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Acute treatment with copoazú fermented extract ameliorates myocardial ischemia-reperfusion injury via eNOS activation

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ABSTRACT

Our aim was to examine the effects of aqueous extracts of fermented (CFE) and non-fermented (CNFE) ¨copoazú (fruit of *Theobroma grandiflorum*) seeds against ischemia-reperfusion injury. Isolated rat hearts were submitted to 30 min of global ischemia (GI) and 60 min of reperfusion (R). Other hearts received CFE or CNFE in absence or in presence of L-NAME (a nitric oxide synthase inhibitor). Infarct size (IS) and post-ischemic myocardial function (PMF) were measured. Lipid peroxidation, reduced glutathione (GSH) and the expression of phosphorylated forms of eNOS, Akt, GSK-3β and PKCε were assessed. The response of isolated mitochondria to Ca^{2+} (MR) was also determined. CFE but not CNFE decreased IS, increased PMF and the expression of P-eNOS, P-Akt, P-GSK-3β and P-PKCε, partially preserved GSH and improved MR. These effects were lost in presence of L-NAME.

These data demonstrate that the acute treatment with CFE protects the heart against ischemia-reperfusion damage through NOS-dependent mechanisms.

1. Introduction

Cardiovascular diseases and ischemic heart disease in particular, remain the leading cause of death and a significant cause of long-term morbidity worldwide. Although reperfusion is an absolute criterion for the survival of ischemic tissues, the notion has developed that it is not without hazard, and reperfusion-induced pathological changes may occur and further aggravate the previously ischemia-induced damage. An inadequate perfusion of myocardium despite removal of coronary artery occlusion, defined as ¨no-reflow phenomenoñ, is one of the consequences to revascularization that causes left ventricular dysfunction, and even cardiac death (Chan et al., 2012).

Oxidative stress- imbalance of reactive oxygen species (ROS) production and antioxidants in favor of the former- and calcium overload appear as the most important contributors to the reperfusion injury (Das, 2001). Both factors impact on mitochondria promoting the formation and/or opening of mitochondrial permeability transition pore (mPTP) leading to cell death (Halestrap, Clarke, & Javadov, 2004).

The relationship between dietary factors and coronary heart disease has been and remains a matter of intense study and debate. Several studies show that an increase in fruit and vegetable consumption promotes cardiovascular health and reduces the ischemic stroke, the ischemic heart disease mortality and cardiovascular events (Gardener et al., 2011; Hlebowicz et al., 2013).

At experimental level, much attention has been focused on the beneficial actions against ischemia-reperfusion alterations achieved by plant extracts containing the polyphenols as predominant ingredients (Ma et al., 2013; Sakanashi et al., 2013; Saravanan, Ponmurugan, Sathiyavathi, Vadivukkarasi, & Sengottuvelu, 2013). In this sense, we recently showed that the acute treatment with an extract of *Teobroma cocoa* (González Arbeláez et al., 2016) was able to limit the infarct size and mPTP opening through nitric oxide synthase (NOS) activation by reperfusion injury salvage kinases (RISK) pathways.

Theobroma grandiflorum is specie from the Amazon region that belongs to the Malvaceae family, as the cocoa tree (*Theobroma cacao*). The fruit pulp is used in the industry for candy, ice cream, liquor, and juice production while the seeds are used for the manufacturing of a product similar to chocolate called ¨cupulatë.

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Phytochemical studies show that the seeds and pulp of ¨copoazú¨ contain flavone, flavan-3-ols and proanthocyanidin as principal polyphenols (Pugliese, Tomas-Barberan, Truchado, & Genovese, 2013; Yang et al., 2003). There are scarce studies about ¨copoazú¨ and these are mainly focused on the fruit pulp (Gonçalves, Lajolo, & Genovese, 2010). Thus, it was previously demonstrated that daily administration of ¨copoazú¨ liquors to diabetic rats ameliorated body weight gain, improved the plasma antioxidant capacity and reduced lipid peroxidation (Pinent et al., 2016). Recently, the antioxidant effects of a proanthocyanidin-rich extract from ¨copoazú¨ seeds in intestinal cells were shown (de Oliveira & Genovese, 2013). However, the potential benefits against ischemia-reperfusion injury of the chocolate-like product obtained from ¨copoazú¨ seeds have not yet been explored.

The present study was designed to (i) evaluate, in isolated and Langendorff perfused rat heart, the effects of ¨copoazú¨ seeds aqueous extracts obtained at time 0 and after 10 days of traditional fermentation, against ischemia-reperfusion injury and (ii) identify the intracellular cascades responsible for the cardiac effects achieved by the extracts.

2. Materials and methods

2.1. Plant material

The fruit of *Teobroma grandiflorum* (copoazú), constitutes the material used for the fermentation in this research. Mature fruit were collected at the Center for Research of University of Amazonia CIMAZ-Macagual (voucher specimen number HUAZ 9030) located in the department of Caquetá, Colombia.

2.2. Fermentation process

The maceration was performed in the traditional fashion, punching 800 grams of ¨copoazú¨ seeds with 100% of pulp and the fermentation was carried out using baskets placed in the form of a ladder. The material was transferred from one basket to another and this removal was performed every 24 h after the start of the process for 10 days. After that the seeds were dried, toasted and crushed (Krysiak, 2006).

2.3. Preparation of ¨copoazú¨ extracts

50 g of ¨copoazú¨ seeds obtained at 0 (CNFE) and 10 days (CFE) of fermentation process were solubilized in aqua and cooked for 20 min. Both aqueous extracts were filtered, lyophilized, and maintained at −30 °C until used.

2.4. HPLC analysis

HPLC-DAD analysis was performed on a Shimadzu LC-2010HT system equipped with a pump LC-2010AHT, UV Detector LC-2010AHT, autosampler LC2010 HT, and column RP-C18 (150 × 3 mm, 4 μm, Restek-Pinnacle). The data were collected and processed with the software LC- Solutions (Shimadzu). The experimental protocol was suggested by Hatzidimitriou et al. (2007) with some modifications. The eluting system used was: 90% solvent A (water with 2% acetic acid, v/v) and 10% solvent B (acetonitrile) in isocratic conditions. Peak identification was based on retention times and spiking with standard solutions. Seven-point calibration curves of theobromine, caffeine, (+)-catechin and (−)-epicatechin were used for quantification. The content of these compounds was expressed as mg/100 g of dry weight of the extract.

2.5. Determination of total phenols

Total phenolic content was measured by using the Folin-Ciocalteu method. Results were expressed as mg gallic acid equivalents/100 g of dry weight of the extract (Singleton & Rossi, 1965).

2.6. Total antioxidant activity

2.6.1. Ferric reducing-antioxidant power (FRAP)

The FRAP assay was carried out to determine the reducing ability of the extracts with a method adapted from Benzie and Strain (1996). Results were expressed as μmol of ascorbic acid equivalents/100 g of dry weight of the extract.

2.6.2. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) scavenging activity

The reduction of DPPH stable free radical was determined according to the modified version of the method described by Cavin, Hostettmann, Dyatmyko, and Potterat (1998). Results are expressed in μmol Trolox/100 g of dry weight of the extract.

2.7. Isolated heart preparation

All procedures followed during this investigation were approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Medicine, University of La Plata (P-05-2014).

Hearts from male Wistar rats were isolated and Langendorff perfused with Ringer's solution containing (in mmol/L) 118 NaCl, 5.9 KCl, 1.2 $MgSO_4$, 1.35 $CaCl_2$, 20 $NaCO_3H$ and 11.0 glucose (gassed with 95% O_2-5% CO_2, pH 7.4, 37 °C). The conductive tissue in the atrial septum was damaged with a fine needle to achieve atrioventricular block, and the right ventricle was paced at 280 ± 10 beats/min. A latex balloon tied to the end of a polyethylene tube was passed into the left ventricle through the mitral valve; the opposite end of the tube was then connected to a Statham P23XL pressure transducer. The balloon was filled with water to provide a left ventricular end-diastolic pressure (LVEDP) of 8–12 mmHg, and this volume was unchanged for the rest of the experiment. Coronary perfusion pressure (CPP) was monitored at the point of cannulation of the aorta and was adjusted to approximately 60–70 mmHg. Coronary flow (CF), which was controlled with a peristaltic pump, was 11 ± 2 ml/min. Left ventricular pressure (LVP) and CPP data were acquired by using an analog-to-digital converter and acquisition software (Chart V4.2.3 AD Instruments).

2.7.1. Experimental protocols

After 20 min of stabilization, the following experimental protocols were performed (Fig. 1):

Non-ischemic control hearts (NIC; n = 7): Hearts were perfused for 110 min without any treatment.

Ischemic control hearts (IC, n = 8): Hearts were subjected to 30 min of normothermic global ischemia followed by 60 min of reperfusion. Global ischemia was induced by stopping the perfusate inflow line, and the heart was placed in a saline bath held at 37 °C.

Copoazú non-fermented extract (CNFE n = 7): Hearts were treated during the first 10 min of reperfusion with a dose of 0.30 mg/min of CNFE.

Copoazú fermented extract (CFE n = 8): Hearts were treated during the first 10 min of reperfusion with a dose of 0.30 mg/min of CFE. The final concentration in the perfusate was 30 μg/ml.

L-NAME (n = 7): Hearts received 1 mM of L^G nitro-L-arginine methyl ester (L-NAME) -a non-selective nitric oxide synthase (NOS) inhibitor- from 10 min before ischemia and during all the reperfusion time.

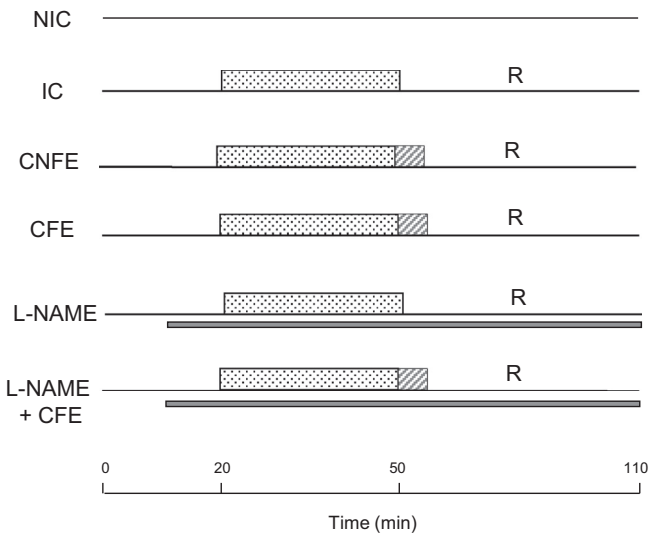

Fig. 1. Scheme of the experimental protocols. NIC: non-ischemic control; IC: ischemic control; CNFE: copoazú non-fermented extract; CFE: copoazú fermented extract; L-NAME: L^G-nitroL-arginine methyl ester, inhibitor of NOS and L-NAME + CFE.

L-NAME + CFE (n = 7): Hearts received L-NAME in a similar manner than L-NAME group and CP was administered at the time and dose above indicated.

2.7.2. Infarct size determination

Infarct size was assessed by the widely validated triphenyltetrazolium chloride (TTC) staining technique (Fishbein et al., 1981). This technique relies on the ability of dehydrogenase enzymes and cofactors in the tissue to react with tetrazolium salt to form a formazan pigment (red). It is a recognized method to discriminate between dead and viable tissue. At the end of reperfusion, atrial and right ventricular tissues were excised and left ventricle (LV) was frozen. The freeze LV was cut into six transverse slices, which were incubated for 5 min at 37 °C in a 1% solution of TTC. To measure myocardial infarction, the slices were weighed and scanned. The infarcted (pale) and viable ischemic/reperfused (red) areas were measured by computed planimetry (Scion Image 1.62; Scion Corp., Frederick, Maryland, USA). Infarct weights were calculated as $(A1 \times W1) + (A2 \times W2) + (A3 \times W3) + (A4 \times W4) + (A5 \times W5) + (A6 \times W6)$, where A is the area of infarct for the slice and W is the weight of the respective section. Infarct size was expressed as a percentage of total area (area at risk, AR).

2.7.3. Systolic and diastolic function

The systolic function was assessed by the left ventricular developed pressure (LVDP)- calculated by subtracting LVEDP from the left ventricular (LV) peak pressure values- and the maximal velocity of rise of LVP ($+dP/dt_{max}$). The diastolic function was evaluated through the maximal velocity of decrease of LVP ($-dP/dt_{max}$) and LVEDP.

2.7.4. Coronary resistance (CR)

CR was calculated as a quotient between CPP and CF.

2.7.5. Assessment of lipid peroxidation

We used the TBARS spectroscopic technique to evaluate lipid peroxidation. At the end of the reperfusion period, a portion of LV was homogenized in physiological saline solution and centrifuged at 770g to allow measuring TBARS in the supernatant. Absorbance at 535 nm was measured and TBARS expressed in nmol/mg protein using an extinction coefficient of $1.56 \times 105\ M^{-1}\ cm^{-1}$ (Buege & Aust, 1974).

Table 1
Content of phenolic compounds and antioxidant activity of aqueousextracts of copoazú.

	CNFE	CFE
Phenolic compounds		
Total phenols (mg GA/100 g)	1216 ± 59	189 ± 3**
Catechin (mg/100 g)	6.2 ± 0.7	3.6 ± 0.4**
Epicatechin (mg/100 g)	1.8 ± 0.2	1.0 ± 0.1**
Theobromine (mg/100 g)	2.7 ± 0.3	1.6 ± 0.2**
Caffeine (mg/100 g)	3.6 ± 0.4	2.1 ± 0.2**
Antioxidant activity		
FRAP (μmol AA/100 g)	920 ± 130	130 ± 2**
DPPH (μmol TROLOX/100 g)	805 ± 97	144 ± 3**

GA: Gallic acid; FRAP: Ferric reducing ability of plasma; AA: Ascorbic acid; DPPH: α, α-diphenyl-β-picrylhydrazyl.
** $p < 0.01$.

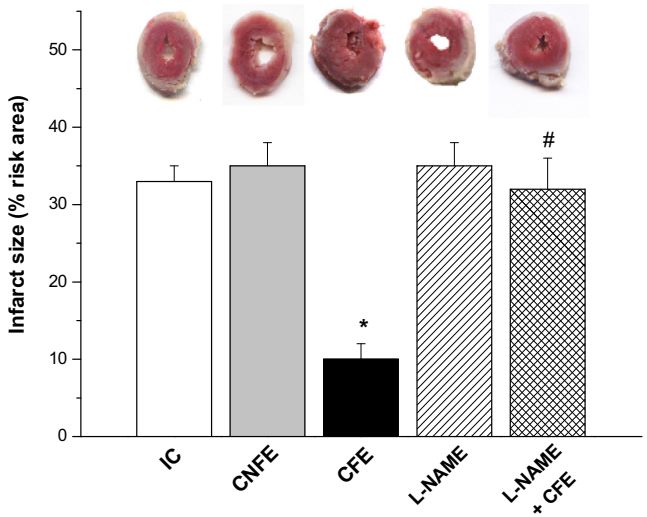

Fig. 2. Infarct size (IS), expressed as a percentage of risk area, in ischemic control (IC) and in hearts treated with aqueous extracts of copoazú fermented (CFE) and non-fermented (CFE) or L-NAME, inhibitor of NOS and the combination of L-NAME + CFE. Observe that CFE treatment but not CNFE decreased the IS obtained in IC hearts and that this action was abolished by L-NAME treatment. *$p < 0.05$ vs. IC; #$p < 0.05$ vs. CFE.

2.7.6. Reduced glutathione

GSH was determined by Ellman's method, which is based on the reaction of non-protein sulfhydryl groups with 5,5′-dithiobis (2-nitrobenzoic acid) to give a compound that absorbs at 412 nm. GSH levels were expressed as μg/mg protein (Sedlak & Lindsay, 1968).

2.7.7. Immunoblotting

Other portion of LV was homogenized and cytosolic fraction was isolated by differential centrifugation. Briefly, LV were homogenized in ice-cold RIPA buffer (300 mmol/L sacarosa, 1 mmol/L DTT, 4 mmol/L EGTA, 20 mmol/L Tris pH 7.4, 1% Triton X, 10% protease cocktail, 25 μmol/L FNa, 1 μmol/L ortovanadate) and centrifuged at 12,000g for 15 min at 4 °C. From supernatant proteins (60 μg) were resolved on SDS-PAGE and transferred to PVDF membrane (2 h). Equal loading of samples was confirmed by Ponceau S staining. Membranes were blocked with 5% non-fat milk in Tris-buffered saline (pH 7.5) containing 0.1 % Tween (TBS-T), and probed overnight at 4 °C with antibodies against phosphorylated

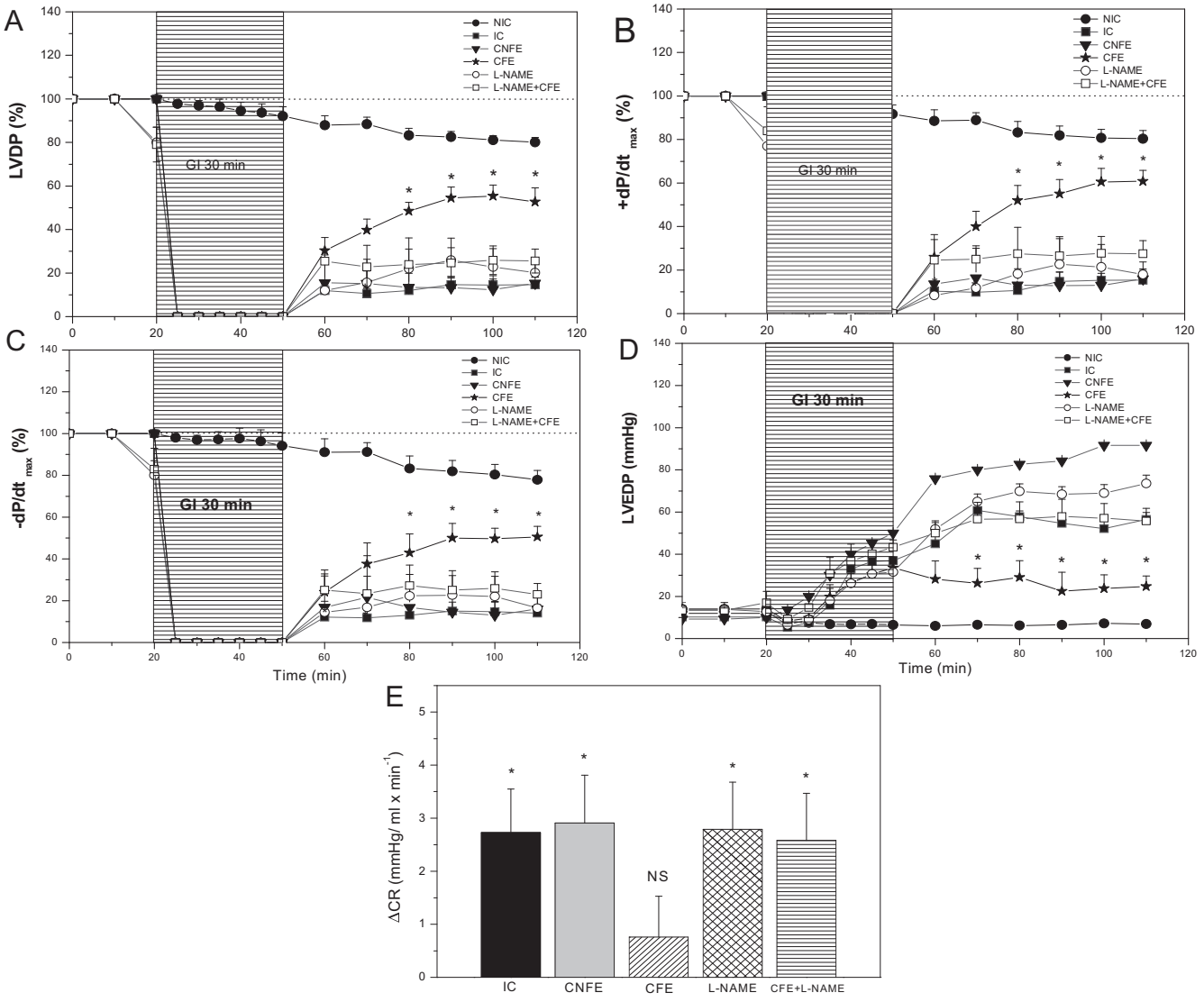

Fig. 3. Time course of left ventricular developed pressure (LVDP, A panel), maximal velocity of rise of left ventricular pressure (+dP/dt$_{max}$, B panel), maximal velocity of decrease of left ventricular pressure (−dP/dt$_{max}$, C panel), left ventricular end diastolic pressure (LVEDP, D panel), and reperfusion changes of coronary resistance (ΔCR, E panel), in ischemic control (IC) and in hearts treated with CNFE, CFE, L-NAME or L-NAME + CFE. Note that CFE but not CNFE, significantly improved the postischemic recovery of myocardial function, attenuated the cardiac stiffness and annulled the increase of CR detected in IC hearts. The NOS blockade with L-NAME abolished these beneficial actions of CFE. $^{*}p < 0.05$ vs. IC.

forms of GSK-3β-Ser9 (1:1000, Santa Cruz Biotechnology), Akt (1:1000, Santa Cruz Biotechnology), PKCε ((1:1000, Santa Cruz Biotechnology) and eNOS-Ser1177 (1:1000, Sigma-Aldrich). Membranes were washed four times for 10 min in TBS-T prior to addition of anti-rabbit secondary antibody (1:5000, Santa Cruz Biotechnology) and protein bands were analysed by a chemiluminescent system (ECL Plus; GE Healthcare Life Sciences). GAPDH signal was used as a loading control.

2.8. Isolation of mitochondria

LV of non-perfused rat hearts were washed and homogenized in ice-cold isolation solution (S) consisting of 75 mM sucrose, 225 mM mannitol, and 0.01 mM EGTA neutralized with Trizma buffer at pH 7.4. After the tissue pieces were settled, the entire supernatant was discarded and fresh IS (5 mL) was added, and the mixture was transferred to a hand homogenizer. Proteinase (0.8 mg, bacterial, type XXIV, Sigma-Aldrich, formerly called Nagarse) was added just before starting the homogenization procedure. The whole homogenization procedure took no longer than 14 min in two steps of 7 min each (with 5 mL addition of fresh S each). The homogenate was carefully transferred after each step to a polycarbonate centrifuge tube. After 5 min of 480*g* of centrifugation to discard unbroken tissue and debris, the supernatant was centrifuged at 7700*g* for 10 min to sediment the mitochondria. The mitochondrial pellet was washed twice with IS and the last one with suspension solution (IS without EGTA) at 7700*g* for 5 min each.

2.8.1. Ca^{2+}-induced mPTP opening

The ability of mitochondria to resist swelling was assessed by incubating 0.3 mg/mL of isolated mitochondria in a buffer containing (in mmol/L): 120 KCl, 20 MOPS, 10 Tris HCl, and 5 KH_2PO_4 adjusted to pH = 7.4. After 5-min preincubation, the mitochondria energized with the addition of 5 mmol/L succinate were induced to swell with 100 μmol/L $CaCl_2$. If mPTP is open in the presence of Ca^{2+} loading, solutes will be free to enter the inner matrix, causing the mitochondria to swell. These changes are observed as

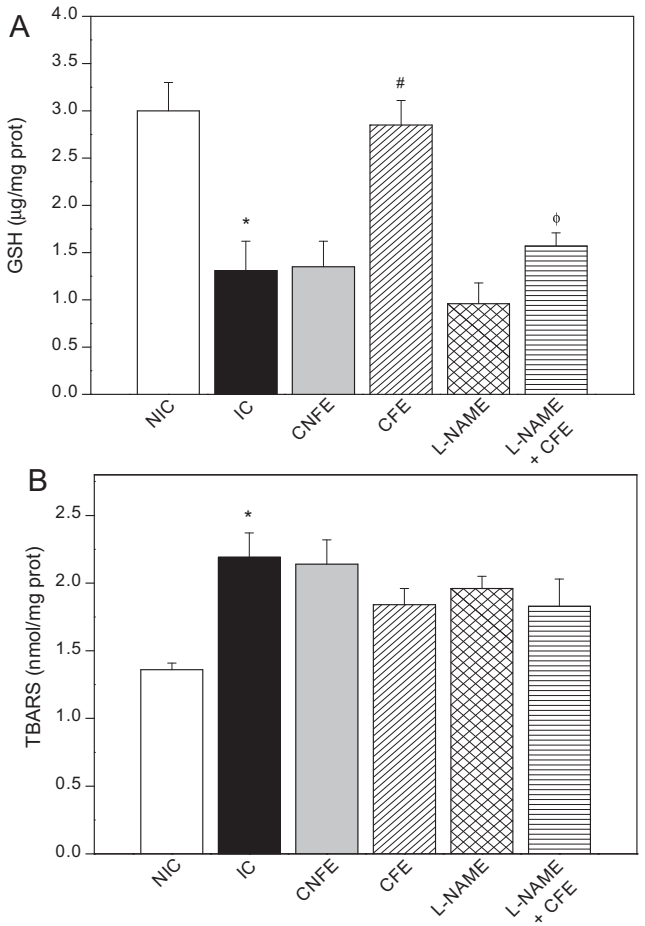

Fig. 4. Reduced glutathione content (GSH, A panel) and thiobarbituric acid reactive substances concentration (TBARS, B panel) in ischemic control hearts (IC) and in hearts treated with CNFE, CFE, L-NAME or L-NAME + CFE. GSH content decreased in IC hearts, CFE partially preserved it and L-NAME abolished the CFE action. The TBARS increased in IC hearts and were not modified by any treatment. $^{*}p < 0.05$ vs. NIC; $^{\#}p < 0.05$ vs. IC; $^{\phi}p < 0.05$ vs. CFE.

decreases of light scattering and followed using a temperature-controlled Hitachi F4500 spectrofluorometer operating with continuous stirring at excitation and emission wavelengths of 520 nm (Facundo, de Paula, & Kowaltowski, 2007). LSD was calculated for each sample by taking the difference of scattered light between before and after the addition of $CaCl_2$. LSD was assessed in samples without any treatment and in those treated with CFE (7.5 µg/mL), L-NAME (1 mM) and the combination of both (L-NAME + CFE). In order to relate mPTP opening to decreased light scattering, we added cyclosporine A 0.5 µM to inhibit mPTP or abolish any observed reduction.

2.9. Statistical analysis

Data were expressed as means ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Newman-Keul's post-test used for multiple comparisons among groups. Values of $p < 0.05$ were considered to indicate statistical significance.

3. Results

Table 1 shows the content of phenolic compounds and antioxidant activity of both ¨copoazú¨ extracts (CNFE and CFE). The data show that CFE, probably as a consequence of the fermentation process, has a smaller amount of catechin, epicatechin, theobromine and caffeine and a lesser antioxidant activity than CNFE.

Thirty min of global ischemia followed by 60 min of reperfusion caused an infarct size (IS) of ~30% of risk area. This value was not modified by the addition of CNFE or L^G-nitro-L-arginine methyl ester (L-NAME) but a significant reduction was observed when CFE 30 µg/mL was added to the perfusate (Fig. 2). This beneficial effect was annulled by L-NAME treatment.

The time course of contractility during ischemia and reperfusion of all experimental groups is shown in Fig. 3. In IC hearts, LVDP decreased to 15 ± 4% from pre-ischemic value at the end of the reperfusion period. The treatment with CFE but not with CNFE improved post-ischemic recovery of myocardial function. At the end of reperfusion, LVDP reached a value of approximately 50% of pre-ischemic value (Fig. 3, A panel). A similar pattern was observed when $+dP/dt_{max}$ was analysed (Fig. 3, B panel). An increase of $-dP/dt_{max}$ was also evident after CFE treatment (Fig. 3, C panel). The LVEDP (an index of diastolic stiffness) was approximately 10 mmHg at the end of stabilization period and significantly increased during ischemia-reperfusion reaching a value of approximately 50 mmHg at the end of reperfusion. After CNFE or L-NAME addition, LVEDP acquired higher values than those observed in IC. However, the treatment with CFE significantly reduced the LVEDP values detected in untreated hearts (Fig. 3, D panel). These beneficial effects were lost when NO synthesis was inhibited by L-NAME. Ischemic contracture was not modified by CFE but increased in presence of L-NAME.

No-reflow phenomenon, a disorder that interrupts the microcirculation during reperfusion, is also involved in reperfusion injury (Kloner, 2011). In our experimental preparation, at constant coronary flow (CF), changes of coronary perfusion pressure (CPP) produce changes in coronary resistance (CR). These changes are indicating the incidence of treatments on vasomotor tone. The ischemia-reperfusion produced an increase of CR leading to an inadequate coronary perfusion endangering myocardial cell life and promoting cell death. This increase was not observed after CFE treatment indicating than an improving of post-ischemic coronary perfusion occurred in presence of the extract. This beneficial action was abolished by NOS inhibition with L-NAME, highlighting the NO-mediated CFE vasodilator effect (Fig. 3, E panel).

Untreated hearts (IC) also showed a diminution of GSH level and an increase of TBARS. The treatment with CFE partially preserved the GSH content and did not modify the TBARS concentration. The beneficial action on GSH was attenuated when CFE was administered in presence of L-NAME (Fig. 4).

A significant increase of the expression of phosphorylated forms of Akt, GSK-3β, e-NOS and PKCε at the end of reperfusion period was observed in hearts treated with CFE. This increase was significantly attenuated when CFE was added in presence of L-NAME (Fig. 5).

Fig. 6 shows typical traces of swelling experiments (A) and the mean values of light scattering decrease (LSD, B) produced by the addition of 100 µmol/L Ca^{2+} to samples of mitochondrial suspension obtained from untreated and treated hearts. The CFE treatment significantly decreased the LSD produced by Ca^{2+} (0.26 ± 0.10 vs. 1.40 ± 0.14 a.u.) and L-NAME abolished this change (0.80 ± 0.10 a.u.). The addition of CsA significantly attenuated the LSD produced by Ca^{2+} (0.10 ± 0.02 a.u.) (data not shown).

4. Discussion

The present study demonstrates for the first time that the treatment at the onset of reperfusion with ¨copoazú¨ fermented extract (CFE) limits the infarct size and improves postischemic recovery of

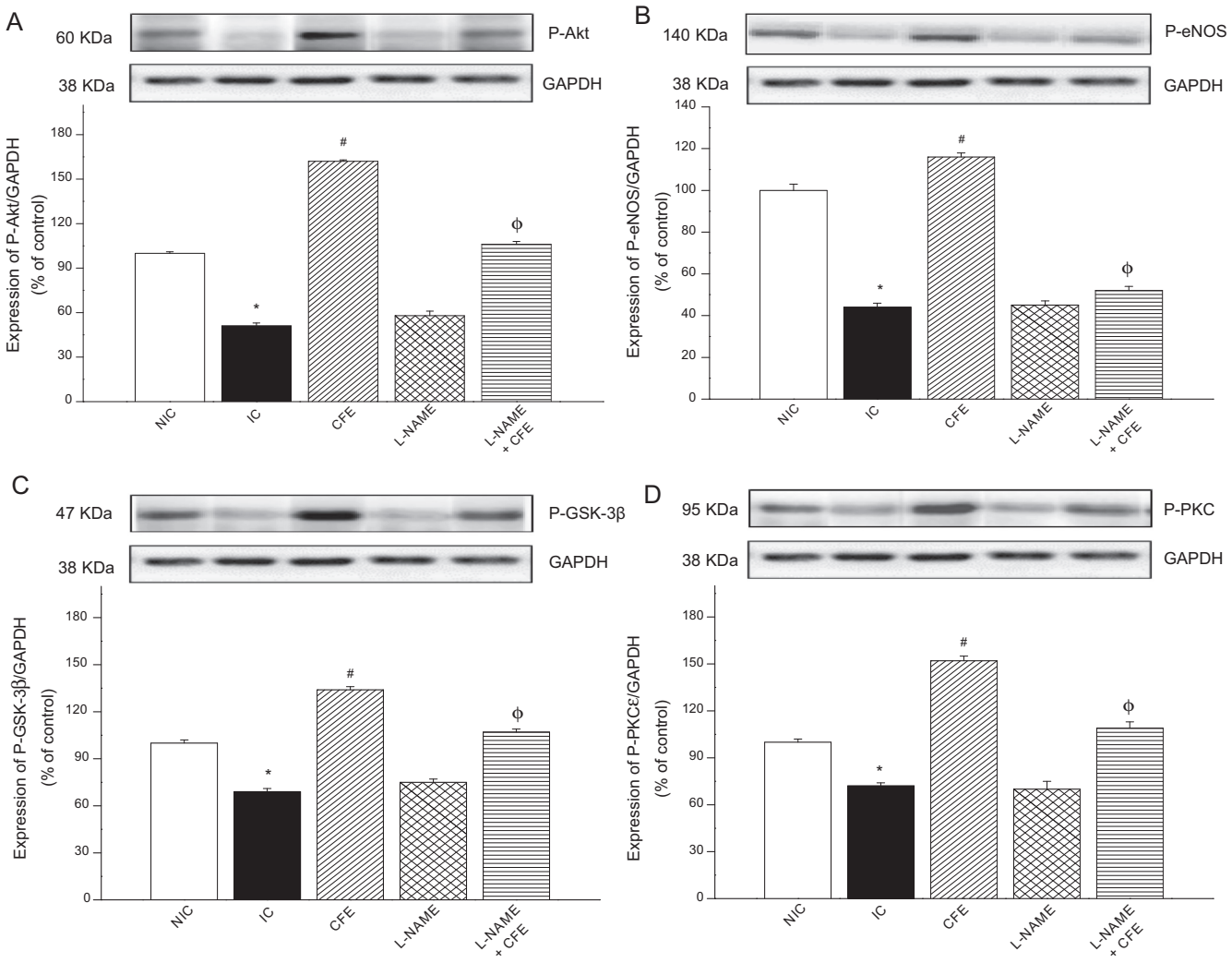

Fig. 5. Representative immunoblots of phosphorylated forms and summary of densitometry data of phospho-Akt (P-Akt, A panel), phospho-eNOS (P-eNOS, B panel), phospho-GSK-3β (P-GSK-3β, C panel) and phospho-PKCε (P-PKCε, D panel), in cardiac homogenate of non-ischemic (NIC) and ischemic control (IC), and in hearts treated with CFE, L-NAME or L-NAME + CFE. CFE increased the expression of all the examined proteins and the NOS inhibition with L-NAME significantly decreased it, being eNOS the most affected. $^{*}p < 0.05$ vs. NIC; $^{\#}p < 0.05$ vs. IC; $^{\Phi}p < 0.05$ vs. CFE.

myocardial function and coronary perfusion. In the same experimental conditions, the administration of a non-fermented extract of ¨copoazú¨ did not exert beneficial actions on myocardium.

An improvement of coronary perfusion is an indication of the protection against ¨non-reflow¨ phenomenon. Previous results suggest that polyphenols are able to improve endothelium-dependent vasodilation, in which NO plays a crucial role (Andriantsitohaina et al., 2012). In our experimental preparation, we detected an attenuation of coronary resistance (vasodilator effect) in hearts treated with CFE. This effect was abolished when NOS was inhibited by L-NAME. Therefore, this result reinforces the hypothesis that an increased NO production via eNOS activation achieved by CFE is an important mechanism contributing to the attenuation of coronary perfusion post-ischemic deficiency.

Of the various pathological events that lead to ischemia-reperfusion injury, the abrupt ROS production, the increased cytosolic calcium and the cross talk between both events play key roles (Peng & Jou, 2010). Although ROS production occurs in several cellular locations, the mitochondrial production predominates in ischemia-reperfusion-mediated oxidant injury in the heart. Under these conditions, the antioxidant effect should be relevant. In our experiments, the partial preservation of GSH detected in hearts treated with CFE would be an indication of a lesser ROS production. What would be the mechanism to explain this possible ROS diminution? Could it be due to the antioxidant action of the extract? There is accumulated evidence that show that cardioprotective mechanisms triggered by natural antioxidant go further than their antioxidant action (Akhlaghi & Bandy, 2009). Examining our data this premise comes true. The¨in vitrö analysis of CFE shows that the fermentation process, drying and roasting of ¨copoazú¨ seeds, decreases the total polyphenols content and the antioxidant activity compared to CNFE. At this point it should be remembered that fermentation is an old technology used to improve the useful life and nutritional and organoleptic qualities of food and to extract its bioactive compounds (Martins et al., 2011). This process alters the relationship between nutritive and non-nutritive components of food, affecting the bioactivity and digestibility of the products (Zhang et al., 2012). In our case, cardioprotective actions against ischemia-reperfusion injury were only detected when CFE was administered. Therefore, we demonstrated that the product obtained by the fermentation process was efficient to protect the heart while that unfermented was not. Moreover, our results are suggesting that the antioxidant action of CFE is not the principal mechanism responsible of the

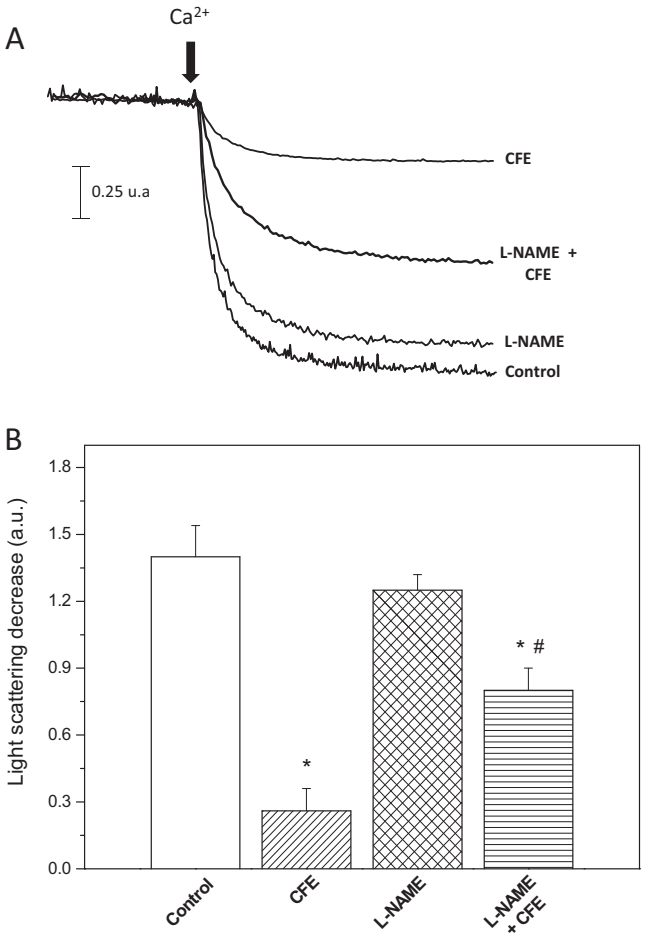

Fig. 6. Typical traces (A) and mean values of the light scattering decreases (B) produced by 100 μM Ca^{2+} addition to mitochondrial suspensions, in absence and in presence of ¨copoazű fermented extract (CFE), L-NAME or L-NAME + CFE. The response of isolated mitochondria to Ca^{2+} was significantly attenuated by CFE and partially restored after NOS inhibition with L-NAME. $^{*}p < 0.05$ vs. C; $^{\#}p < 0.05$ vs. CFE.

beneficial actions afforded by the extract against ischemia-reperfusion injury. Hence, others mechanisms would be implicated. Thus, the modulation of intracellular signalling pathways has been long recognized as crucial for cardioprotection being the mitochondria and specifically the mPTP the end target. The formation/and opening of mPTP is linked to cell death (Halestrap et al., 2004) and the attenuation of that process promote the cell survival. In relation to the kinases, Juhaszova et al. (2009) showed that inhibition/phosphorylation of GSK-3β alters the phosphorylation of target substrates associated to mPTP thus delaying its opening time. Our data show that CFE increased the level of phosphorylated form of GSK-3β which was attenuated in presence of L-NAME.

What kinases are able to phosphorylate GSK-3β? It was previously recognized that this action is mediated by PI3K/Akt dependent pathway (Tong, Imahashi, Steenbergen, & Murphy, 2002). In this study the Akt expression followed the same pattern than GSK-3β, i.e. decreased in IC, increased in CFE treated hearts and it was attenuated when NOS was inhibited.

On the other hand, it has been previously documented the protective role of NO during ischemia-reperfusion (Bolli, 2001). This radical combines with $O_2^{-\cdot}$ at a very fast rate to form peroxynitrite ($ONOO^-$). NO is produced by nitric oxide synthase (NOS) activated by phosphorylation. Although there are many potential phosphorylation sites on the endothelial isoform of NOS (eNOS), functionally most is known about the phosphorylation of the Ser1177 residue (induces eNOS activation) and the Thr495 residue (produces eNOS inhibition). In our experimental conditions, an increase of $eNOS^{Ser1117}$ phosphorylation was detected in CFE treated compared to untreated hearts and was annulled in presence of L-NAME. Who activates eNOS? Previously, it was shown that Akt is able to activate eNOS increasing NO production (Fulton et al., 1999). Other kinases and phosphatases, such as PKC, ERK1/2, protein phosphatase 1 and protein phosphatase 2A participate in the control of eNOS phosphorylation (Dudzinski & Michel, 2007).

Protein kinase C ε (PKCε) is a pivotal signalling element in the myocardial protection against ischemia-reperfusion injury (Baines, Pass, & Ping, 2001). An essential feature of the activation of PKCε is its subcellular redistribution. The translocation to specific subcellular compartments is thought to be an important mechanism for PKCε to direct downstream signalling cascades and orchestrate protection. In this study, we showed an increase of the phosphorylated form of PKCε in hearts treated with CFE and a reduction in presence of L-NAME.

The decreased level of Akt and PKCε in presence of L-NAME could be explained considering the ability of NO/cGMP in the translocation/activation of PKC (Costa et al., 2005; Ping et al., 1999) and Akt phosphorylation (Kawasaki et al., 2003). A physical and functional coupling of PKCε, Akt, and eNOS constituting signalling modules, as was previously reported (Zhang et al., 2005) could be contributing to the cardiac protection afforded by CFE.

Mitochondria and specifically the mPTP has received considerable attention in the development of cardioprotection. In isolated mitochondria we observed an attenuation of mPTP response to Ca^{2+} after CFE addition. This response was abolished in presence of L-NAME suggesting that NO plays a crucial role in the protective effects of CFE on mitochondria. This action could explain the CFE-evoked infarct size limitation.

Given the complex composition of herbal extracts, the beneficial actions observed in hearts treated with CFE would be the result of the synergistic action of all components and not attributable only to polyphenols (Wagner & Ulrich-Merzenich, 2009; Yang et al., 2014).

5. Conclusion

This study demonstrate in an¨ex vivö heart model that the treatment only during reperfusion with an aqueous extract of fermented ¨copoazű seeds protects the myocardium against ischemia-reperfusion injury through Akt/GSK-3β, Akt/NO/Akt, Akt/NO/PKCε-dependent pathways- in which NO plays a central role- targeting on mitochondria. Other interesting finding is that there is no direct relationship between antioxidant effect and cardioprotection. Taking into account the growing interest in designing food-derived tools for health improvement and for preventing and treating several pathologies, the knowledge of the beneficial effects of ¨copoazű fermented extract may be of great relevance. However, our findings could not be extrapolled directly to human. Multiple models including large animals and models with co-morbidities should be performed prior to clinical translation.

Since that the combined therapy is used to improve the patient's overall health status, the utilization of herbal medicine, which is considered as a "multi-target herb" should be optimized.

Conflict of interest

None.

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