textsuperscript{-}) is generated by a diffusion-limited reaction between $\text{O}_2^-$ and Nitric Oxide.\textsuperscript{14,18,20}

Protective effects of NO are mediated through the production of cGMP. NO donors given during ischemia possibly protect the myocardium by increasing tissue cGMP and decreasing cytosolic Ca\textsuperscript{2+} overload. Increases in cAMP levels associated with ischemia would increase Ca\textsuperscript{2+} levels and exacerbate ischemic and reperfusion injury.\textsuperscript{21} In this regard it is possible that cGMP may attenuate this type of injury by inhibiting the cAMP induced slow inward calcium current thus leading to a decrease in cytosolic calcium levels.\textsuperscript{22} Because little is known about the protective effect of RPO and the mechanisms involved, our aim was to determine whether dietary RPO-supplementation protect against the consequences of ischemia and reperfusion.

**Material and methods**

**Experimental model**

Male Long-Evans rats were divided into two groups: a control group receiving normal rat chow (see composition below) and an experimental group receiving normal chow plus 7g RPO/kg diet (main fatty acid composition: C16:0 ± 44%; C18:1 ± 39%; C18:2 ±10.5%) for six weeks. The rats were anesthetized with diethyl ether and intravenously injected with 400 units of heparin, before the hearts were rapidly excised and placed in ice-cold Krebs-Henseleit buffer. Hearts were transferred to the standard working heart perfusion apparatus where they were perfused with a Krebs-Henseleit buffer equilibrated with 95% O\textsubscript{2} and 5% CO\textsubscript{2} at 37°C (121.5 mmol/l NaCl; 3.8 mmol/l KCl; 1.2 mmol/l MgCl\textsubscript{2} x 6 H\textsubscript{2}O; 2.5 mmol/l CaCl\textsubscript{2}; 15.5 mmol/l NaHCO\textsubscript{3}; 1.2 mmol/l KH\textsubscript{2}PO\textsubscript{4}; 11.0 mmol/l glucose) at a perfusion pressure of 100 cmH\textsubscript{2}O. The aorta was cannulated and a retrograde perfusion with Krebs-Henseleit buffer was 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-minute stabilisation period in the Langendorff mode, hearts were switched to the working mode (see study design). Hearts were perfused in a non-recirculating manner and were enclosed in a water-jacketed chamber. The temperature of the perfusate as well as that of the air surrounding the heart was thermally controlled and checked at regular intervals to ensure that the temperature was maintained at 37°C irrespective of coronary flow. A cannula, connected to the pressure transducer, was inserted into the left ventricle. Left ventricular systolic and diastolic pressure, coronary flow (CF), heart rate and aortic output (AO) were measured at 5, 10 and 20 minutes before ischemia. After 20 minutes, hearts were subjected to 25 minutes of global ischemia. At the end of ischemia, hearts were reperfused in the Langendorff mode for 10 minutes with a 2% Lignocaine solution for the initial three-minute period, followed by the working mode for 15 minutes. Cardiac function was measured at 15, 17, 20 and 25 minutes during reperfusion. To assess fatty acid composition and cGMP pathway activity, hearts were freeze-clamped with Wollenberger clamps pre-cooled in liquid nitrogen and freeze-dried to analyze for tissue cyclic nucleotide level changes.

**Study design**

![Study design diagram]

TC=Total cholesterol; AO=Aortic Output; HDL=High density lipoprotein; Phospholipids=Myocardial total phospholipid fatty acids

**Diet**

Composition of the control diet was as follow:

<table>
<thead>
<tr>
<th>Substance</th>
<th>g/kg Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>180 g/kg (min)</td>
</tr>
<tr>
<td>Moisture</td>
<td>120 g/kg (min)</td>
</tr>
<tr>
<td>Fat</td>
<td>25 g/kg (min)</td>
</tr>
<tr>
<td>Fibre</td>
<td>60 g/kg (max)</td>
</tr>
<tr>
<td>Calcium</td>
<td>18 g/kg (max)</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>7 g/kg (min)</td>
</tr>
</tbody>
</table>

**Left ventricular developed pressure (LVDevP) (mmHg)**

LVDevP (the difference between systolic and diastolic pressure) was used to measure mechanical function of the heart. It was calculated by comparing the LVDevP before and after ischemia.

**Aortic output recovery (AO) (%)**

Coronary flow and aortic flow rates were measured by collecting one-minute samples of the respective effluent. Aortic Output Recovery was calculated by dividing the AO after ischemia with AO before ischemia and expressing these values as a percentage recovery.

**Biochemical analysis**

The cAMP and cGMP levels were determined using radioimmunoassay kits obtained from Amersham Corp.
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(Amersham, UK). For cGMP assays, freeze-clamped hearts were freeze-dried and 20-25 mg of dry 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 cGMP assay. The IC50 for the cGMP assay was 25 pmol/tube. For the cAMP assays, 20-25 mg freeze-dried tissue were extracted with perchloric acid, neutralized and assayed. The IC50 for this assay was 1.92 mmol/tube.

Heart muscle total phospholipid fatty acids

Hearts isolated from rats fed standard rat chow or standard rat chow and RPO for six weeks were perfused, freeze clamped and freeze-dried tissue was used to determine the influence of the supplementation on the composition of myocardial phospholipid fatty acids.

Serum lipids

Animals were weighed weekly during the RPO supplementation period and blood was collected before and after the six-week period for both the control and the RPO supplemented groups. The blood was centrifuged for ten minutes at 3000 rpm to obtain serum and analysed for lipid profiles i.e. total cholesterol (TC), HDL-cholesterol and triglycerides. Aliquots of the supernatant were analyzed for lipid parameters. The serum lipid profile was determined using a Ciba Corning Express 550 instrument and Ciba Corning reagents employing enzymatic and colorimetric methods.

Statistical methods

Values are presented as mean ± SEM. Significance between groups was determined by ANOVA. For paired comparisons the Student’s t test was used. P <0.05 was considered significant.

Results

Left ventricular developed pressure (LVDevP)

The pre-ischemic LVDevP of RPO-supplemented and control hearts were similar. After 25 minutes of global ischemia and 25 minutes of reperfusion, the LVDevP recovery of hearts from the RPO-supplemented group was 89.0 ± 8.1% as compared to the 81.0 ± 6.0 % LVDevP of hearts obtained from rats fed the normal diet (Fig. 1).

Aortic output recovery (%)

After 25 minutes of global ischemia and 25 minutes of reperfusion, percentage AO recovery of hearts from the RPO-supplemented group were 72.9 ± 3.4% vs 55.4 ± 2.5% for the control group, P<0.05 (Fig. 2).

Effects of RPO-supplementation on ischemic cAMP and cGMP levels

Myocardial cAMP levels were not affected by RPO supplementation (Fig. 3). The cGMP levels of the RPO-supplemented group were higher than the control group 10 minutes into ischemia. cGMP levels at 10 minutes ischemia were 26.5 ± 2.78 pmol/g in hearts of the RPO-supplemented group, and 10.1 ± 1.78 pmol/g in hearts of the control group (P<0.05) (Fig.4).

Serum lipids

The mean baseline concentrations of total cholesterol in the control group was 1.19 ± 0.04 mmol/l and after 6 weeks RPO-supplementation it was 1.12 ± 0.06 mmol/l. For the RPO-supplemented group, the mean baseline value was 1.42 ± 0.04 mmol/l and after 6 weeks it was 1.40 ± 0.03 mmol/l. The mean baseline concentrations of serum HDL-cholesterol in the control group were 0.99 ± 0.03 mmol/l and after 6 weeks it was 1.01 ± 0.07 mmol/l. In the RPO-supplemented group the mean baseline value was 1.17 ± 0.05 mmol/l and after 6 weeks it was 1.26 ± 0.04 mmol/l. Serum concentrations of triglycerides were significantly increased in the RPO supplemented group (N=10) after 6 weeks [from 0.56 ± 0.07 mmol/l to 0.87 ± 0.08 mmol/l (P<0.05)]. In the control group it was 0.54 ± 0.07 mmol/l before and 0.76 ± 0.10 mmol/l after the 6-week diet (Fig.5).

Heart muscle total phospholipid fatty acids composition (%)

Our results show that supplementation with RPO caused significant changes in fatty acid composition of myocardial tissue with a significant increase in myocardial total SFA composition (37.95 ± 0.95%) versus control hearts (34.42 ± 0.37%). No significant changes occurred in MUFA and PUFA composition. During the perfusion protocol, the event of ischemia altered fatty acid composition. The myocardial total SFA composition decreased significantly from 37.95 ± 0.95% before ischemia to 33.59 ± 0.24% after ischemia in hearts of the group supplemented with RPO (P<0.05). Concurrently, myocardial total PUFA composition in hearts of the group supplemented with RPO increased from 54.45 ± 1.11% before ischemia to 59.03±0.30 % after ischemia (P<0.05), with no changes in total myocardial MUFA composition.

Discussion

Our results demonstrate that RPO-supplementation offered protection against ischemia/reperfusion injury in the isolated perfused working heart as reflected by improved aortic output recovery. These data support the findings of
**Figure 2.** % Aortic output recovery of hearts in the RPO-supplemented group versus hearts of the control group ($N = 10$) (*$P < 0.05$)

**Figure 3.** Myocardial cAMP levels for hearts of rats on RPO-supplemented diet versus hearts of control diet ($N = 5$).

**Figure 4.** Myocardial cGMP levels for hearts of rats on RPO-supplemented diet versus hearts of control diet ($N = 5$) (*$P < 0.05$)
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Serbinova et al., who showed that palm oil vitamin E was more effective in the protection against ischemia/reperfusion injury in the isolated Langendorff perfused heart than tocopherol alone. Based on our results we propose that the protective effect of RPO may be associated with either its antioxidant characteristics and/or changes in the fatty acid composition of the myocardium during ischemia/reperfusion. Abeywardena and co-workers also argued that RPO protection lies in alliance of fatty acids and endogenous antioxidants during ischemia/reperfusion.

The protective effect against ischemia/reperfusion of the individual fatty acids in RPO needs consideration. Although myocardial total MUFA composition was unchanged over the 25 minute period of ischemia, myocardial PUFA content increased during the same period. This was associated with improved reperfusion aortic output recovery and indicates that dietary RPO-supplementation for six weeks may increase the bioavailability of myocardial PUFAs. Normally, linoleic acid (LA) undergoes a series of elongations and desaturations to yield Arachidonic acid (AA). To our knowledge no data exist on the effect of ischemia on Elongase and Desaturase activity. Many studies have focused on the effect of FA on arrhythmias. Contradictory results exist on the on the effect of AA on the development of arrhythmias. Li and co-workers found that free AA is able to prevent arrhythmias, but the major cyclooxygenase metabolites (PGD2, PGE2, PGF2 and TXA2) derived from AA are arrhythmogenic. However, prostacyclins (PGI2), also synthesized from AA, are anti-thrombotic agents that act as a vasodilator in blood vessels and have an anti-arrhythmogenic effect. No clear relationship exists between the availability of AA in myocardial phospholipids and eicosanoid profile. Abeywardena and co-workers showed that a chemically refined palm oil-supplementation for twelve months had no effect on prostacyclin production from AA in rat myocardial.
tissue. Concurrently, thromboxane A2 production was inhibited. Another palm oil supplement (with a near-identical fatty acid profile) in the same study showed no thromboxane A2 inhibition. This argues that thromboxane A2 production is unlikely to be mediated via fatty acids. In another study (Abeywardena et al., 1999), myocardial prostacyclin production was increased after ischemia with refined, bleached and deodorized palm oil (RBDPO) supplementation for nine months. However, Abeywardena and co-workers, 2003 argues that this increase in prostacyclin production may not be mediated by fatty acids alone but that endogenous antioxidants may play a role.

The increase in PUFAs during ischemia/reperfusion in the current study, suggest that PUFAs may be involved in the protection against ischemia/reperfusion injury. The mechanism of fatty acid protection remains elusive and needs further investigations. Generally, the mechanism of eicosanoid action is to bind to membrane receptors, leading to generation of second messengers such as cAMP and Ca2+. As myocardial cAMP levels were not significantly affected throughout the protocol in the RPO supplemented hearts, prevention of fatal cardiac arrhythmias may depend on the net effect of these metabolites, which is determined by the status of AA metabolism and the contents of n-6 and other fatty acids.

Interaction between cardiac endothelium derived prostaglandins and NO determines myocardial performance. Our results suggest that the NO-cGMP pathway is involved in the protective effect of RPO. The elevated cGMP levels early in ischemia may suggest that RPO-supplementation protected the isolated heart against the consequences of ischemia/reperfusion injury via the NO-cGMP pathway. NO is known to increase myocardial cGMP, and it can be speculated that the protective effect of NO is related to a mechanism secondary to the stimulation of guanylyl cyclase within the vascular wall or in ventricular myocytes. Besides its effects on myocardial contractility, the NO-cGMP pathway stimulation during ischemia may protect the heart against ischemia/reperfusion induced calcium overload. In this regard it is possible that cGMP may attenuate this type of injury by inhibiting the cAMP induced slow inward calcium current thus leading to a decrease in cytosolic calcium levels. Another protective pathway might include the NO-cGMP dependent pathway in which the sarcolemmal-K ATP channels are opened and the cystolic calcium levels are lowered. Therefore, cGMP appears to be an endogenous intracellular cardioprotectant.

Studies have shown that reactive oxygen species (ROS) can oxidize lipids and proteins and contribute to myocardial injury. Peroxynitrite (ONOO⁻) is generated by a diffusion-limited reaction between O₂⁻ and Nitric Oxide. Palm oil and palm oil products are naturally occurring sources of the antioxidant vitamin E constituents, tocopherol and tocotrienols, which act as scavengers of these damaging oxygen free radicals which may lessen oxidative damage to the heart. This may suggest that NO, synthesized from L-arginine by NO-synthase (NOS), results in enhanced synthesis of cGMP. NO synthase activity in the heart is rapidly stimulated by ischemia and this stimulation is maintained during the whole ischemic episode. Our data show that RPO-supplementation increases cGMP levels that may confer some of the cardioprotection to the ischemic and reperfused heart.

Our results showed no increase in total serum cholesterol in the RPO supplemented group. These data are in agreement with other studies, which showed that not all saturated dietary fats raise total serum cholesterol. Triglycerides were increased in all the groups after the six-week period with a significant difference in the RPO-supplemented group. The baseline difference between the control and RPO-supplemented groups cannot be explained and requires further investigation, since groups were randomly divided without preference.

Currently little documented data exist on the effects of dietary RPO-supplementation and post ischemic recovery linked to the NO-cGMP pathway, heart muscle total phospholipid fatty acid composition and antioxidant status. The findings of this study create opportunities for further investigations to elucidate mechanisms involved in cardiac protection.

Conclusion

Dietary RPO-supplementation protects against the consequences of global ischemia in the isolated perfused rat heart. The mechanism of protection may involve the NO-cGMP pathway and/or myocardial fatty acid compositional changes during ischemia/reperfusion.

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饮食补充红棕榈油对大鼠离体心脏完全缺血再灌注损伤的保护作用

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一氧化氮-环鸟苷一磷酸（NO-cGMP）途径的激活与心肌缺血保护有关。心肌缺血时，该途径的功能被扰乱。膳食补充剂，如红棕榈油对心肌 NO-cGMP 信号传输途径的作用了解得还很少。红棕榈油含有饱和脂肪酸、单不饱和脂肪酸和多不饱和脂肪酸，而且是富含天然的β-胡萝卜素和维生素E（生育酚和生育三烯酚）的抗氧化剂。本课题研究了膳食补充红棕榈油是否具有保护心肌缺血损伤的作用及其可能机制。大鼠饲喂对照组饲料或对照组饲料+红棕榈油 7 克/天，喂养 6 周后，剥离大鼠心脏并固定在正在运行的心脏灌注装置上。测心脏完全缺血 25 分钟前后的心脏功能，包括左心室的收缩压（LVSP）和舒张压（LVDP）、冠状血流量（CF）、心率（HR）和大动脉输出量（AO）。在相同条件下，将心脏组织冷夹（freeze-clamped），采用放射免疫方法测心脏组织环腺苷一磷酸（cAMP）和环鸟苷一磷酸（cGMP）含量，以评价 NO-cGMP 途径的活性。此外，用气相色谱测定了心肌磷脂的脂肪酸组成，收集血液样本，测定了血清脂质组成。补充红棕榈的大鼠心脏大动脉输出量恢复率为 72.9 ± 3.43 %，对照组的恢复率为 55.4 ± 2.48 %。缺血 10 分钟时，补充红棕榈组大鼠心肌 cGMP 水平显著高于对照组（26.5 ± 2.78 pmol/g vs 10.1 ± 1.78 pmol/g）。补充红棕榈组大鼠心肌多不饱和脂肪酸（PUFA）含量由缺血开始前的 54.45 ± 1.11% 增加至缺血结束后 59.03 ± 0.30 %。结果表明，膳食补充红棕榈油能保护大鼠心肌缺血再灌注引起的损伤。这些发现表明，膳食补充红棕榈油的保护作用是通过 NO-cGMP 途径和/或改变心肌缺血再灌注过程中 PUFA 的组成实现的。

关键词：红棕榈油，缺血，再灌注，大动脉输出量恢复，环鸟苷一磷酸，磷脂脂肪酸