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Comparative effects of natural products on ischemia-reperfusion injury: relation to their ¨*in vitro*¨ antioxidant capacity

[Efectos comparativos de productos naturales sobre la injuria por isquemia-reperfusión: relación con la capacidad antioxidante ¨*in vitro*¨]

Juliana Fantinelli[1], Luisa González-Arbeláez[1], Alejandro Ciocci-Pardo[1], Guillermo Schinella[2] & Susana M Mosca[1]

[1]*Centro de Investigaciones Cardiovasculares, Universidad Nacional de La Plata, La Plata, Buenos Aires, Argentina*
[2]*Cátedra de Farmacología Básica, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, CIC, Provincia de Buenos Aires, La Plata, Argentina*
Contactos | Contacts: **Susana M MOSCA - *E-mail address:*** smosca@med.unlp.edu.ar

Abstract: Our aim was to compare the effects of a non-alcoholic Cabernet-Sauvignon (CS), Malbec (M), Merlot blend (BW) red wine extracts, *Ilex paraguariensis* (Ip) or *Ilex brasiliensis* (Ib) aqueous extracts, *Vaccinium meridionale* Swartz (mortiño) fermented extract (FE), berry juice (BJ) and polyphenols-riched fractions of cocoa(PFC) against reperfusion injury. Isolated rat hearts were submitted to 20 min of global ischemia (GI) and 30 min of reperfusion (R). Other hearts were treated 10 min before GI and first 10 min of R with the extracts. CS, M, Ip, Ib and FE attenuated the myocardial dysfunction and oxidative damage whereas BW, BJ and PFC were ineffective. Paradoxically, PFC had the highest and BW similar scavenging activity than protective extracts. The beneficial actions were lost when nitric oxide synthase (NOS) was inhibited. These data indicate that ¨*in vitro*¨ antioxidant capacity of natural products is not primarily responsible for the cardioprotection being involved NO-dependent pathways...

Keywords: natural products extracts, antioxidant capacity, ischemia-reperfusion, nitric oxide

Resumen: Nuestro objetivo fue comparar los efectos de extractos no alcohólicos de los vinos tinto Cabernet-Sauvignon (CS), Malbec (M) y Merlot (BW), de extractos acuosos de *Ilex paraguariensis* (Ip) e *Ilex brasiliensis* (Ib), de un extracto fermentado (FE) de *Vaccinium meridionale* Swartz (mortiño), del jugo del mortiño (BJ) y de fracciones enriquecidas en polifenoles de cacao (PFC) sobre las alteraciones miocárdicas producidas por isquemia-reperfusión. Para ello, corazones aislados de rata fueron sometidos a 20 min de isquemia global (GI) y 30 min de reperfusión (R). Otros corazones fueron tratados 10 minutos antes de GI y durante los primeros 10 minutos de la R con los extractos. CS, M, Ip, Ib y FE atenuaron la disfunción contráctil postisquémica y el daño oxidativo mientras que BW, BJ y PFC fueron ineficaces. Paradójicamente, PFC mostró la más alta y BW similar actividad antioxidante que los extractos protectores. Las acciones beneficiosas fueron abolidas cuando la óxido nítrico sintasa (NOS) fue inhibida. Estos datos indican que la capacidad antioxidante ¨*in vitro*¨ de los productos naturales no es el principal responsable de la cardioprotección estando involucradas vías dependientes del NO.

Palabras clave: Extractos de productos naturales, capacidad antioxidante, isquemia-reperfusión, óxido nítrico

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INTRODUCTION

Reactive oxygen species (ROS) are characteristically unstable and very reactive oxygen-derived small molecules. In the cardiovascular system, ROS can be produced by several different cellular sources, including mitochondria, xanthine oxidases, lipoxygenases, cyclooxygenases, uncoupled nitric oxide synthase (NOS), peroxidases, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, as well as other heme-containing proteins (Zhang *et al.,* 2012). Superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase- as enzymatic antioxidant- and reduced glutathione (GSH), ascorbic acid, and tocopherol, are involved in the ROS detoxification (Baines *et al.,* 2005). When the endogenous antioxidant defences are not enough to counterbalance the abrupt ROS production the ¨oxidative stress¨ takes place. This misbalance has been implicated in many pathophysiological conditions one of which is during ischemia and reperfusion.

After a short episode of ischemia (< 20 min) a transient myocardial dysfunction takes place without histological signs of irreversible injury. This phenomenon was called 'myocardial stunning' (Braunwald, 1991). In this situation the abrupt ROS production occurring early on reperfusion is one of the responsible factors. According to this theory it would be possible to limit oxidative damage and ameliorate myocardial disease progression by supplementing antioxidants. Prevention rather than treatment of heart disease can significantly improve patients' quality of life and reduce health care costs.

A significant reduction in coronary artery disease incidence has been linked the consumption of herbal flavonoids (Keli *et al.,* 1996; Arts *et al.,* 2001; Mann *et al.,* 2007). This beneficial action is based on several clinical studies that positively correlate flavonoid intake to a reduced incidence of the disease. A meta-analysis of prospective cohort studies concluded that high flavonoid intake from fruits, vegetables, tea, and red wine is associated with a reduced risk of ischemic heart disease (Huxley & Neil, 2003; Li *et al.,* 2010). This effect has been associated to the high antioxidant power of those compounds. Indeed, numerous experimental studies show that natural antioxidants present in herbal extracts such as *Salvia miltiorrhiza*, *Dracocephalum moldavica L.* and *Scutellaria baicalensis* exert a cardioprotective effect against ischemia-reperfusion injury (Wang *et al.,* 2011; Ge *et al.,* 2014; Jiang *et al.,* 2014). In this sense, experiments performed in our laboratory show the efficacy of a non-alcoholic extract of red wine (Mosca & Cingolani, 2000; Mosca & Cingolani, 2002), aqueous extracts of *Ilex paraguariensis, Ilex brasiliensis* (Schinella *et al.,* 2009), and *Vaccinium meridionale* Swartz (Lopera *et al.,* 2013) in a model of a short period of ischemia inducing myocardial stunning. Unfortunately, most of the clinical trials carried out to test the ¨in vivo¨ efficacy of antioxidants could not measure any benefit of their administration (Levraut *et al.,* 2003; Halliwell, 2011). Thus, recent studies indicate that the radical scavenger property is unlikely to be the only reason for their cardioprotective actions and in fact, a wide spectrum of cellular signalling events may well account for their biological actions (Li *et al.,* 2013; Wu *et al.,* 2013).

Then, the aim of this study was to get insight about the mechanisms involved in the cardioprotective effects of different red wine varieties, *Ilex paraguariensis, Ilex brasiliensis*, *Vaccinium meridionale* Swartz, and polyphenol-rich cocoa extracts, assessing the contribution of their ¨in vitro¨ antioxidant properties. A high polyphenols content with high antioxidant capacity are the common factors of the natural products extracts selected for this investigation.

MATERIAL AND METHODS

Cabernet-Sauvignon (CS), Malbec (M) and CS-M-Merlot blend (BW) non-alcoholic red wine extracts, aqueous extracts of *Ilex paraguariensis* (Ip) and *Ilex brasiliensis* (Ib), fermented extract (FE) and berry juice (BJ) of *Vaccinium meridionale Swartz* and polyphenol-rich cocoa extracts (A, B and C) were selected as natural products.

Preparation of extracts

Red wine

Red wine (200 ml) was vacuum evaporated (< 30° C) to obtain 3-5 g of jelly-like extract. Then, this extract was solubilized in deionized double-distilled water and filtered. This resulting fluid was used in the experiments. CS, M and BW red wine were obtained from local supermarkets. The three red wines were from Mendoza (Argentina) and the year of production was 1998 or 1999.

Ip and Ib

Aerial parts of *Ilex brasiliensis* (Sprengel) Loes and *Ilex paraguariensis* St. Hilarie, both species of Aquifoliaceae family, were collected from Cerro Azul (Misiones, Argentina) in April 2002. A vouche specimen of both samples were deposited in the herbarium of the Museo de Botánica y Farmacognosia ¨Carlos Spegazzini¨ (Universidad Nacional de La Plata, Argentina) under the numbers LPE 1005 and 938, respectively. The dried and powdered leaves of both Ilex species were extracted with hot water (90° C), left standing for 20 min, filtered and lyophilised. The dry matter was maintained at -20° C until it was used. Both extracts were dissolved in distilled water immediately before performing all tests (Schinella *et al.,* 2009).

Vaccinium meridionale Swartz

The berry fruits were harvested at the beginning of December 2009 in Colombia, zone ¨El Retiro¨ (2175 m.a.s.l.) into Antioquia region. The berry juice (BJ) was transferred into stainless steel tank and the fermentation was carried out at 25 ± 2° C for 10 days and stopped by the addition of SO_2. This extract (FE) was decanted, treated with albumin, filtered and vacuum-evaporated. BJ was used as control. Both extracts were dissolved in distilled water immediately before performing all tests (Lopera *et al.,* 2013).

Polyphenol-rich cocoa extracts

A, B and C fractions of fresh cocoa pods of the Amazonic-Trinitary variety (CCN51 clone) from the Quevedo region in Ecuador were obtained following the instructions detailed in our recent publication (Schinella et al., 2010).

In vitro assays

All determinations were performed by spectrophotometric methods using Beckman DU®640 spectrophotometer.

Determination of total phenol content

Total phenol content of the extracts was determined using Folin Ciocalteu reagent (Singleton & Rossi, 1965). Gallic acid was used as standard and the calibration curve was prepared in the range 2-20 µg/mL (R^2 =0.9983, $p < 0.01$), and the results were expressed as gallic acid equivalents/mg extract.

Scavenging activities

1-Diphenyl-2-picryl-hydrazyl (DPPH)

Reduction of the stable free radical DPPH was determined with the aid of a modified version of the method described by Cavin *et al.* (1998).The results are expressed in μg caffeic acid equivalents/mg of dry weight of the extract.

Superoxide ($O_2^{\cdot-}$)

Superoxide -generated by enzymatic oxidation of hypoxanthine with xanthine oxidase- was determined following the nitroblue tetrazolium reduction at 560 nm in presence of the extracts as was previously described by Schinella *et al.* (2009).

Peroxynitrite Anion ($ONOO^-$)

$ONOO^-$ was synthesized in a quenched flow reactor in accordance with the method described by Koppenol *et al.* (1996). The pyrogallol red bleaching assay was carried out as reported by Balavoine and Geletii (1999).

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) 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/UE.

Male Wistar rats of 5-6 months of age were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg bodywt). The heart was excised and perfused through the aorta by the nonrecirculating Langendorff technique with Ringer's solution (pH 7.4) and at 37º C. Heart rate was maintained at 280 ± 10 beats/min. A latex balloon was placed inside the left ventricle (LV) and connected to a Statham P23XL pressure transducer. The balloon was filled with water to provide an end-diastolic pressure (LVEDP) of 8-12 mm Hg, and this volume was unchanged for the rest of the experiment. Coronary perfusion pressure was adjusted to approximately 60-70 mm Hg and coronary flow was 11 ± 2 mL/min. Left ventricular pressure (LVP) was acquired by using an analog to-digital converter and acquisition software (Chart V4.2.3AD Instruments Sudamérica, Santiago de Chile, Chile).

Experimental Protocols
After 10 min of stabilization, the following experimental protocols were performed:

Ischemic control (IC)
Hearts were subjected to 20 min of normothermic global ischemia followed by 30min 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.

Treated hearts
Hearts were treated 10 min before ischemia and the initial 10 min of reperfusion with 30 µg/ml of each one of the described extracts.

Other groups of hearts received 1mM of NG-nitro-L-arginine methyl ester (L-NAME), a nonselective nitric oxide synthase (NOS) inhibitor, 20 min before ischemia and during the reperfusion period.

Systolic and Diastolic Function
Myocardial contractility was assessed through the left ventricular developed pressure (LVDP), obtained by subtracting LVEDP to the LVP peak values and the maximal rise velocity of the left ventricular pressure ($+dP/dt_{max}$). Data were expressed as percentages of their respective preischemic values. The diastolic function was evaluated through the isovolumic LVEDP.

Oxidative damage of cardiac tissue
At the end of reperfusion, hearts were frozen in liquid N2 and kept at −70º C until the moment of assays.

Assessment of thiobarbituric acid reactive substances (TBARS) concentration
A portion of LV was homogenized in a solution composed by 25 mM KH_2PO_4-140 mM KCl. Then the samples were centrifuged. In the supernatant TBARS -an index of lipid peroxidation- was determined. This assay is based on the reaction of 2-thiobarbituric acid with malondialdehyde (MDA) to yield a chromophore with absorbance at 535nm (Buege & Aust, 1978). Data were expressed as nmol MDA/g tissue weight.

Reduced Glutathione (GSH)
Aliquots of homogenate were used to assess GSH according to Ellman's method (Sedlak & Lindsay, 1968) and expressed as µg GSH/g tissue weight.

Western Blot Analysis
Other portion of LV was homogenized in ice-cold RIPA buffer, centrifuged at 10000 × g for 15 min at 4º C. The supernatant was collected and subjected to SDS-PAGE. The samples were transferred to a PVDF membrane (2 h). Equal loading of samples was confirmed by Ponceau S staining. Membranes were blocked with 5% nonfat milk in Tris-buffered saline (pH 7.5) containing 0.1% Tween (TBS-T) and probed overnight at 4∘C with antibodies anti-eNOS (Sigma-Aldrich, St. Louis, MO, USA) and anti-Akt (Calbiochem, Merck Millipore Darmstadt, Germany). Membranes were washed four times for 10min in TBS-T prior to the addition of anti-rabbit secondary antibody (1 : 1000 dilution) and the antibody-antigen complexes were developed using a chemiluminescent system (ECL Plus; GE Healthcare, Buckinghamshire, UK).

Statistical Analysis
Data were expressed as means ± SE. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple comparisons test. Differences were considered significant at $P < 0.05$.

RESULTS
The extracts herein considered possess different phenolic compounds. Thus, the non-alcoholic extracts of red wine (RWE) possess a high content of flavonoids (anthocyanins and tannins) and non-flavonoids compounds, such as phenolic acids (Fanzone *et al.,* 2010; Granato *et al.,* 2011). The aqueous extracts of *Ilex* species (Ib and Ip) have phenylpropanoids and flavonols (Filip *et al.,* 2001; Filip *et al.,* 2008; Schinella *et al.,* 2009), cocoa extracts (A, B and C) flavanol monomers and dimeric procyanidins (Schinella *et al.,* 2010) and the extract obtained from *Vaccinium meridionale* Sw is enriched in anthocyanins (Garzón *et al.,* 2010; Lopera *et al.,* 2013).

Table 1 shows that the three red wine non-alcoholic extracts (CS, M and BW) exerted a similar DPPH reduction and $O_2^{\cdot-}$ scavenging and non-detectable $ONOO^-$ scavenging activity. These similarities were associated and/or attributed to a similar total phenols (TF) content found in the red wine extracts. Ib aqueous extract and B and C cocoa fractions showed the highest TF content whereas that Ib and C cocoa fraction showed the highest $ONOO^-$ scavenging activity. The berry juice (BJ) exhibited

the lowest values of all parameters, indicating the scarce antioxidant activity of that preparation. The B and C cocoa fractions also exhibited the highest values of $O_2^{\cdot -}$ scavenging activity.

Table 1
Total phenol content and ¨in vitro¨ assessment of antioxidant properties of the extracts
TF: Total phenol content; Scavenging activity of DPPH (2,2-diphenyl-1-picrylhydrazyl radical), $O_2^{\cdot -}$ (superoxide) and $ONOO^-$ (peroxynitrite). RWE: samples of non-alcoholic red wine extract (CS: Cabernet-Sauvignon, M: Malbec and BW: CS-M-Merlot blend; Ip: *Ilex paraguariensis* and Ib: *Ilex brasiliensis* aqueous extracts; FE and BJ: fermented extract and berry juice of *Vaccinium meridionale* Swartz; A, B and C: polyphenol-rich cocoa extracts.

Sample	TF (a)	DPPH (a)	$O_2^{\cdot -}$ (a)	$ONOO^-$ (b)
RWE				
CS	109.4 ± 17.2	27.0 ± 1.6	28,6 ± 2.0	ND
M	103.4 ± 9.9	23.4 ± 2.1	22,6 ± 2.8	ND
BW	115.5 ± 10.8	26.8 ± 1.9	26.7 ± 2.2	ND
Ilex				
Ib	350.0 ± 27.8	159.8 ± 12.3	25.9 ± 2.1	58,3 ± 6.3
Ip	230.0 ± 23.7	229.6 ± 19.1	31.1 ±2.8	8,3 ± 0.9
VMS				
FE	59.5 ± 3.7	23.0 ± 1.5	5.6 ± 0.4	5.0 ± 0.2
BJ	14.0 ± 0.8	5.0 ± 0.3	2.3 ± 0.3	1.3 ± 0.3
Cocoa				
A	106.5 ± 9.1	63.5 ± 5.1	8.0 ± 0.6	14.9 ± 0.9
B	759.3 ± 6.4	202.9 ± 16.8	68.4 ± 7.9	31.6 ± 2.7
C	1007.6 ± 15.7	456.7 ± 22.2	74.7 ± 5.8	63.8 ± 5.1

(a) Equivalent µg gallic acid/mg of extract
(b) Equivalent µg ascorbic acid/mg of extract
ND not determined

Figure 1 shows typical traces of LVP in untreated (upper panel) and treated (lower panel) hearts during the three steps of the experiment (stabilization, global ischemia and reperfusion). Treated heart showed an improvement of postischemic myocardial function (characterized by an increase of LVDP and a decrease in LVEDP) after the treatment with an extract with protective action.

LVDP and $+dP/dt_{max}$ -expressed as percentage of preischemic values- and LVEDP -expressed in mm Hg- at the end of reperfusion period in all experimental groups are depicted in Figure 2. Both parameters favourability changed when CS, M, Ip, Ib and FE extracts were administered compared to ischemic control hearts. Thus, in hearts treated with those extracts LVDP significantly increased reaching values of approximately 100 % in comparison to untreated ischemic hearts (58 ± 4%). The $+dP/dt_{max}$ showed a similar pattern. The increase of LVEDP detected in IC hearts was significantly attenuated by the treatments (6 to 19 for treated hearts vs. 44 mmHg in IC). However, when BW or BJ extracts were infused it was unable to mitigate the myocardial postischemic alterations. Severe arrhythmias and abnormalities in myocardial function were registered in hearts treated with A, B and C cocoa fractions.

An attenuation of cardiac oxidative stress submitted to ischemia-reperfusion was also detected after acute administration of the mentioned extracts. Thus, a decrease in TBARS concentration and a partial preservation of GSH was observed after CS, M, Ip, Ib and FE extracts administration (Figure 3). BW or BJ did not modify the values of those parameters observed in IC hearts.

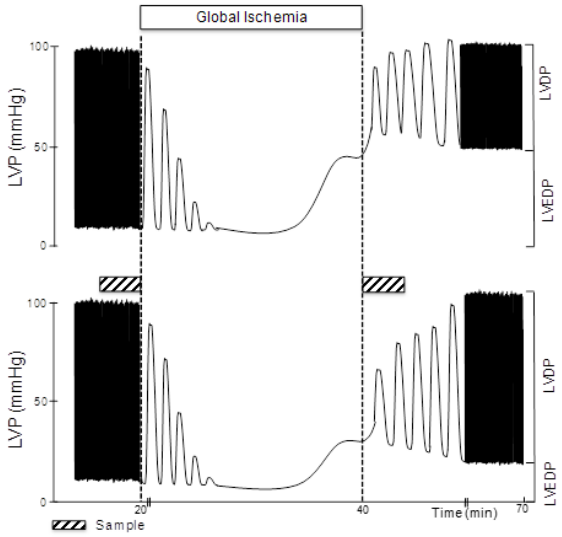

Figure 1
Typical traces of left ventricular pressure (LVP) during ischemia and reperfusion in untreated (upper panel) and treated hearts (lower panel). Note that the sample produced an increase of left ventricular developed pressure (LVDP) and a decrease of left ventricular end diastolic pressure (LVEDP).

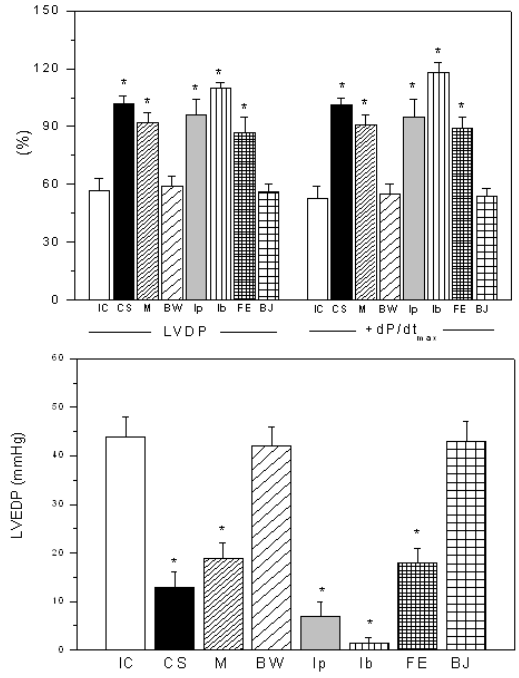

Figure 2
Values of left ventricular developed pressure (LVDP) and +dP/dt$_{max}$ -expressed as percentage of preischemic values- and left ventricular end diastolic pressure (LVEDP)- expressed in mmHg- at the end of reperfusion, in ischemic control hearts (IC) and hearts treated with extracts of CS, M, BW, Ip, Ib, FE and BJ.
*** p < 0.05 vs IC**

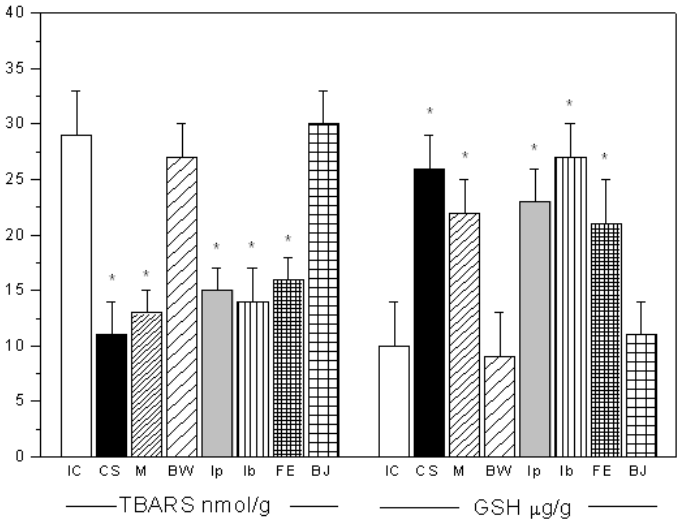

Figure 3
Thiobarbituric acid reactive substances (TBARS) concentration and reduced glutathione content (GSH) in IC and in hearts treated with CS, M, BW, Ip, Ib, FE and BJ. *p < 0,05 vs IC

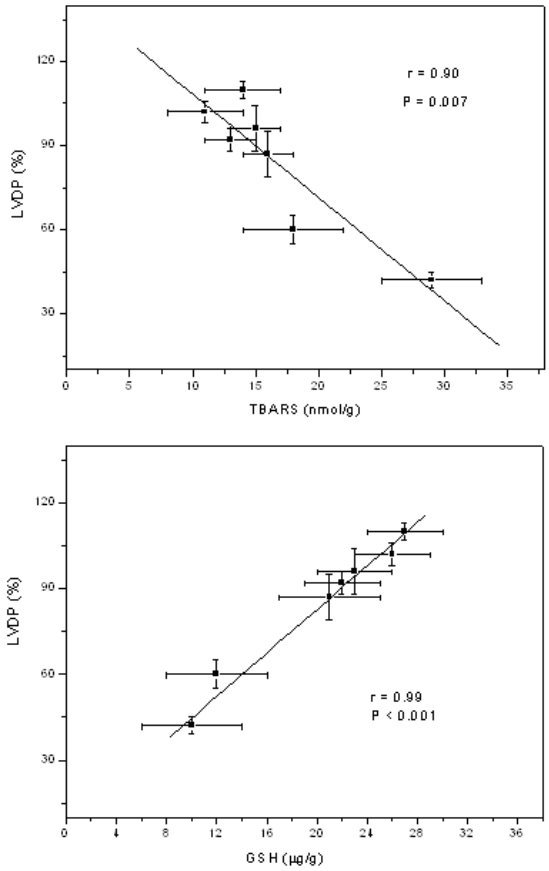

Figure 4
Relationship between left ventricular developed pressure (LVDP) and TBARS (upper panel) and GSH (lower panel) in all experimental situations. Data were fitted to straight line by linear regression and significant correlations were found.

Figure 4 shows the relationships between TBARS or GSH and LVDP. The analysis of data demonstrated the existence of a negative correlation between postischemic recovery of systolic function, assessed by LVDP and TBARS (an index of lipid peroxidation). Also, a positive correlation was found between GSH content and LVDP indicating that higher levels of GSH are associated to an improvement of recovery of myocardial function (lower panel).

A diminution of P-Akt and eNOS concentration was detected in IC hearts and this level increased after the treatment with CS, M, Ip, Ib and FE extracts (Figure 5) and were not modified when BW and BJ were added.

The blockade of NOS with L-NAME abolished the improvement of postischemic recovery of systolic function and the attenuation of diastolic stiffness afforded by the extracts. In this condition, a decrease of eNOS and P-Akt content was also evident (data not shown).

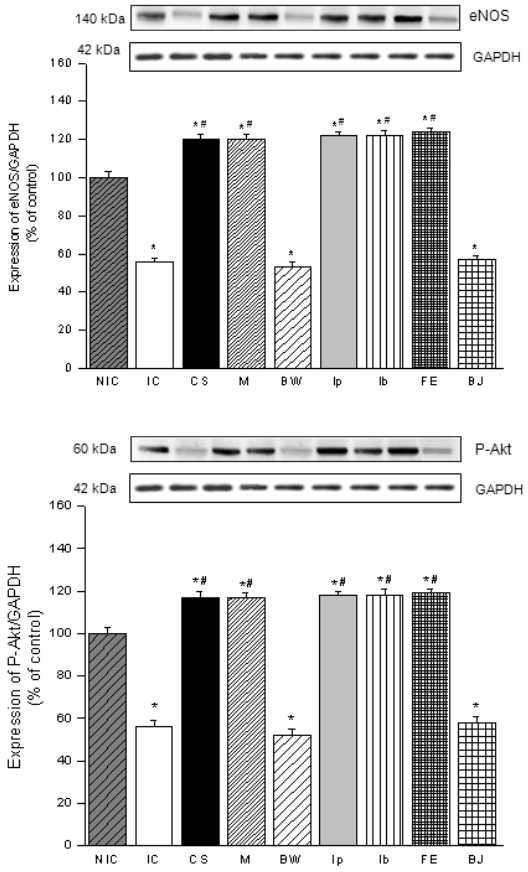

Figure 5
Representative immunoblots and summary of densitometry data of eNOS (A panel), and phospho-Akt (P-Akt, B panel) in cardiac homogenate of ischemic control (IC) and hearts treated with CS, M, BW, Ip, Ib , FE and BJ extracts. * p< 0.05 vs. IC.

DISCUSSION

Our data show that non-alcoholic extracts of CS and M red wine, aqueous extracts of two *Ilex* species (Ip and Ib) and *Vaccinium meridionale* Sw fermented extract attenuate the myocardial contractile dysfunction and oxidative damage produced by ischemia and reperfusion. However, the BW, BJ and polyphenols-riched fractions of cocoa are not beneficial in the same conditions.

It is recognized that Ca^{2+} overload linked to a large burst of ROS which takes place early during reperfusion are responsible of the transitory postischemic dysfunction (Takano *et al.,* 2003; Yellon & Hausenloy, 2007) having to the

mitochondrial permeability transition pore (mPTP) as the end target (Wong *et al.,* 2012). Therefore, any treatment able to reduce the intracellular Ca^{2+} concentration or ROS would be beneficial. All the extracts used in this study exhibited a significant ¨in vitro¨ antioxidant capacity. Then, the improvement of postischemic myocardial function observed after the acute treatment with the extracts could be ¨a priori¨ attributed to their antioxidant properties. This conclusion is invalid because the cocoa fractions which possesses the highest phenols content and $O_2^{\cdot-}$ and $ONOO^-$ scavenging activity were not effective to attenuate the myocardial dysfunction. These data constitute a first and strong evidence of a lack of relationship between both actions. Thus, a high ¨*in vitro*¨ antioxidant capacity of an extract is not a ¨sine qua non¨ condition to protect the heart against reperfusion injury.

Our data also show that the improvement of myocardial dysfunction was accompanied by an attenuation of oxidative stress evidenced by a diminution of TBARS (as index of lipid peroxidation) and a significant increase of GSH content. These results suggest that a low ROS production or release could be occurring when those protective extracts are administered to the hearts before and after ischemia.

It is known that NO is released during ischemia and at the beginning of reperfusion by activation of NOS, particularly the endothelial isoform (Schulz *et al.,* 2004). Also, the ROS-mediated oxidation of eNOS essential cofactor tetrahydrobiopterin (BH4) turns to the enzyme in a system of $O_2^{\cdot-}$ production (Dumitrescu *et al.,* 2007). The interaction of $O_2^{\cdot-}$ with NO produces ONOO- which causes nitration of tyrosine residues. Thus, both process -protein nitration and lipid peroxidation- are contributing to the postischemic oxidative damage (Valko *et al.,* 2007). In this sense, it was previously described that the balance between NO and ROS generation, the so-called ‘‘nitroso/redox balance’’ plays a crucial role in the modulation of ischemic alterations (Heusch *et al.,* 2008; Nediani *et al.,* 2011). In this study, the western blot analysis showed an increased eNOS expression in hearts treated with protective extracts but was not observed with non-protective extracts (BW and BJ).

Current evidence suggest that the protective effect occurs activating pro-survival signalling cascade which involve among others the phosphatidylinositol 3-kinase/Akt (PI3K/Akt), the extracellular signal-regulated kinase (ERK1/2) and adenosine monophosphate-activated kinase (AMPK) (Rusell *et al.,* 2004; Hausenloy & Yellon, 2007). Recently, Akt and ERK1/2 appeared involved in the green tea-mediated beneficial effects (Kim *et al.,* 2014). On the other hand, it has been previously reported the ability of plant-derived agents to activate AMPK (Srivastava *et al.,* 2012) and the AMPK-mediated eNOS phosphorylation and activation (Young, 2008). In our experimental conditions, an activation of Akt in treated hearts with the protective extracts was also observed. This change occurred simultaneously with those of eNOS indicating that Akt-eNOS pathway could be involved in the cardioprotection.

Which are the targets of NO? Previous reports show that the beneficial actions of NO are mediated by an attenuation of mPTP opening GMP_C - dependent or independent and probably through the mitochondrial ATP-dependent K channels (mitoKATP) opening (Sasaki *et al.,* 2000; Schulz *et al.,* 2004). The participation of these channels in the cardioprotection exerted by a non-alcoholic extract of CS red wine was previously demonstrated by us (Mosca & Cingolani, 2002). These actions could explain a lesser mitochondrial ROS release and/or production and the consequent lesser oxidative damage observed after the effective treatments.

All data obtained in this study indicate that the protective effects of the extracts on the alterations derived from ischemia and reperfusion are mediated by NO-dependent cascades.

Which compounds would be responsible of the cardioprotection? Although the ¨*in vitro*¨ experiments performed in this study are crucial to screen the effects, security, efficiency, and other biochemical parameters, the extract-derived bioactive molecules cannot be identified. Furthermore, these compounds possess several modes of action, establishing synergic, antagonist, and polyvalent relationships with other compounds, besides suffering chemical changes due to organic metabolism. In our experimental conditions we can only ensure that the principal components of the polyphenol-rich cocoa extracts (procyanidinB2, epicatechin) are not participate in the cardioprotection.

CONCLUSION

The present study demonstrates that although the extracts have significant antioxidant capacities not all are beneficial against ischemia-reperfusion injury. This fact is the first indication that ¨in vitro¨ antioxidant capacity does not keep strict correlation with the protective effect on postischemic myocardial function. Therefore, we propose that some components present in the extracts of natural products by interaction with membrane receptors are able to activate eNOS via PI3K/Akt. The consequent increase of NO production could exert its beneficial actions limiting the mPTP opening (Figure 6).

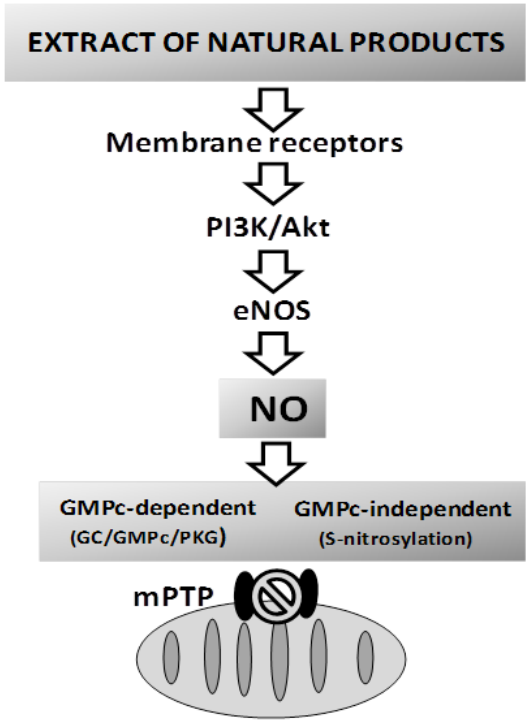

Figure 6
Proposed mechanism for the NO-dependent cardioprotection against ischemia-reperfusion injury afforded by the extract of natural products

Finally, the discrepancies in the outcome of intervention studies may be understood if, instead of considering the simple paradigm of bad oxidants and good antioxidants, scientists will start to talk about the real molecular function of such compounds in each particular situation. Hence, although contradictory results were obtained in the trials about the effects of antioxidant supplementation, the recommendation of a healthy diet, rich in fruits and vegetables and whole brain foods, is still standing.

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