ORIGINAL ARTICLE

Cardioprotective effect of eicosapentaenoic acid during ischemia and reperfusion

Efeito cardioprotetor do ácido eicosapentaenóico durante a isquemia e reperfusão

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Abstract

Objective: In this work, rats isolated hearts were infused EPA before the ischemia period and during reperfusion for available get well in parameter relatives to redox reactions. **Methods**: The effect of EPA was tested on isolated hearts induced to ischemia and reperfusion, treatment occurred at different times (ischemia or reperfusion). Antioxidant capacity against peroxyl radicals, glutathione cysteine ligase activity, glutathione concentration, lactate dehydrogenase, and creatine kinase concentration was analyzed. **Results**: Hearts treated with eicosapentaenoic acid had the minor generation of species reactive oxygen and lipid damage after reperfusion. The GSH concentration was higher when the hearts were treated with eicosapentaenoic acid in the period of reperfusion. **Conclusion**: In conclusion, this study demonstrates that the dose of EPA (20μM) used before ischemia can act as a cardioprotective antioxidant molecule, prevented damage heart from ischemic and reperfusion injury.

Keywords: Heart; Infarction; Myocardium; Omega 3; Oxidative Stress; Ischemia and Reperfusion.

Resumo

Objetivo: Neste trabalho, corações isolados de ratos foram infundidos com EPA antes do período de isquemia e durante a reperfusão para obtenção de melhora em parâmetros relativos às reações redox. **Métodos:** O efeito do EPA foi testado em corações isolados induzidos a isquemia e reperfusão, o tratamento ocorreu em diferentes momentos (isquemia ou reperfusão). A capacidade antioxidante contra os radicais peroxil, atividade da glutationa cisteína ligase, concentração de glutationa, lactato desidrogenase e concentração de creatina quinase foi analisada. **Resultados**: Corações tratados com ácido eicosapentaenóico tiveram a menor geração de espécies reativas de oxigênio e danos lipídicos após a reperfusão. A concentração de GSH foi maior quando os corações foram tratados com ácido eicosapentaenóico no período de reperfusão. **Conclusão:** Em conclusão, este estudo demonstra que a dose de EPA (20μM) utilizada antes da isquemia pode atuar como uma molécula antioxidante cardioprotetora, prevenindo danos ao coração por isquemia e lesão de reperfusão.

Palavras-chave: Coração; Infarte; Myocardio; Omega 3; Estresse Oxidativo; Isquemia e reperfusão.

INTRODUCTION

Myocardial ischemia and reperfusion (IR) is a major contributor to morbidity and mortality in the world[1,2]. The injury induced by myocardial IR may range from a small insult resulting in limited tissue damage to a large one leading to cellular death[3]. The ischemia may be total when the blood flow is insufficient to maintain tissue or cell life, or partial, which maintains cell viability, but with the risk of progression to cell death, depending on the tissue and time[4]. During reperfusion, with the return of mitochondrial activity and the consequent reactivation of the electron transport chain occur a large production of reactive oxygen species (ROS) and reactive nitrogen species occurs[5]. These reactive species, in turn, promote an attack on cardiolipin, a phospholipid located in the internal mitochondrial membrane, altering the membrane fluidity, ionic permeability, structure, and function of the components of the mitochondrial electron transport chain, resulting in reduced efficiency of mitochondrial oxidative phosphorylation[6]. In addition, ROS and reactive nitrogen species also cause the activation of ryanodine receptors, located in the sarcoplasmic reticulum, which culminates in a large release of Ca2+ and further contributes to the overload of this ion in the cytosol[7].

Omega-3 fatty acids like eicosapentaenoic acid (20:5n3 - EPA) and docosahexaenoic acid (22:6n3 - DHA) are polyunsaturated carboxylic acids (PUFA n-3), wherein the double bond is the third carbon from the opposite end to carboxyl[8]. The PUFAs n-3 are not only used in the clinical prevention of death from heart disease but also has a protective role in cardiac physiology, cardiovascular health, and prevention of heart diseases[9]. Among cardioprotective roles, many studies indicate that regular intake of PUFA n-3 improves some risk factors such as blood pressure, platelet reactivity and thrombosis, triglycerides levels, vascular function, arrhythmias, heart rate variability, and inflammation[10, 11, 12, 13]. The daily recommendation is about 1g n-3 PUFAs/day as fish or fish oils as secondary prevention who has a coronary vascular disease; 2 to 4g/day for the treatment of hypertriglyceridemia; and fish oil three times/week (0.5g / day) for healthy individuals[14].

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Most studies that seek to evaluate the effects of PUFAs on ischemia and reperfusion used as a source of omega 3 fish oils or make use of a mixture of EPA and DHA[15,16]. Few articles seek to establish individual relationships of these substances with cardiac damage caused during ischemia and reperfusion. Considering this background, the objective of this study was to evaluate the imbalance of redox owing to acute treatment with eicosapentaenoic acid in different moments of ischemia and reperfusion in an ex vivo model of rat heart.

METHODS AND MATERIALS

Animals

This research protocol was approved by the Animal Care and Ethical Use Committee of the Federal University of Rio Grande (P019/2013), RS, Brazil. Thirty-five male rats (Wistar, weighting 300-400g) were obtained from the Central Animal House of the Federal University of Rio Grande do Sul, RS, Brazil. They were maintained on a 12h light/12h dark cycle in constant temperature 21±1°C and received commercial rodent food (25g/animal/day) and water "*ad libitum*".

Preparation of Eicosapentaioc Acid (EPA) solution stock

To prepare the stock solution cis-[5,8,11,14,17]-Eicosapentaenoic acid (Sigma-Aldrich), 100mg/ml ethanol P.A., remaining at -15°C. Aliquots were used and diluted in Krebs-Heinselet buffer to have a final work solution of 20µM of EPA to treat isolated hearts before the ischemia and during reperfusion.

Ex vivo model of ischemia and reperfusion in isolated heart

Ischemia/Reperfusion was conducted in a Langendorff system[17]. Rats were ended by decapitation and the hearts were removed and immediately the ascending aorta was cannulated. The heart was subjected to retrograde perfusion with Krebs-Henseleit buffer (118mM NaCl, 4.7mM KCl, 1.2mM KH2PO4, 1.2mM MgSO4.7H2O, 25mM NaHCO3, 2.5mM CaCl2, and 11.1mM Glucose) equilibrated with 95% oxygen and 5% carbon dioxide to a pH of 7.4. Non-recycling perfusion was initiated at a constant flow rate of 10ml/min with 90mmHg of basal pressure, at 37°C. The perfusion buffer was previously filtered through a 0.22µm membrane to remove any aggregated particles. Hearts were perfused with Krebs buffer for 10 min (stabilization period), then underwent a period of global ischemia (15 min), followed by 15 min of reperfusion.

The isolated heart submitted to Langendorff preparation and after 10 minutes of stabilization was divided into the following groups (n = 7 / group): C - control (perfusion for 45 minutes), IR - ischemia and reperfusion (15 minutes of stabilization, 15 minutes of ischemia and sequentially 15 minutes of reperfusion), Epa - eicosapentaenoic acid (15 minutes of stabilization after infusion with EPA for another 15 minutes followed by 15 minutes of infusion), treatment with Eicosapentaenoic acid EpaIR immediately before ischemia and reperfusion (perfusion) with EPA for 15 minutes followed by 15 minutes of ischemia and 15 minutes of reperfusion) and IEpa-R (15 minutes of stabilization followed by 15 minutes of ischemia and 15 minutes of reperfusion with EPA) (for details, see Table 1).

Table 1. Experimental design. Eicosapentaenoic acid (EPA) treatment in ex vivo heart during ischemia and reperfusion.

Groups	Stabilization period 15 min	Ischemia period 15 min	Reperfusion period 15 min
Control	Buffer infusion	Buffer infusion	Buffer infusion
I/R	Buffer infusion	Ischemia	Buffer infusion
Epa	Buffer infusion	Epa infusion	Buffer infusion
EpaIR	Epa infusion	Ischemia	Buffer infusion
IEpaR	Buffer infusion	Ischemia	Epa infusion

Measurement of lactate dehydrogenase (LDH) and creatine kinase (CK) enzymes activities

For the determination of the activity of these enzymes, the perfusate was collected during the reperfusion period, then the kit was used to determine LDH activity (LabTeste) and Creatine kinase (CK) levels (from the CK-MB Liquiform-Labtest kit) at 340 nm in a spectrophotometer according to the manufacturer's instructions.

The amount of CK produced was expressed as U / L tissue and the factor of 8121 was used for the calculation.

Measurement of total antioxidant capacity (ACAP)

Total antioxidant competence against peroxyl radicals was analyzed through ROS determination in hearts treated or not with a peroxyl radical generator. Peroxyl radicals were produced by thermal (35oC) decomposition of 2, 2´-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4mM; Aldrich) e. Readings were carried through in a fluorescence microplate reader (Victor 2 Perkin), in a medium containing 30 mM HEPES (pH 7.2), 200 mM KCl, 1 mM $MgCl_2$, 40 µM DCF-DA. The results were expressed as area difference of FU x min in the same sample with and without ABAP addition and standardized to the ROS area without ABAP (background area)[18].

Glutamate cysteine ligase activity and glutathione concentration

Glutamate cysteine ligase (GCL) activity and baseline glutathione (GSH) level were determined based on the reaction of naphthalenedicarboxialdehyde (NDA) with GSH or γ-glutamylcysteine (γ-GC) to form cyclized products that are highly fluorescent. Briefly, samples for GCL activities and GSH levels were kept on ice until transferred, at 15 s time intervals, to the corresponding 96-well reaction late (25°C) containing

Fonte: Dados da pesquisa, 2019.

25µL of the reaction solution. After a pre-incubation of 5 min, the GCL reaction was initiated by adding 25µL of cysteine (2mM). The plate was incubated for 60 min and the reaction stopped by adding 25µL of 5-sulfosalicylic acid (SSA, 200mM). The plate was then centrifuged for 5 min at 2.000 G and 20µL aliquots of supernatant were transferred to a new 96-well plate. GSH reaction was initiated by adding 180µL of naphthalene carboxaldehyde (NDA). NDA-GSH and NDA-GCL fluorescence intensities were measured on a fluorescence microplate reader at 450 nm excitation/535 nm emission, respectively (FilterMax F5, Molecular Devices).

Statistical Analysis

Data were expressed as mean ± S.E. ANOVA assumptions of normality and homoscedasticity were tested by Kolmogorov-Smirnov and Levene´s tests, respectively, and when they were not reached, data were mathematical transformed (square root) and performed by one-way ANOVA or were tested by non-parametric analysis of variance of Kruskal-Wallis. Significant differences were tested by a posteriori Tukey HSD test. Statistical significance was accepted if p<0.05.

RESULTS

Lactate dehydrogenase (LDH) activity and creatine kinase levels (CK)

The EpaIR group had a minor activity LDH and CK when compared to the IR group (p <0.05) (Figure 1). The treatment with EPA during reperfusion did not show significant alterations in activity LDH and CK in relation to IR (p>0.05). (Figure 1).

Total antioxidant capacity

In respect of total antioxidant capacity against peroxyl radicals (ACAP), it was observed that isolated hearts did show significant differences between Control and Epa groups (p>0.05, Figure 2). However, the IR group demonstrates a depletion in antioxidant capacity when compared to the control (p <0.05). When Epa treatment was administered in the pre-ischemic period it was also observed a decrease in ACAP (p <0.05). When Epa was administered during reperfusion no change in the myocardium antioxidant capacity was observed (Figure 2).

Glutamate cysteine ligase activity and glutathione concentration

The GCL activity does not show a significant difference between groups control, IR, Epa, and Epa PI. However, an increase was observed in EPA R when compared to control and IR p<0.05). The GSH concentration was not different among control, IR, Epa, and EpaPI groups (Figure 3B), but when compared with other groups, the EpaR presented a higher GSH concentration (Figure 3B) (p<0.05).

Figure 1. The activity LDH (A) and CK (B) in perfusate after reperfusion treated with Eicosapentaenoic acid (Epa). Figure A. LDH activity in control hearts (Control), comparing to ischemia/reperfusion (IR) and Epa treated with Epa before ischemia (EpaIR) and during reperfusion (IEpaR) related to IR. Figure B. CK activity in control hearts (control), comparing to ischemia/reperfusion (IR) and Epa treated with Epa before ischemia (EpaIR) and during reperfusion (IEpaR) related to IR. Different letters mean statistically significant differences (p <0.05).

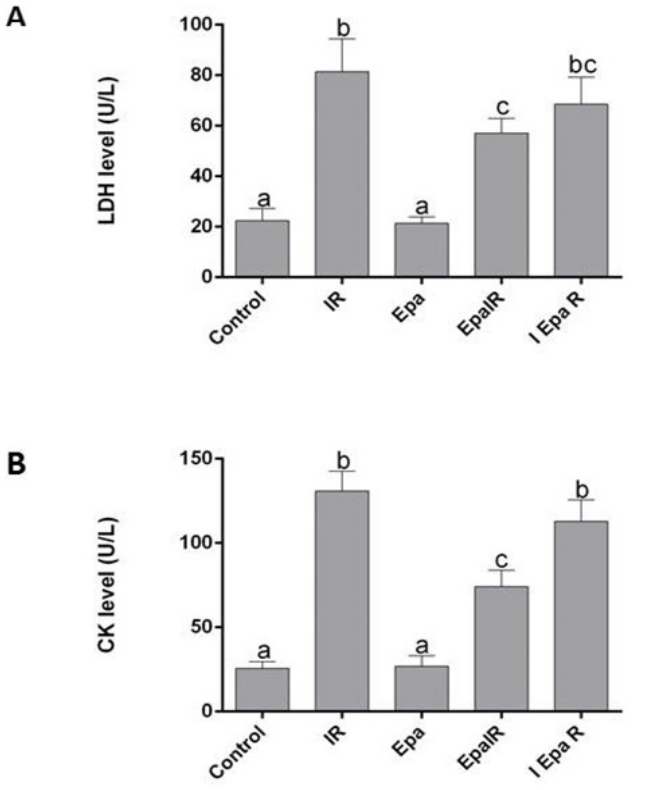


Figure 2. Antioxidant capacity against peroxyl radical (ACAP) from isolated hearts during ischemia and reperfusion treated with Eicosapentaoic acid (Epa). ACAP in control hearts (Control), comparing to ischemia/reperfusion (IR) and Epa, antioxidant capacity with Epa treatment before ischemia (EpaIR) and during reperfusion (IEpaR) in relation to IR. Different letters mean statistically significant differences (p<0.05).

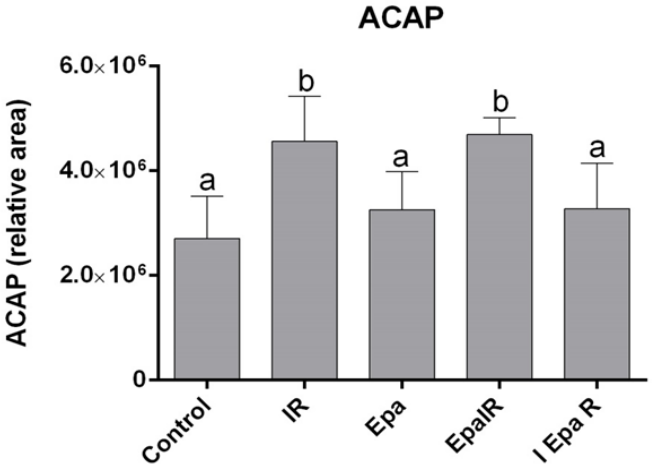
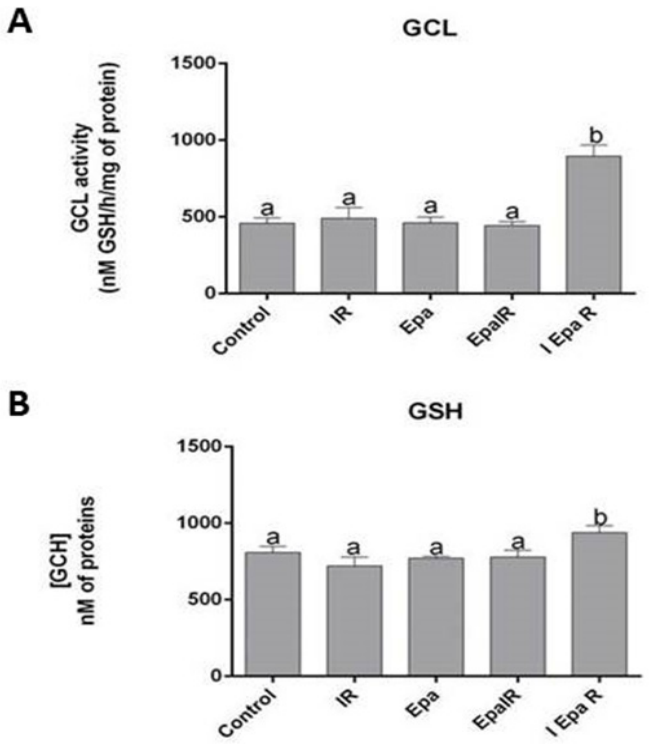


Figure 3. Glutamate cysteine ligase activity and glutathione concentration in isolated hearts during ischemia and reperfusion treated with Eicosapentaoic acid (Epa). Figure A. GCL activity in control hearts (Control), comparing to ischemia/reperfusion (IR) and Epa, GCL activity in hearts treated with Epa before ischemia (EpaIR) and during reperfusion (IEpaR) related to IR. Figure B. GSH concentration in control hearts (control), comparing to ischemia/reperfusion (IR) and Epa, GSH concentration in hearts treated with Epa before ischemia (EpaIR) and during reperfusion (IEpaR) related to IR. Different letters mean statistically significant differences (p<0,05).



DISCUSSION

Diets rich in n-3 polyunsaturated fatty acids are considered effective in preventing many diseases[19,20]. However, its susceptibility to oxidation of free radicals was directly correlated to the degree of unsaturation of the molecule, but it is not common to know whether or which PUFA has pro or anti-oxidant effects[20]. Fatty acids linoleic acid (LA), alpha-linolenic acid (ALA), arachidonic acid (ARA), EPA, and DHA (60 µM) compared to each other, in a study with HepG2 (human hepatoma cells) demonstrate a reduction in cell viability (except for DHA). Increased activity of antioxidant enzymes (SOD, glutathione s-transferase, and glutathione peroxidase) by DHA, LA, and ALA, and high levels of glutathione (by DHA and ALA) have also been observed, indicating a related pro-oxidant or differential antioxidant capacity to fatty acid type[20]. None of the tested fatty acids modified the cell's total antioxidant activity.

High levels of oxidative stress have been observed in rats with intracerebral hemorrhagic stroke, along with decreased superoxide dismutase activity and glutathione concentration and increased lipid peroxidation, when supplemented with 1% EPA and DHA of total energy consumption[21]. In addition, increased consumption of fish oil produced increased lipid peroxidation in adult males with diet-rich salmon[22] and increased oxidative DNA damage in rat hepatocytes[23]. In contrast, our results suggest that treatment with EPA during reperfusion managed to have a protective effect since it kept the ACAP values equal to the control group. Treatment with EPA (20µM) during reperfusion also caused an increase in glutathione content. Both results confirm a positive EPA cardioprotective action.

When myocardial cells, containing LDH and CK-MB are damaged due to deficient oxygen supply or glucose, the cell membrane becomes permeable or may rupture, which results in the leakage of enzymes. Richard et al.[15] reported a reduction in lipid peroxidation and infarct size and CK activity when the isolated heart was pre-treated and pre and pos-treated with DHA. In the present study, the administration of EPA (20µM) before IR (EpaIR Group) significantly decreased LDH and CK levels.

Literature has a lot of examples of similar antioxidant effects from polyunsaturated fatty acids in different target tissues, generally administered as a mixture of PUFAs n-3, and from natural or synthetic sources. Working with chronic treatment using DHA and EPA mixture during renal ischemia and reperfusion in rats, a decrease in the lipid peroxidation and increase in the activity of catalase (CAT) and superoxide dismutase (SOD) was verified[24]. There are pieces of evidence that dietary fish oil may decrease brain stroke volume by means of increase the activity of antioxidant enzymes and reduce lipoperoxidation25. Few articles present effects of isolated fatty acids in myocardium infarction26 or endothelial cells[33, 34]. Infusion of DHA isolated protects against myocardial infarction in a study with a similar methodology[26]. This research also utilizing isolated rat heart preparation, tested DHA (2.5µM) 10 min before a protocol of 20 min of global ischemia and 30min of reperfusion or infusion DHA 10 min pre-ischemia plus another infusion of 10 min after ischemia. These authors observed that DHA lowered oxygen and ATP myocardium consumption, reduced infarct size, release creatine kinase, and diminished lipid peroxidation[26]. Fish oil consumption has been indicated because of its proprieties to stimulate neovasculogenesis, mainly through the effects of EPA, exerting prevention to ischemic injury, as saw in vitro human endothelial progenitor cells[27]. Chronic treatment of human coronary endothelial cells with low physiological concentrations of DHA enhanced endothelial nitric oxide synthase and Akt activity, and increase de NO bioavailability, improving endothelial function28. This work predicts that only Epa, especially when administered before ischemia or during reperfusion, acts as an antioxidant molecule, reducing oxidative damage in the isolated heart of rats.

CONCLUSION

The present ex vivo IR protocol did not activate the ACAP system, or the GCL-GSH cycle, in addition to increasing CK and

LDH levels, a classic change in infarction.

Acute treatment with EPA alone (20 μM) was first tested in isolated IR hearts, and the results indicate that pre-ischemia and during reperfusion treatment can reduce cardiac damage. These findings indicate that the accuracy of acute therapy depends on the time of administration. Our hypothesis is that higher doses/treatment time during reperfusion should be necessary to protect the heart from damage to IR. In conclusion, the time, dose, and the most effective time for cardioprotection in the isolated heart of rats were determined as 20 μM eicosapentaenoic acid, delivered fifty minutes before the ischemia and reperfusion induction.

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