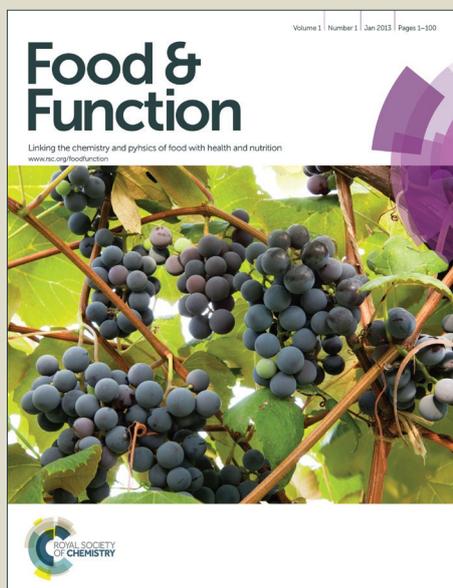


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**EFFECT OF *ILEX PARAGUARIENSIS* (YERBA MATE) EXTRACT ON INFARCT
SIZE IN ISOLATED RAT HEART: MECHANISMS INVOLVED**

Luisa F González Arbeláez^{a*}, Juliana C Fantinelli^{a*}, Alejandro Ciocci Pardo^a, Claudia I Caldiz^a, José Luis Ríos^b, Guillermo R Schinella^c, Susana M Mosca^a

^aCentro de Investigaciones Cardiovasculares, Universidad Nacional de La Plata-CONICET, La Plata, Argentina.

^b Departament de Farmacologia, Facultat de Farmàcia, Universitat de València, Spain

^c Cátedra de Farmacología Básica, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, CIC, Provincia de Buenos Aires, La Plata, Argentina.

* These authors collaborated equally to the work

Corresponding author: Dr Susana M Mosca, Centro de Investigaciones Cardiovasculares, Universidad Nacional de La Plata, 60 y 120, 1900 La Plata, Argentina.

E-mail: smosca@med.unlp.edu.ar

Phone and Fax: 54-221-4834833

Abstract

Ilex paraguariensis (IP) dried and minced leaves made into a brewed tea are a beverage widely consumed by large populations in South America as a source of caffeine (stimulant action) and for its medicinal properties. However, there is little information about the actions of IP on myocardium in ischemia-reperfusion situation. Therefore, the objective of this study was to examine the effects of an aqueous extract of IP on infarct size in a model of regional ischemia. Isolated rat hearts were perfused by Langendorff technique and submitted to 40-min of coronary artery occlusion followed by 60 min of reperfusion (ischemic control hearts). Other hearts received IP 30 $\mu\text{g}/\text{mL}$ during the first 10 min of reperfusion in absence or presence of L^G-nitro-L-arginine methyl ester [L-NAME, a nitric oxide synthase (NOS) inhibitor]. Infarct size was determined by triphenyltetrazolium chloride (TTC) staining. Postischemic myocardial function and coronary perfusion were also assessed. Cardiac oxidative damage was evaluated by the thiobarbituric acid reactive substances (TBARS) concentration and reduced glutathione (GSH) content. To analyze the mechanisms involved the expression of phosphorylated forms of eNOS and Akt were measured. In isolated mitochondria the Ca²⁺-induced mitochondrial permeability transition pore (mPTP) opening was determined. IP significantly decreased the infarct size and improved postischemic myocardial function and coronary perfusion. TBARS decreased, GSH was partially preserved, levels of P-eNOS and P-Akt increased and mPTP opening diminished after IP addition. These changes were abolished by L-NAME. Therefore, our data demonstrate that the acute treatment with IP only during reperfusion was effective to reduce myocardial postischemic alterations. These actions would be mediated by a decrease of mitochondrial permeability through IP-activated Akt/eNOS-dependent pathways.

Key words: *Ilex paraguariensis*, ischemia-reperfusion, TBARS, GSH, P-Akt/P-eNOS, mPTP

1. Introduction

Mate tea, or simply “mate”, is a traditional beverage prepared as infusions or decoctions of the dried and minced leaves and twigs of the native South America plant known as yerba mate, *Ilex paraguariensis* (IP) A.St.-Hil. (Aquifoliaceae). Mate is the most widely consumed non-alcoholic beverage in Argentina, Brazil, Paraguay, and Uruguay. Its consumption surpasses its psycho-stimulant properties; it is a cultural phenomenon that has different forms of consumption according to geographic regions and social groups.

Yerba mate contains caffeine (0.3-1.7%) as principal xanthine, tannins, essential oils, triterpenes, saponins, resin, and phenolics, principally flavonoids and caffeoyl derivatives, being chlorogenic, isochlorogenic and neochlorogenic acids the most relevant compounds of this last group^{1,2}. The caffeine confers psycho-stimulant properties as well as cardiovascular and respiratory stimulant properties, which are the base of its anti-fatigue and stimulatory effects. The second relevant group of compounds is the phenolics, which confer antioxidant properties are closely related with the protection against LDL lipoperoxidation, anti-mutagenic, anti-tumoral and anti-obesity actions²⁻⁴. According to the “antioxidant hypothesis” it would be possible to limit oxidative damage and ameliorate pathologies that involve free radicals generation by supplementing antioxidants. Unfortunately, most of the clinical trials carried out to test the “in vivo” efficacy of antioxidants could not measure any benefit of their administration^{5, 6}. On the other hand, recent studies indicate that some antioxidants, such as polyphenols, exert their biological actions through a wide spectrum of cellular signalling events⁶⁻¹⁰.

Cardiovascular diseases cause nearly one-third of all deaths worldwide being ischemic heart disease (IHD) the most common cause¹¹. A combination of primary prevention (improvements in risk factors) and secondary prevention (improved treatment) are participating in the reduction of mortality incidence by IHD. Research in the nutrition field has recently aroused considerable interest based on the potential of natural products to

counteract or attenuate cardiovascular diseases. Few studies have identified yerba mate as an excellent candidate to limit postischemic alterations. In this sense, previous data from our laboratory ¹² show that IP treatment before and after a short ischemic period improved the postischemic recovery of myocardial function. If IP is able to reduce cell death has not yet been demonstrated.

Therefore, our aim was to assess the effects of an aqueous extract of commercially available "yerba mate" on infarct size, myocardial contractile function, coronary perfusion and oxidative damage produced by 40 min of coronary artery occlusion and 60 min of reperfusion examining the involved pathways.

2. Materials and Methods

2.1. Plant material

A sample of commercial mate of IP produced in Las Marías (Corrientes, Argentina) was obtained for this study and a voucher specimen was deposited in the herbarium of the Museo de Botánica y Farmacognosia “Carlos Spegazzini” (Universidad Nacional de La Plata, Argentina) under the number LPE 1005.

2.1.1. Preparation of the extract

The IP extract was the same to that used by Schinella et al¹³ and in this paper the HPLC-UV profiles were showed. Briefly, the dried and powdered leaves of IP was extracted (5 %w/v) with hot water (90 °C), left standing for 20 min, filtered and lyophilised (yield 9.0%, w/w). The dry matter was maintained at -20 °C until use.

2.2. 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 following the Guide for the Care and Use of Laboratory Animals published by the Nacional Research Council, National Academy Press, Washington DC 2010 and/or European Union Directive for Animal Experiments 2010/63/EU.

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 MgSO₄, 1.35 CaCl₂, 20 NaHCO₃ and 11.0 glucose (gassed with 95% O₂-5% CO₂, 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 LVEDP of 8-12 mmHg, and this volume was unchanged for the rest of the experiment. CPP was monitored at the point of cannulation of the aorta and was adjusted to

approximately 60-70 mmHg. CF, which was controlled with a peristaltic pump, was 11 ± 2 mL/min. Left ventricular pressure (LVP) and its first derivative (dP/dt) were recorded with a direct writing recorder.

2.2.1. Experimental protocols

After 20 min of stabilization, the following experimental protocols were performed (Fig. 1). Non-ischemic control hearts (NIC; n = 6): hearts were perfused for 120 min without any treatment. Ischemic control hearts (IC, n = 9): hearts were subjected to 40 min of occlusion of the left anterior descending coronary artery followed by 60 min of reperfusion. IP (n = 8): hearts were treated during 10 min at the beginning of reperfusion with a dose of 0.30 mg/min of an aqueous extract of IP. The final concentration of IP in the perfusate was 30 μ g/mL. L^G-nitro-L-arginine methyl ester (L-NAME, n = 5): hearts received 1 mM L-NAME (NOS inhibitor), from 10 min before ischemia and during all the reperfusion time. L-NAME + IP (n = 7): hearts received L-NAME in a similar manner than L-NAME group and IP was added at the onset of reperfusion.

2.2.2. Infarct size determination

Infarct size was assessed by the triphenyltetrazoliumchloride (TTC) staining technique. At the end of reperfusion, the left anterior descending coronary artery was occluded again and the myocardium was perfused during 1 min with a 0.1% solution of Evans blue. This procedure delineated the non-ischemic myocardium as dark blue. After staining, the hearts were frozen and cut into six transverse slices, which were incubated for 15 min 37 °C in 1% solution of TTC. All atrial and right ventricular tissues were excised. Infarct and area at risk were determined following the instructions detailed in a previous paper¹⁴.

2.2.3. 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 ($-dP/dt_{\max}$), half-time relaxation (t_{50}), the time constant of relaxation (τ)- assessed using a monoexponential model with asymptote¹⁵- and LVEDP. CR was calculated as a quotient between CPP and CF.

2.2.4. 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 $770 \times g$ to allow measuring TBARS in the supernatant. Absorbance at 535nm was measured and TBARS expressed in nmol/mg protein using an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ ¹⁶.

2.2.5. 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 $\mu\text{g}/\text{mg}$ protein¹⁷.

2.2.6. 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 $\mu\text{mol}/\text{L}$ FNa, 1 $\mu\text{mol}/\text{L}$ ortovanadate) and centrifuged at $12000 \times g$ 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 and total forms of GSK-3 β -Ser⁹ (1:1000, Santa Cruz Biotechnology), Akt (1:1000, Santa Cruz Biotechnology), ERK1/2 (1:1000, Millipore), p90^{RSK} (1:1000, Millipore) 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.

2.3. 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.

2.3.1. Ca²⁺ 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 KH₂PO₄ 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 CaCl₂. If mPTP is open in the presence of Ca²⁺ loading, solutes will be free to enter the inner matrix, causing the mitochondria to swell. These changes are observed as decreases of light scattering and followed using a temperature-controlled Hitachi F4500 spectrofluorometer operating with continuous stirring at excitation and emission wavelengths of 520 nm¹⁸. LSD was calculated for each sample by taking the difference of scattered light between before and after the addition of CaCl₂. LSD was assessed in samples without any treatment and in those treated

with IP (7.5 $\mu\text{g}/\text{mL}$), L-NAME (1 mM) and the combination of both (L-NAME + IP). 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.

2.4. Statistical analysis

Data were expressed as means \pm 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

Forty minutes of regional ischemia followed by 1 h of reperfusion in rat hearts without any treatment caused an infarct size of ~40% of the risk area. The risk area for all interventions was similar and represented ~32% of the left ventricle. The addition of L^G-nitro-L-arginine methyl ester (L-NAME) did not modified the IS observed in ischemic control hearts. A significant reduction in IS was obtained when 30 µg/mL IP was added to the perfusate during the first 10 min of reperfusion (Fig. 2). This protection was annulled by L-NAME treatment detecting an IS similar to untreated hearts. In IC hearts, LVDP decreased to $35 \pm 6\%$ from baseline at the end of the reperfusion period. The addition of 30 µg/mL of IP improved post-ischemic recovery reaching LVDP values of approximately 60%. A similar pattern was observed when $+dP/dt_{max}$ was analyzed. Examining $-dP/dt_{max}$ an improvement of relaxation velocity after treatment with IP was also evident ($64 \pm 8\%$ vs. $35 \pm 7\%$ in untreated hearts). In IC group, t_{50} values at the end of reperfusion were similar to those obtained in preischemic period (63.8 ± 2.0 vs. 65.3 ± 4.9 msec). This pattern was not modified by IP treatment (66.7 ± 5.0 vs. 67.2 ± 4.6 msec). Contrarily, in IC group τ significantly increased at the end of reperfusion compared to preischemia (44.8 ± 2.0 vs. 29.9 ± 3.0 msec). This increase was avoided by IP. In this group, τ values at the end of reperfusion were significantly lower than those detected in preischemia (24.9 ± 0.9 vs. 30.6 ± 1.3 msec). The lusitropic effect of IP was blunted when NOS was inhibited by L-NAME. In these conditions, τ values at the end of reperfusion and preischemic periods were similar (30.2 ± 2.5 vs. 33.3 ± 1.3 msec, respectively). LVEDP, as index of diastolic stiffness, was approximately 13 mmHg at the end of the stabilization period and significantly increased reaching a value of approximately 40 mmHg at the end of reperfusion. IP treatment significantly reduced the LVEDP increase. These beneficial effects were lost when NO synthesis was inhibited by L-NAME (Fig. 3, A and B panels). Ischemic contracture was not modified by IP but was significantly increased when nitric oxide synthase (NOS) was inhibited with L-NAME.

No-reflow phenomenon, a disorder that interrupts the microcirculation during reperfusion, is also involved in reperfusion injury¹⁹. In our experimental preparation, at constant coronary flow (CF) changes of coronary perfusion pressure (CPP) produce changes in CR. Therefore, an increase of CPP and consequent increase of CR as occurs in ischemic control hearts would be an indication of sub-perfusion of myocardium and this could contribute to infarct generation. The increase of CR was significantly attenuated by IP treatment and abolished by NOS inhibition (Fig. 4).

Given that reactive oxygen and species (ROS) generation and the consequent tissue damage may be responsible for myocardial reperfusion injury, we next determined the impact of IP treatment on myocardial thiobarbituric acid reactive substances (TBARS) concentration –an indirect index of lipid peroxidation– and level of reduced glutathione (GSH). In control ischemic hearts TBARS increased and GSH decreased. These changes were significantly attenuated by IP treatment and reversed by NOS inhibition with L-NAME (Fig. 5).

Since ROS may stimulate the ERK1/2–p90^{RSK} pathway, the expression of the phosphorylated forms of those kinases at the end of the reperfusion period was determined. The IP treatment did not modify the level of P-ERK1/2 and P- p90^{RSK} (data not shown) indicating that both kinases are not involved in the cardioprotection achieved by the herbal extract.

Hearts treated with IP showed a significant increase of the expression of phosphorylated and total forms of e-NOS (P-eNOS/GAPDH = 55 ± 2% and P-eNOS/eNOS = 35 ± 1%) at the end of reperfusion period and both decreased in presence of L-NAME. In these hearts, it was also observed increases of P-Akt and P-GSK-3β, which were not significantly modified by NOS inhibition with L-NAME (Fig. 6). The total content of both enzymes (Akt and GSK-3β) did not change in the different interventions.

Figure 7 shows typical traces of swelling experiments (A) and the mean values of light scattering decrease (LSD, B panel) produced by the addition of 100 μmol/L Ca²⁺ to samples of mitochondrial suspension non-treated and treated with IP, L-NAME or L-NAME + IP. The

IP treatment decreased the LSD produced by Ca^{2+} addition (0.76 ± 0.05 vs. 1.28 ± 0.04 a.u.) and L-NAME abolished this change (1.08 ± 0.01 a.u.). The addition of cyclosporine A significantly attenuated the LSD produced by Ca^{2+} (0.10 ± 0.02 a.u., data not shown).

4. Discussion

The present study reinforces the concept of cardioprotection against ischemia-reperfusion injury exerted by an aqueous extract of IP showing by the first time its ability to decrease the cell death produced by 40 min of coronary artery occlusion and 60-min reperfusion in isolated heart. Simultaneously, an improvement of postischemic recovery of myocardial systolic and diastolic function was observed in hearts treated with IP. Additionally, an attenuation of non-reflow phenomenon, a reduction of oxidative stress and a preservation of mitochondrial integrity were also detected after IP treatment.

Different signalling pathways have been involved in the cardioprotection against ischemia-reperfusion injury²⁰⁻²². An abrupt oxidants and free radicals production takes place during the first minutes of reperfusion²³⁻²⁶. These molecules produce structural changes and functional alterations contributing to irreversible cell injury. During reperfusion, the production of the oxidants species exceeds scavenging capacity, resulting in oxidation of proteins and lipids leading first to reversible damage, and eventually to necrosis and/or apoptosis. On the other hand, Dumitrescu et al²⁷ provided evidence about that overproduction of O_2^- and hydroxyl radicals occurring during reperfusion as much as their secondary oxidant products could readily oxidize the essential cofactor of eNOS (tetrahydrobiopterin, BH4) converting NOS to an O_2^- producing enzyme. NO combines with O_2^- at a very fast rate to form peroxynitrite ($ONOO^-$) which acts on a wide variety of molecules producing oxidative damage²⁸. In the present study, the homogenate of hearts treated with IP exhibited higher GSH levels and lower lipid peroxidation than untreated hearts, evidencing the antioxidant action of IP. Thus, the diminution of oxidative stress found in hearts treated with IP could contribute to BH4 stabilization and to a higher NO bioavailability in that hearts compared to ischemic control hearts.

Several papers have demonstrated the protective role of NO during ischemia-reperfusion condition^{29, 30}, which is principally produced by phosphorylation of Ser1177 residue eNOS.

Several kinases and phosphatases control eNOS phosphorylation, including protein kinase C (PKC), Akt, ERK1/2, protein phosphatase 1 and protein phosphatase 2A³¹. In our experimental conditions, an increase of eNOS^{Ser1117} phosphorylation was detected in IP-treated compared to ischemic untreated hearts. The increase of the total form of eNOS by IP could be an indication of a transcriptional action herbal extract-mediated. Simultaneously, an increase of P-Akt and unchange of P-ERK1/2 and P-p90^{RSK} was obtained in hearts treated with IP, indicating that Akt is the kinase responsible of the activation/phosphorylation of eNOS. In addition, an increase of P-GSK-3 β was observed in hearts treated with IP. The L-NAME treatment abolished the cardioprotective effects of IP, showing greater infarct size and lesser content of P-eNOS without changes in P-Akt and P-GSK-3 β levels in comparison to IP treated hearts. Therefore, the Akt/eNOS-dependent pathway is primarily responsible for the beneficial action of IP.

Oxidants and free radicals are one of the triggers of mitochondrial permeability transition pore (mPTP) opening event associated to cell death^{32,33}. Our data in isolated mitochondria show that IP attenuates mPTP opening. This beneficial effect was annulled by L-NAME treatment. This result can be attributed to the inhibition of eNOS docked to outer mitochondrial membrane followed by a diminution of NO production³⁴. This finding is also indicating that the attenuation of mPTP opening achieved by IP is mediated by NO which exerts direct action on the pore³⁵. This action could explain the IP-evoked infarct size limitation.

A prolonged ischemic period followed by reperfusion also produces damage of coronary microvasculature which leads to lack of adequate tissue perfusion referred to as no-reflow phenomenon¹⁹. The oxidants species are implicated in that phenomenon³⁶. In animal models, extensive no-reflow was associated with worse infarct expansion³⁷. In a recent report, the eNOS phosphorylation was involved in the protection against no-reflow phenomenon³⁸. An increase of healthy blood flow (without ischemia) in patient after oral

administration of yerba mate tea was recently demonstrated³⁹. Our data show that the increase of CR detected in ischemic control hearts was significantly attenuated by IP. This result indicates that the herbal extract improves the cardiac perfusion, thus contributing to the decrease of cell death. This action could be attributed to an increased NO production via eNOS activation. An increase of the protein S-nitrosylation, previously described as a protective mechanism⁴⁰, could also be happening in the hearts treated with IP. On the other hand, the $O_2^{\cdot-}$ and $ONOO^-$ scavenging activity afforded by IP⁴¹ could increase the NO bioavailability, thus contributing to cardioprotection.

In summary, protective actions of IP could be mediated by stimulation of G-protein coupled receptor (GPCR)-dependent Akt/eNOS, transcriptional regulation or /and post-translational activation of eNOS and $O_2^{\cdot-}$ and $ONOO^-$ scavenging (Fig. 8).

Limitations

In the current study we demonstrated in a model of heart "ex vivo" the beneficial action of an *Ilex paraguariensis* extract against reperfusion injury. However, the complex composition of the extract and the low intestinal absorption of its constituents determine that our findings could not be extrapolated directly to human.

Conclusion

This study reveals that the treatment with "yerba mate" beverage- as an interesting source of phytochemicals- reduces infarct size, improves postischemic myocardial function and coronary perfusion, attenuates oxidative stress, and decreases the mitochondrial permeability. All these actions are mediated by an Akt/eNOS-dependent signaling pathway.

Despite of the low intestinal absorption of non-metabolized or biotransformed compounds of "mate", the bioavailability of them could be enough to interact with different targets promoting "in vivo" cardioprotection. Although future experiments are necessary for recognition and acceptance to IP extract in the prevention and/or therapy of human coronary heart disease, our results are encouraging.

Conflict of interests

None

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Legends

Figure 1: Scheme of the experimental protocols. NIC: non-ischemic control; IC: ischemic control; IP: aqueous extract of *Ilex paraguariensis*; L-NAME: inhibitor of NOS and L-NAME + IP.

Figure 2: Infarct size (IS), expressed as a percentage of risk area, in ischemic control (IC) and in hearts treated with IP, L-NAME or L-NAME + IP. Observe that IP treatment 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. IP

Figure 3: A) Changes of left ventricular developed pressure (LVDP), maximal velocity of rise ($+dP/dt_{max}$) and decrease ($-dP/dt_{max}$) of left ventricular pressure, expressed as percentage of preischemic values, at the end of reperfusion period, in ischemic control group (IC) and in hearts treated with IP, L-NAME or L-NAME + IP. B) Time reperfusion course of left ventricular end diastolic pressure (LVEDP, mmHg) in the groups mentioned above. Note that IP significantly improved the postischemic recovery of myocardial function and the NOS blockade with L-NAME abolished these changes. * $p < 0.05$ vs. IC; # $p < 0.05$ vs. IP.

Figure 4: Changes of coronary resistance (CR) expressed as $\text{mmHg/mL} \times \text{min}^{-1}$ in ischemic control hearts (IC) and in hearts treated with IP, L-NAME or L-NAME + IP. The treatment with IP significantly reduced the increase of CR detected in IC hearts and the NOS inhibition with L-NAME abolished this change. * $p < 0.05$ vs. IC; # $p < 0.05$ vs. IP

Figure 5: A) Thiobarbituric acid reactive substances concentration (TBARS), and B) reduced glutathione content (GSH) in ischemic control hearts (IC) and in hearts treated with IP, L-NAME or L-NAME + IP. The treatment with IP decreased TBARS and partially preserved the level of GSH. These beneficial effects were abolished by L-NAME. $p < 0.05$ vs. IC; # $p < 0.05$ vs. IP

Figure 6: Representative immunoblots of phosphorylated forms and summary of densitometry data of phospho-eNOS (P-eNOS, A panel), phospho-Akt (P-Akt, B panel) and

phospho-GSK-3 β (P-GSK-3 β , C panel) in cardiac homogenate of ischemic control (IC) and hearts treated with IP, L-NAME or L-NAME + IP. IP increased the expression of the three examined proteins. In L-NAME + IP the level of P-eNOS decreased and the contents of P-Akt and P-GSK-3 β were not significantly changed. * $p < 0.05$ vs. IC; # $p < 0.05$ vs. IP.

Figure 7: 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 IP, L-NAME or L-NAME + IP. The response of isolated mitochondria to Ca^{2+} was significantly attenuated by IP and restored when IP was administered after NOS inhibition with L-NAME. * $p < 0.05$ vs. IC; # $p < 0.05$ vs. IP.

Figure 8: Possible mechanisms of cardioprotection against reperfusion injury IP-mediated.

