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Omega 3 chronic supplementation attenuates myocardial ischaemia-reperfusion injury through reinforcement of antioxidant defense system in rats

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Currently, controversial clinical data about the protective effects in the consumption of n-3 polyunsaturated fatty acids (PUFAs) in ischaemic heart diseases exist. Improved myocardial resistance to ischaemia-reperfusion (IR) injury results in non-lethal myocardial infarction, which is a relevant factor in the myocardial function. We hypothesized that chronic supplementation with PUFAs reduced infarct size (IS) and induced an improvement on oxidative stress-related parameters in IR model. Rats were supplemented with two doses of PUFAs D1 ($n = 7$) ($0.6\,g\,kg^{-1}\,d^{-1}$) and D2 ($n = 7$) ($1.2\,g\,kg^{-1}\,d^{-1}$) for 8 weeks. Control group ($n = 7$) received only standard diet. In *ex vivo* model, all rat hearts were subjected to 30 min of global ischaemia followed by 120 min of reperfusion. The IS and left ventricular function were assessed. Lipid peroxidation, reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio and antioxidant enzyme activity were measured in the whole heart. The results show a reduction in IS in a dose-dependent manner with PUFAs D1 (30.6%) and D2 (48.5%) and higher values of left ventricular developed pressure, at the end of the reperfusion, for each dose, respectively ($p < 0.05$). The two PUFAs groups showed higher values of GSH/GSSG ratio and lipid peroxidation, and higher values of activity of antioxidant enzymes catalase, superoxide dismutase and glutathione peroxidase at basal condition ($p < 0.05$). At the end of reperfusion, the GSH/GSSG ratio and antioxidants enzyme activity did not show a significant drop in their values ($p > 0.05$). These findings suggested that the supplementation with PUFAs induces cardioprotection against IR injury, associated with reinforcement of the antioxidant defense system. Copyright © 2013 John Wiley & Sons, Ltd.

KEY WORDS—oxidative stress; omega 3; ischaemia-reperfusion; heart

INTRODUCTION

Dietary fatty acids supplementation plays an important role in coronary heart disease complications.[1,2] The biological effects of these compounds included a decreased in the triglyceride plasma levels, improvement in the endothelial function and prevention in the development and progression of heart failure. These effects could be determined by the dietary fatty acid profile and in this way modulate the resistance of the myocardium to ischaemia-reperfusion (IR) injury.[3] This is a critical issue because myocardial resistance to IR is a major determinant of myocardial infarct size (IS). This factor causes the development of cardiac heart disease complications, such as cardiac pump failure and ventricular arrhythmias, which are the main causes of cardiac death in humans.[4]

The IR cycle in the myocardium is associated with the activation of an injurious cascade, thus leading to new myocardial challenges, which account for up to 50% of IS. Some evidence implicates a reactive oxygen species (ROS) as a probably causes of myocardial injury in prooxidant clinical settings.[5] The damage occurs during both ischaemia and post-ischaemic reperfusion in animal and human models. The mechanisms that contribute to this damage included the increase cellular calcium concentration and induction of ROS sources during reperfusion.[5] In this regard, a pharmacological preconditioning, which includes pharmacological strategies that counteract the ROS burst and calcium overload followed to IR cycle in the myocardium, could be effective.

The supplementation with ω-3 polyunsaturated fatty acids (PUFAs), docosahexaenoic acid [DHA (22:6n3)] and eicosapentanoic acid [EPA (20:5n3)] from fish oils are used experimentally and clinically for the prevention of IR injury in the heart. The mechanisms for these effects are not clear, and clinical evidence is still a controversy.[6] The experimental evidence indicates that these effects may be dependent upon changes in cardiac phospholipid composition and improved mitochondrial tolerance to stress.[7] Experimental studies involved to several signaling pathways such as MAPKs, PKC and PI3K in antiapoptotic and antioxidant response induced by PUFAs.[8,9] Therefore, it is critical to

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identify which specific fatty acid profile may be optimal in improving myocardial resistance to IR injury.

The purpose of this study was to determine the effect of PUFAs supplementation on the IS and oxidative stress-related parameters in a model of IR in isolated rat hearts.

MATERIALS AND METHODS

Animals and surgical procedures

This study conformed to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (Publication No. 85–23, revised in 1996), and was approved by the Institutional Ethics Review Committee at the Universidad de Chile. All experiments were conducted on *ex vivo* heart models of adults, male Wistar rats (200 g). All animals received a standard laboratory solid diet and water ad libitum.[10] The composition of the experimental diet is shown in Table 1. Daily fluid intake was measured with graduated Richter tubes. Food intake was also estimated by gravimetry. All rats were housed under conditions of constant temperature, humidity and standard light–dark cycle and were supplemented by PUFAs (Acolest TG, 720 mg, DHA:EPA = 1.1:1.0) on a daily doses via gavage for 8 weeks. The animals were divided in three groups: control rats, which did not received PUFAs supplementation ($n = 7$); PUFAs D1 ($n = 7$), rats supplemented with PUFAs dose 1 ($D1 = 0.6\ g\ kg^{-1}d^{-1}$); and PUFAs D2, which received PUFAs dose 2 ($n = 7$) ($D2 = 1.2\ g\ kg^{-1}d^{-1}$). PUFAs D1 and D2 groups received omega 3 once a day for 8 weeks.

Table 1. Composition of experimental diet ($g\ kg^{-1}$ diet)

Casein	200
DL-methionine	3
Corn starch	297
Sucrose	225
Canola oil	100
Potato starch	25
Water-soluble vitamins*	30
Fat-soluble vitamins†	20
Mineral mixture‡	50
Fibre	50
Energy ($MJ\ kg^{-1}$)	16.3II

*The water-soluble vitamin composition was ($g\ kg^{-1}$ diet): choline chloride 0.945, *p*-aminobenzoic acid 0.473, inositol 0.094, niacin 0.047, calcium pantothenate 0.024, riboflavin 0.024, thiamine hydrochloride 0.019, pyridoxine hydrochloride 0.005, folic acid 0.005, biotin and cyanocobalamin 0.0005.

†The fat-soluble vitamins composition was (per kilogram diet): DL-α-tocopherol acetate 66 mg, all-*trans-retinyl* acetate 2.5 mg (7000 IU), menadione 60 µg and cholecalciferol 7.5 µg.

‡The mineral composition was (per kilogram diet): $CaCO_3$ 15.75, $CaH_2PO_4 \cdot 2H_2O$ 3.35 g, K_2HPO_4 16.40 g, NaCl 8.52 g, $MgSO_4.7H_2O$ 5.18 g, iron citrate 0.51 g, $MnSO_4$ 0.25 g, $CuSO_4.5\ H_2O$ 25.3 mg, $ZnCl_2$ 5.0 mg, KI 1.2 mg, sodium selenite 5.0 mg and NaF 5.0 mg.

II 3900 $kcal\ kg^{-1}$.

Modified: Araya *et al*., 2001.

The rats were anaesthetized with pentobarbital ($50\ mg\ kg^{-1}$ IP). Subsequently, a sternotomy was performed, and heparin $100\ U\ kg^{-1}$ intravenous (IV) was administered. The heart was rapidly excised, mounted in a temperature-regulated heart chamber and perfused retrograde via the ascending aorta using a peristaltic infusion pump (Gilson Minipuls3, France) at a constant flow of 10–14 ml min^{-1} to generate an initial mean coronary (aortic) perfusion pressure of 60–70 mmHg with physiological modified Krebs Henseleit Buffer solution containing (in mM) NaCl (128.3), KCl (4.7), $CaCl_2$ (1.35), $NaHCO_3$ (20.2), NaH_2PO_4 (0.4), $MgSO_4$ (1.1), glucose (11.1) and pH 7.4 at 37 °C when equilibrated with a mixture of 95% O_2/5% CO_2. Perfusate and bath temperatures were maintained at 37 °C by a thermostatically controlled water circulator (B. Braun Thermomix 1420, Germany). Then, a latex balloon inserted in the left ventricle through the mitral valve was connected to a pressure transducer (Bridge Amp ML221 AD Instruments, Australia) and filled with normal saline to produce a left ventricle end-diastolic pressure (LVEDP) of 5–10 mmHg. The volume of the balloon was maintained constant throughout the experiment. After 15 min stabilization (basal conditions), hearts with a left ventricular developed pressure (LVDP) less than 60 mmHg and a heart rate (HR) less than 180 bpm were excluded from the study. The remaining hearts were subject to 30 min of global ischaemia followed by 120 min reperfusion. The scheme of protocol was showed in Figure 1.

Measurement of infarct size

After the 120-min reperfusion, the heart was perfused with 15 ml 2,3,5-triphenyltetrazolium chloride 1% (Sigma Chemical) in phosphate buffer adjusted to pH 7.4, for 15 min at 37 °C. After overnight storage in 10% formaldehyde, five to six slices uniform 2 mm covered by a glass. Then, a digital photography of the mounted tissue was taken. For each slice, measuring the size was performed by planimetry using the Image J program. The IS was expressed as a percentage of the total ventricular volume.[11]

Measurement of left ventricular function

The left ventricle and diastolic pressure, coronary perfusion pressure were measured and continuously recorded throughout the entire experiment on a personal computer using PowerLab (ML866 ADInstruments, Australia). LVDP was calculated as follow: LVDP = LVSP − LVEDP (mmHg).

Figure 1. Schematic representation of the experimental protocol

Oxidative stress-related parameters

The intracellular redox status in atrial tissue was assessed by a fluorometric method in order to measure the oxidized glutathione (GSSG) and reduced glutathione (GSH).[12] The inter-assay and intra-assay coefficient of variations (CV) for GSH and GSSG were 3.1% and 4.2%, and 2.7% and 3.5%, respectively. The GSH/GSSG ratio was then calculated. Lipid peroxidation was assessed by the thiobarbituric acid reaction at pH 3.5, followed by solvent extraction with a mixture of n-butanol/pyridine (15:1, v/v).[13] Tetramethoxypropane was used as the external standard, and the levels of lipid peroxides were detected spectrophotometrically at 532 nm and were expressed as mmol thiobarbituric acid reactive substances (TBARS) per milligram protein. The inter-assay and intra-assay CV for TBARS were 10.5% and 4.8%, respectively.

Antioxidant enzymes

Cardiac lysates were homogenized in 0.25 mol l^{-1} sucrose in order to determine Cu-Zn superoxide dismutase (SOD) activity or in 1.15% KCl 10 mmol l^{-1} l^{-1} Tris/HCl buffer (pH 7.4) for catalase (CAT) and glutathione peroxidase (GSH-Px) activity. The Cu-Zn SOD assay is based on the SOD-mediated increase in the rate of auto-oxidation of catechols in an aqueous alkaline solution in order to yield a chromophore with a maximum absorbance at 525 nm.[14] One Cu-Zn SOD unit is defined as the activity that doubles the auto-oxidation background, and the results are expressed as units per milligram of protein. CAT activity was assayed by the kinetic of breakdown of H_2O_2 at 240 nm by an aliquot of the 2400 *g* supernatant and are expressed as the first-order reaction rate constant (k) per milligram of protein.[15] Soluble GSH-Px activity was measured spectrophotometrically in the cytosolic fraction (100 000 *g* supernatant) by the reduction of glutathione disulfide coupled to nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidation by glutathione reductase.[16] One GSH-Px unit is defined as the activity that oxidizes 1 μmol of NADPH per minute and is expressed as units per milligram of protein.

Statistical analysis

A sample size calculation was performed on the basis of the IS reduction. The assumptions used for this purpose included an expected 30% of reduction in IS at the end of reperfusion in the supplemented rats with PUFAs, compared with rats that did not receive any supplementation. The sample size calculation was based on the differences in the mean value between two groups with equal sample size, prespecified 5% alpha error and 80% power. The resulting sample size was 7.3 rats in each treatment group (Stata 10.0 for Windows). The infarct areas were analysed at the end of reperfusion using one-way ANOVA. A Tukey post-hoc test was used to assess the differences in biomarkers of oxidative stress among groups. Haemodynamic data at different times were analysed with repeated-measures two-way ANOVA (mixed model). Bonferroni post-hoc test was used to assess the recovery differences among groups. A value of $p < 0.05$ was considered for significant statistical difference. All data are expressed as mean ± SD.

RESULTS

General characteristics, infarct size and haemodynamic parameters

The rats in the three groups did not show difference in body weight, fluid and energy consumption after 8 weeks of supplementation. None of the rats died during the study at the doses used. There are no significant differences between control and both PUFA groups in blood levels of total cholesterol, high density lipoprotein and low density lipoprotein (LDL). However, the triglycerides and very LDL show 26.4% and 30.5%; 27.8% and 33.3% lower levels at the end of 8 weeks supplementation in PUFA D1 and D2, with respect to the control values ($p < 0.05$).

Polyunsaturated fatty acids induce cardioprotection against global IR injury. The means of myocardial infarction size in the control, PUFAs D1 and PUFAs D2 were 30.6% and 48.5% lower with respect to the control in PUFA D1 and D2, respectively ($p < 0.05$) (Figure 2).

The cardiac performance using the Langendorff control system, PUFAs D1 and PUFAs D2 groups at basal conditions, and following 30 min of ischaemia and 120 min of reperfusion (End Rep) are show in Figure 3. Control hearts subjected to IR cycle exhibited a significant reduction in LVDP (Figure 3A and B) with respect to basal values at end of reperfusion. Control and D1 hearts subjected to IR

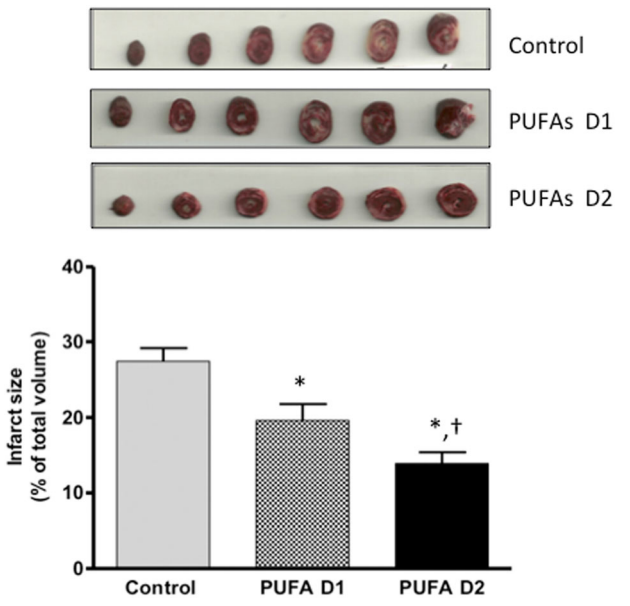

Figure 2. Infarct size in rats supplemented with polyunsaturated fatty acids (PUFAs) D1 (0.6 g kg^{-1} d^{-1}) and D2 (1.2 g kg^{-1} d^{-1}) after 8 weeks of supplementation. Values are expressed as mean ± SD of total ventricular volume ($n = 7$ per group). Significant differences: *$p < 0.05$ versus control; †$p < 0.05$ versus PUFA D1

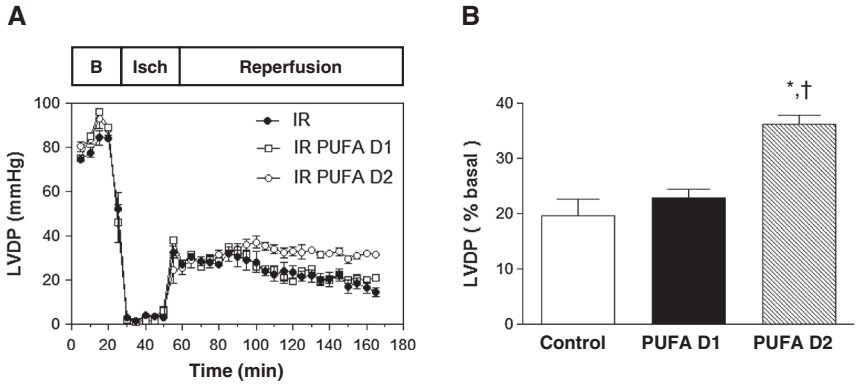

Figure 3. Measurement of left ventricular developed pressure: (A) through the protocol and (B) at the end of reperfusion, in rats supplemented with polyunsaturated fatty acids (PUFAs) D1 (0.6 g kg^{-1} d^{-1}), D2 (1.2 g kg^{-1} d^{-1}) and control (without PUFA). Values are expressed as mean ± SD ($n = 7$ per group). Significant differences: *$p < 0.05$ versus control; †$p < 0.05$ versus PUFA D1

cycle did not show significant differences in LVDP; on the other hand, PUFA D2 group showed an 18.1% and 14.2% higher values of LVDP than control group and PUFA D1, at the end of the reperfusion, respectively ($p < 0.05$).

Effects of polyunsaturated fatty acids in oxidative stress-related parameters

Cardiac lipid peroxidation showed a 191% and 353% higher levels of TBARS in PUFA D1 and D2, at basal time, following 8 weeks of supplementation with respect to contol group ($p < 0.05$). With respect to each group, TBARS levels at the end of reperfusion were 65.8% and 27.1% higher in control and PUFAs D1, respectively ($p < 0.05$). PUFAs D2 group has not given a significant difference in TBARS values. (Figure 4A).

With respect to the GSH/GSSG ratio (Figure 4B), this biomarker showed a drop in 35.7% and 25% in control and PUFA D1 groups, compared with the basal values with respect to the values at the end of reperfusion ($p < 0.05$), respectively. The groups that were supplemented with PUFAs D2 did not show a significant difference in the basal GSH/GSSG ratio and values at the end of reperfusion. On the other hand, the GSH/GSSG ratio was a 30.1% and 76.7% higher in PUFA D1 and D2 groups at the end of reperfusion, compared with basal values, respectively ($p < 0.05$).

The activities of antioxidant enzymes SOD, CAT and GSH-Px in the three study groups were shown in Figure 5. The activity of SOD (Figure 5A) was 42.7% and 27.8 % lower in control and PUFAs D1 groups at the end of reperfusion, compared with the basal values of activities ($p < 0.05$). There are no significant differences between basal and in PUFA D2 at the end of reperfusion. The basal SOD activity in PUFA D1 and D2 groups with respect to the control rats was 35.3% and 80.5% higher values, respectively ($p < 0.01$). Similar values of CAT and GSH-Px activity at basal condition were show in Figure 5B and C. A significant increase of over 200% of the activity values is present in groups receiving PUFAs D2 compared with D1 and control rats, respectively ($p < 0.01$).

DISCUSSION

This study confirms that ischaemia reperfusion induced in *ex vivo* model is associated with oxidative and functional damage in cardiac tissue. The omega 3 supplementation protects the heart against IR injury, which may be mediated by a reinforcement of the myocardial antioxidant defence system.

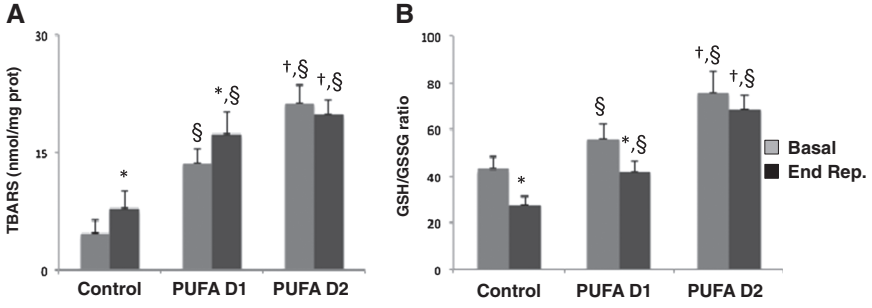

Figure 4. Lipid peroxidation (A) and reduced glutathione/oxidized glutathione ratio (B) in rats supplemented with polyunsaturated fatty acids (PUFAs) D1 (0.6 g kg^{-1} d^{-1}) and D2 (1.2 g kg^{-1} d^{-1}) and control (without PUFA) at basal condition and at the end of reperfusion (120 min). Values are expressed as mean ± SD ($n = 7$ per group). Significant differences: *$p < 0.05$ versus basal; †$p < 0.05$ versus PUFAs D1; § versus control

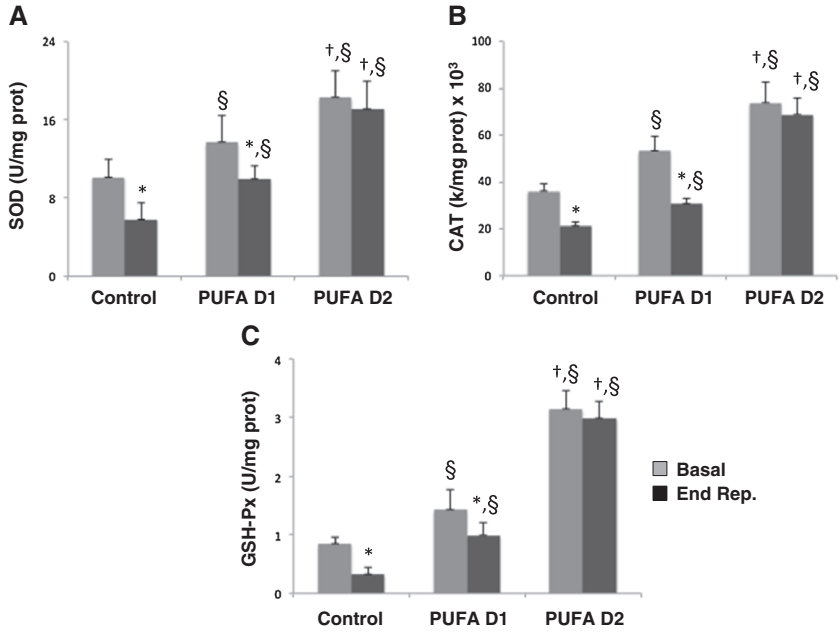

Figure 5. Activities of superoxide dismutase (A), catalase (B) and GSH-Px (C) in rats supplemented with reduced glutathione/oxidized glutathione (PUFAs) D1 (0.6 g kg^{-1} d^{-1}) and D2 (1.2 g kg^{-1} d^{-1}) and control (without PUFA) at basal condition and at the end of reperfusion (120 min). Values are expressed as mean ± SD ($n = 7$ per group). Significant differences: *$p < 0.05$ versus basal; †$p < 0.05$ versus PUFAs D1; § versus control

Ischaemic heart disease, secondary to acute myocardial infarction, is among the most prevalent health problems in the world and is a major cause of morbidity and mortality. Two major types of myocardial injury occur following myocardial reperfusion: (1) stunning – manifested by reversible contractile dysfunction and (2) infarction – myocardial challenges account for up to 50% of IS. In addition, reperfusion is associated with potentially lethal arrhythmias that are rapidly and predictably induced within seconds of the onset of reflow.[17] Considerable evidence attributes at ROS and reactive nitrogen species, produced either by the myocardium itself or by infiltrating inflammatory cells, as an early event in this process. Once ROS is produced, it can lead to cellular damage through a number of pathways including direct damage to membranes and proteins or indirect damage through the activation of pro-apoptotic pathways.[18] Increase of biomarkers of oxidative stress such as lipid peroxidation products in IR hearts are mainly due to the increased oxidative stress. With respect to TBARS levels, these compounds are associated with the growing evidence that IR-mediated oxidative stress shunts survival signalling pathways.[19] These findings are in agreement with higher lipid peroxidation products such as TBARS and drop in antioxidant compounds as lower levels of GSH/GSSH ratio in control group, which did not receive supplementation with PUFAs (Figure 4). Morphological and biochemical changes in ischaemic myocardium occur rapidly, but they may be considered reversible if reperfusion of the tissue can be effected promptly. However, ischaemia lasting more than 30 min results in a state of irreversible injury that ultimately leads to cell death.[20] Furthermore, it is important to allow a differentiation between partial ischaemia, which may be reversible, and total obstruction. The factors that influence the onset and extent of irreversible injury include the size of the area at risk, the extent of collateral blood flow or residual flow through the infarct-related artery, the duration of ischaemia and myocardial temperature.[21] In our work, the IS following IR in control rats is in agreement with other models of global ischaemia and the effects of protocols that used cardiac pharmacological preconditioning (Figure 2).[22,23]

Glutathione is the primary intracellular redox antioxidant. Some reports show that atrial glutathione content is lower in heart samples from rats subjected to regional and global ischaemia.[24] Moreover, the drop of GSH levels is in agreement with the occurrence of IR cycle, an increased oxidative stress and decreased cardiac contractile function.[25] The finding of these settings is in agreement with the glutathione depletion of the present study at basal time in the control rats. In contrast, the groups that received PUFAs have glutathione repletion associated with higher activity of antioxidant enzymes (Figures 4B and 5).

On the basis of the results from cellular and molecular studies, the cardioprotective effects of supplementation with PUFAs appear not to be due through a single mode of action but to a synergism between multiple mechanism that involve the improvement of cardiac haemodynamic factors, such as blood pressure, left ventricular diastolic filling, HR and endothelial function.[26] Indeed, the molecular effects included proresolving lipid mediators, modulation of cardiac ion channels and downstream cell signalling pathways and synthesis gene expression.[27] Eicosapentaenoic acid and DHA have similar yet very distinctive cardioprotective properties. This aspect is in agreement with the ratio of DHA:EPA used in our protocol. In the case of DHA seems to decrease blood

pressure, HR and the number of total and small dense LDL particles. In this view, lipid-lowering effects are shown after 8 weeks of supplementation in both groups (Table 2); they are in agreement with other studies, which used chronic PUFA administration.[28,29] These effects may be linked to their well-known roles in lowering triglyceride synthesis, very LDL assembly, secretion and in altering the expression of genes involved in lipid and glucose metabolism, as well as adipogenesis via peroxisome proliferator-activated receptors.[30] Moreover, DHA also has higher potency to regulate the activity of several transcriptional factors than EPA.[31] Scientific knowledge regarding the cardioprotective benefits of n-3 PUFA has been translated into nutritional guidelines for improving cardiovascular health, and it has to be used for the optimization of the management and resolution of other chronic diseases.

In relation to haemodynamic and structural parameters, other studies have not found differences in the left ventricular function after ischaemia reperfusion with PUFA supplementation, despite differences in IS.[32] In our study, and because of more severe ischaemia, resistant hearts (with smaller IS) may have developed a kind of myocardial 'hibernation' that may have masked a better recovery of IR function compared with the less resistant hearts with a higher infarct volume. The degree of myocardial stunning, in addition to the duration and severity of reperfusion-induced tachyarrhythmias, was attenuated in hearts of PUFAs groups, compared with the control heart (data not shown).

With respect to the antioxidant properties of PUFAs, these effects are mainly associated with the incorporation into membrane and the antioxidant enzyme activity modulation on different cellular levels. The enhancement of antioxidant defences has been reported in rats supplemented with fish oil rich in n-3 PUFAs. The improvement was revealed by the reduction of plasma lipid peroxidation and the increase in the activity of antioxidant enzymes such as SOD, GSH-Px and xanthine oxidase.[33] In our model, it suggested that n-3 PUFAs could contribute to enhance the cardiac antioxidant status and functional haemodynamic parameters due to long-term effects (Figures 3 and 5). This mechanism is associated with the activation of genomic pathways. The activation of the antioxidant response elements is trigger primarily by the binding of a transcription factor called nuclear factor (erythroid-derived 2)-like 2 (Nrf2). This mechanism is involved in the antioxidant response characterized by an increase in the transcription of phase II detoxification proteins and enzymes for glutathione biosynthesis, such as γ-glutamyl-cysteinyl ligase, glutathione reductase and other antioxidant enzymes. Furthermore, in a pro-oxidant environment, oxidized n-3 PUFA reacted directly with the negative regulator of Nrf2, Keap1 thus initiating a Keap1 dissociation, thereby inducing Nrf2-directed gene expression.[34] Under this background, the increase of expression antioxidant enzymes is described by Jahangiri *et al*.[35] in rats supplemented with a diet rich in omega 3 and in some models of diabetic rats.[4]

In other view, the effects of PUFAs depend on the changes in the properties of the cell membrane as a result of omega 3 incorporation. The type and amount of fatty acids can alter the content of the membrane phospholipids and directly affect cell membrane properties, such as fluidity.[36] Recently, the incorporation of PUFAs can lead to protein functionality and microdomain localization of signalling proteins thus, resulting in the modulation of downstream cellular signalling pathways.[27,37] The probable mechanisms included the reduction of electrical excitability and activity of voltage-dependent Na^+ channels, modulatory action of L-type Ca^+ channels.[38] Indeed, the rapid incorporation of PUFAs such as DHA in cardiac membranes can induce a lower availability of arachidonic acid and pro-inflammatory and pro-oxidant signalling pathways during a period of ischaemic stress.[3,39]

Our data show that the TBARS levels, as index of lipid peroxidation, are higher in isolated hearts samples at the baseline condition than in control levels. In this case, TBARS levels serve as a measurement of the incorporation of PUFAs into the membrane. On the other hand, at the end of IR, we did not observe differences in lipid peroxidation, which evidence the protective effects against IR injury (Figure 4A). This protective effect is supported also by maintaining the values of GSH/GSSG ratio (Figure 4B) and the antioxidant enzyme activities at the end of reperfusion (Figure 5A–C) with PUFA D2.

The limitations in our protocol are dependent of the variability compared with other designs and techniques used. Some studies did not focus in the effects of the global IR, whereas others examine the effect of regional ischaemia and the generation of ventricular arrhythmias and the type of cell death.[3,40] Moreover, other schemes did not assess the effect on IS, because the duration of ischaemia was too short to induce a measurable necrosis area.[41,42] Others, did not show the standard diet or the biochemical changes induced by the tested diets (Table 1).[43,44]

It can be concluded that the antioxidant reinforcement induced by PUFAs supplementation reduced IS, and improved

Table 2. Body weight and blood lipids in groups at 8 weeks

Groups	Control ($n=7$)	After supplementation PUFAs D1($n=7$)	PUFAs D2 ($n=7$)
Body weight, (g)	2.76 ± 1.1	2.72 ± 0.3	2.77 ± 0.8
Fluid consumption (ml day^{-1} 100 g BW)	9.9 ± 1.4	9.2 ± 1.6	8.97 ± 1.3
Energy consumption (kcal day^{-1} 100 g BW)	20.8 ± 2.5	21.6 ± 1.4	21.1 ± 2.1
Blood lipids (mmol L^{-1})			
Total cholesterol	1.45 ± 0.32	1.29 ± 0.32	1.20 ± 0.30
Triglycerides	0.81 ± 0.21	0.51 ± 0.20*	0.48 ± 0.12*
LDL cholesterol	0.47 ± 0.05	0.39 ± 0.07	0.31 ± 0.08
HDL cholesterol	0.63 ± 0.31	0.58 ± 0.09	0.56 ± 0.07
VLDL cholesterol	0.37 ± 0.07	0.23 ± 0.08*	0.22 ± 0.06*

BW, body weight; LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; PUFA, polyunsaturated fatty acids; D1, dose 1 (0.6 g kg^{-1} d^{-1}); D2, dose 2 (1.2 g kg^{-1} d^{-1}).
Variables are expressed as mean ± SD significant differences.
*$p < 0.05$ versus basal.

cardiac functional parameters in a dose-dependent manner. It is suggested that this design could be actively tested in further basic research to determine the molecular pathways for these effects.

CONFLICT OF INTEREST

None of the authors or cooperative members has a proprietary, commercial or other financial interest in any study, procedure or result.

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REFERENCES

1. Castillo R, Rodrigo R, Perez F, *et al*. Antioxidant therapy reduces oxidative and inflammatory tissue damage in patients subjected to cardiac surgery with extracorporeal circulation. *Basic Clin Pharmacol Toxicol* 2011; **108**: 256–262.
2. Masson S, Marchioli R, Mozaffarian D, *et al*. Plasma n-3 polyunsaturated fatty acids in chronic heart failure in the GISSI-Heart Failure Trial: relation with fish intake, circulating biomarkers, and mortality. *Am Heart J* 2013; **165**: 208–215.e4. DOI: 10.1016/j.ahj.2012.10.021
3. Zeghichi-Hamri S, de Lorgeril M, Salen P, *et al*. Protective effect of dietary n-3 polyunsaturated fatty acids on myocardial resistance to ischemia-reperfusion injury in rats. *Nutr Res* 2010; **30**: 849–857. DOI:10.1016/j.nutres.2010.10.010.
4. Xie N, Zhang W, Li J, *et al*. α-Linolenic acid intake attenuates myocardial ischemia/reperfusion injury through anti-inflammatory and anti-oxidative stress effects in diabetic but not normal rats. *Arch Med Res* 2011; **42**: 171–81. DOI:10.1016/j.arcmed.2011.04.008.
5. Rodrigo R, Prieto JC, Castillo R. Cardioprotection against ischemia-reperfusion by vitamins C and E plus n-3 fatty acids: molecular mechanisms and potential clinical application. *Clin Sci (Lond)* 2013; **124**: 1–15.
6. Rizos EC, Ntzani EE, Bik E, Kostapanos MS, Elisaf MS. Association between omega-3 fatty acid supplementation and risk of major cardiovascular disease events: a systematic review and meta-analysis. *JAMA* 2012; **308**: 1024–33.
7. Khairallah RJ, Kim J, O'Shea KM, *et al*. Improved mitochondrial function with diet-induced increase in either docosahexaenoic acid or arachidonic acid in membrane phospholipids. *PLoS One* 2012; **7**: e34402. DOI:10.1371/journal.pone.0034402.
8. Gong P, Hu B, Cederbaum AI. Diallyl sulfide induces heme oxygenase-1 through MAPK pathway. *Arch Biochem Biophys* 2004; **432**: 252–60.
9. Rondeau I, Picard S, Bah TM, Roy L, Godbout R, Rousseau G. Effects of different dietary omega-6/3 polyunsaturated fatty acids ratios on infarct size and the limbic system after myocardial infarction. *Can J Physiol Pharmacol* 2011; **89**: 169–76.
10. Araya J, Rodrigo R, Orellana M, Rivera G. Red wine raises plasma HDL and preserves long-chain polyunsaturated fatty acids in rat kidney and erythrocytes. *Br J Nutr* 2001; **86**: 189–195.
11. Collins TJ. ImageJ for microscopy. *Biotechniques* 2007; **43**: 25–30.
12. Hissin PJ, Hilf R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal Biochem* 1976; **74**: 214–226.
13. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; **95**: 351–358.
14. Nebot C, Moutet M, Huet P, Xu J, Yadan JC, Chaudiere J. Spectrophotometric assay of superoxide dismutase activity based on the activated autoxidation of a tetracyclic catechol. *Anal Biochem* 1993; **14**: 442–51.
15. Aebi H. *Catalase, Methods in Enzymatic Analysis*, Bergmeyer HU (ed.). Academic Press: New York, 1974; 673–677.
16. Flohé L, Günzler WA. Assays of glutathione peroxidase. *Methods Enzymol* 1984; **105**: 114–121.
17. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. *N Engl J Med* 2007; **357**: 1121–1135.
18. Vanden Hoek TL, Li C, Shao Z, Schumacker PT, Becker LB. Significant levels of oxidants are generated by isolated cardiomyocytes during ischemia prior to reperfusion. *J Mol Cell Cardiol* 1997; **29**: 2571–2583.
19. Zhang Y, Sano M, Shinmura K, *et al*. 4-hydroxy-2-nonenal protects against cardiac ischemia-reperfusion injury via the Nrf2-dependent pathway. *J Mol Cell Cardiol* 2010; **49**: 576–586. DOI:10.1016/j.yjmcc.2010.05.011.
20. Inserte J, Barrabés JA, Hernando V, Garcia-Dorado D. Orphan targets for reperfusion injury. *Cardiovasc Res* 2009; **83**: 169–178. DOI:10.1093/cvr/cvp109.
21. Ferdinandy P, Schulz R, Baxter GF. Interaction of cardiovascular risk factors with myocardial ischemia/reperfusion injury, preconditioning, and postconditioning. *Pharmacol Rev* 2007; **59**: 418–458.
22. Adluri RS, Thirunavukkarasu M, Zhan L, *et al*. Cardioprotective efficacy of a novel antioxidant mix vitaepro against ex vivo myocardial ischemia-reperfusion injury. *Cell Biochem Biophys* 2011. [Epub ahead of print]. DOI: 10.1007/s12013-011-9300-7
23. Ibacache M, Sanchez G, Pedrozo Z, *et al*. Dexmedetomidine preconditioning activates pro-survival kinases and attenuates regional ischemia/reperfusion injury in rat heart. *Biochim Biophys Acta* 1822; **2012**: 537–545. DOI:10.1016/j.bbadis.2011.12.013.
24. Ahmed LA, Salem HA, Attia AS, Agha AM. Comparative study of the cardioprotective effects of local and remote preconditioning in ischemia/reperfusion injury. *Life Sci* 2012; **90**: 249–256. DOI:10.1016/j.lfs.2011.11.007.
25. Carnes CA, Janssen PM, Ruehr ML, *et al*. Atrial glutathione content, calcium current, and contractility. *J Biol Chem* 2007; **282**: 28063–28073.
26. Mozaffarian D, Willett WC. Trans fatty acids and cardiovascular risk: a unique cardiometabolic imprint? *Curr Atheroscler Rep* 2007; **9**: 486–493.
27. Adkins Y, Kelley DS. Mechanisms underlying the cardioprotective effects of omega-3 polyunsaturated fatty acids. *J Nutr Biochem* 2010; **21**: 781–792. DOI:10.1016/j.jnutbio.2009.12.004.
28. Cummings BP, Stanhope KL, Graham JL, Griffen SC, Havel PJ. Supplementation with EPA or fish oil for 11 months lowers circulating lipids, but does not delay the onset of diabetes in UC Davis-type 2 diabetes mellitus rats. *Br J Nutr* 2010; **104**: 1628–1634. DOI:10.1017/S0007114510002655.
29. Karsenty J, Landrier JF, Rousseau-Ralliard D, *et al*. Beneficial effects of omega-3 fatty acids on the consequences of a fructose diet are not mediated by PPAR delta or PGC1 alpha. *Eur J Nutr* 2012. [Epub ahead of print]. DOI: 10.1007/s00394-012-0488-0
30. Kaur G, Sinclair AJ, Cameron-Smith D, Barr DP, Molero-Navajas JC, Konstantopoulos N. Docosapentaenoic acid (22:5n-3) down-regulates the expression of genes involved in fat synthesis in liver cells. *Prostaglandins Leukot Essent Fatty Acids* 2011; **85**: 155–161. DOI:10.1016/j.plefa.2011.06.002.
31. Kuang YL, Paulson KE, Lichtenstein AH, Lamon-Fava S. Regulation of the expression of key genes involved in HDL metabolism by unsaturated fatty acids. *Br J Nutr* 2012; **108**: 1351–9. DOI:10.1017/S0007114511006854.
32. Abdukeyum GG, Owen AJ, McLennan PL. Dietary (n-3) long-chain polyunsaturated fatty acids inhibit ischemia and reperfusion arrhythmias and infarction in rat heart not enhanced by ischemic preconditioning. *J Nutr* 2008; **138**: 1902–9.
33. Erdogan H, Fadillioglu E, Ozgocmen S, *et al*. Effect of fish oil supplementation on plasma oxidant/antioxidant status in rats. *Prostaglandins Leukot Essent Fatty Acids* 2004; **71**: 149–152.

34. Gao L, Wang J, Sekhar KR, *et al*. Novel n-3 fatty acid oxidation products activate Nrf2 by destabilizing the association between Keap1 and Cullin3. *J Biol Chem* 2007; **282**: 2529–2537.
35. Jahangiri A, Leifert WR, Kind KL, McMurchie EJ. Dietary fish oil alters cardiomyocyte Ca2+ dynamics and antioxidant status. *Free Radic Biol Med* 2006; **40**: 1592–1602.
36. Leaf A, Xiao YF, Kang JX, Billman GE. Membrane effects of the n-3 fish oil fatty acids, which prevent fatal ventricular arrhythmias. *J Membr Biol* 2005; **206**: 129–39.
37. Chapkin RS, Wang N, Fan YY, Lupton JR, Prior IA. Docosahexaenoic acid alters the size and distribution of cell surface microdomains. *Biochim Biophys Acta* 2008; **1778**: 466–71.
38. Kang JX, Xiao YF, Leaf A. Free, long-chain, polyunsaturated fatty acids reduce membrane electrical excitability in neonatal rat cardiac myocytes. *Proc Natl Acad Sci U S A* 1995; **92**: 3997–4001.
39. Brahmbhatt V, Oliveira M, Briand M, *et al*. Protective effects of dietary EPA and DHA on ischemia-reperfusion-induced intestinal stress. *J Nutr Biochem* 2013; **24**: 104–11.
40. Engelbrecht AM, Engelbrecht P, Genade S, *et al*. Long-chain polyunsaturated fatty acids protect the heart against ischemia/reperfusion-induced injury via a MAPK dependent pathway. *J Mol Cell Cardiol* 2005; **39**: 940–954.
41. Force T, Malis CD, Guerrero JL, *et al*. n-3 fatty acids increase postischemic blood flow but do not reduce myocardial necrosis. *J Physiol* 1989; **257**: H1204–H1210.
42. Pepe S, McLennan PL. Cardiac membrane fatty acid composition modulates myocardial oxygen consumption and postischemic recovery of contractile function. *Circulation* 2002; **105**: 2303–2308.
43. Ogita H, Node K, Asanuma H, *et al*. Eicosapentaenoic acid reduces myocardial injury induced by ischemia and reperfusion in rabbit hearts. *J Cardiovasc Pharmacol* 2003; **41**: 964–969.
44. Ramadeen A, Connelly KA, Leong-Poi H, *et al*. N-3 polyunsaturated fatty acid supplementation does not reduce vulnerability to atrial fibrillation in remodeling atria. *Heart Rhythm* 2012; **9**: 1115–1122. e4. DOI: 10.1016/j.hrthm.2012.02.013