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Ação cardioprotetiva da fração ácida do extrato de *Ilex paraguariensis St. Hil (Aquafoliaceae)* em miocárdio de *Rattus norvegicus* durante a isquemia e reperfusão experimental *in vivo*

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Lista de abreviaturas

5HD – 5-Hidoxydecanoic acid sodium salt

ABAP – 2, 2´-azobis 2 methylpropionamidine dihydrochloride

ACAP – Antioxidant competence against peroxyl radicals – Competência antioxidante contra radicais peroxil

ATP – Trifosfato de adenosina – Adenosine triphosphate

BHT – Butylated hydroxytoluene

EIp – Extrato de *Ilex paraguariensis* – *Ilex paraguariensis* extract

ERN – Espécies reativas de nitrogênio

ERO – Espécies reativas de oxigênio

H_2DCF-DA – 2′,7′-dichloro- dihydrofluorescein diacetate

I/R – Isquemia e reperfusão – ischemia and reperfusion

Ip – *Ilex paraguariensis*

LDL – Low Density Lipoprotein - Lipoproteína de baixa densidade

L-NAME – N^G-nitro-L-arginine methyl ester

LPO – Lipid peroxidation – peoxidação lipídica

MAPK – Mitogen- actived protein kinase

MDA – Malondialdehyde - malondialdeído

NAC – N-acetylcysteine

NO – Óxido nítrico – Nitric oxide

OMS – Organização Mundial da Saúde

PMSF – Phenylmethanesulphonylfluoride

RONS – Reactive nitrogen species

ROS – Reactive oxygen species

TBA – 2-thiobarbituric acid

TBARS – Thiobarbituric acid reactive substances Assay

TMP – Tetramethoxypropane

TTC – Triphenyl tetrazolium chloride – cloreto de trifenil tetrazólio

Resumo Geral

A doença cardiovascular é a causa líder de morte no mundo e no Brasil. Nas últimas décadas, alcançou 32% das causas de mortalidade geral. A isquemia e a reperfusão no miocárdio levam a mudanças metabólicas e da função cardíaca, incluindo depressão na atividade contrátil e mesmo necrose tecidual. É sabido que os habitantes do interior dos estados brasileiros dependem de produtos naturais para os primeiros cuidados de saúde. A erva-mate *Ilex paraguariensis* é comumente utilizada nos estados sul, sudeste e centro-oeste do Brasil para preparação de bebidas alimentícias e estimulantes, como o chimarrão. Vem sendo mundialmente consumida e utilizada para obtenção de produtos fitoterápicos. Considerando que os problemas da isquemia e reperfusão (I/R), estão relacionados a eventos oxidativos e a existência de produtos naturais com potencial para a redução dos danos miocárdicos, este trabalho teve como objetivo avaliar os efeitos de uma fração ácida rica em flavonóides do extrato *I. paraguariensis (Ip)*, no modelo de isquemia e reperfusão induzidas *in vivo* em *Rattus norvegicus*. Ratos foram divididos em 4 grupos: Controle (animais submetidos a toracotomia sem oclusão da coronária anterior descendente esquerda); I/R (submetidos a 15 min de isquemia e 15 min de reperfusão); I/R+Ip (além de I/R o grupo recebia injeção i.v. de fração ácida rica de flavonóides do extrato de *I. paraguariensis*); e I/R+Ip+L-NAME (além do protocolo de I/R+Ip o grupo recebeu o inibidor da óxido nítrico sintase L-NAME). Ao final da I/R foi injetado (i.v.) 0,5mL de Azul dde Evans 10% para a identificação da área de risco. Os corações foram congelados e fatiados para que esta área fosse medida. Após, as fatias foram incubadas em TTC 1% para demarcação do tecido viável e não-viável. Para aumentar o contraste entre as diferenças no tecido as amostras foram incubadas em formalina 10%. As imagens foram analisadas no software Image Tool. Para determinação da geração de espécies reativas de oxigênio amostras do tecido cardíaco foram homogeneizadas, centrifugadas e a concentração de proteínas foi verificada pelo método de Biureto. Após as amostras foram distribuídas em microplacas e foi acrescido H_2DCF-DA que na presença de ERRO gera um composto fluorescente. As placas foram lidas em fluorímetro (488nm de excitação e 525nm de emissão). A capacidade antioxidante contra

radicais peroxil foi avaliada. A metodologia é conduzida como o teste para a geração de ERO. No entanto, neste caso a fluorescência produzida pelo DCF é aumentada por ABAP. As leituras foram realizadas em fluorímetro (485nm de excitação e 520nm de emissão). Para a determinação da peroxidação lipídica amostras do miocárdio foram homogeneizadas e submetidas a um método de reação de MDA com TBA em condições á altas temperaturas e meio ácido. Esta reação gera uma fluorescência que foi lida em fluorímetro (515nm excitação e 553nm de emissão). Animais tratados com a fração ácida de flavonóides de *Ip* demonstraram redução nas áreas de risco e infarto quando comparados ao ratos I/R. A avaliação do estado oxidativo demonstrou que os animais submetidos a I/R e tratados com o extrato não apresentaram os clássicos efeitos antioxidantes dos flavonóides de redução da condição de estresse oxidativo do tecido. Ainda assim o efeito cardioprotetor observado na redução da área de lesão, nos leva a sugerir que outros mecanismos que não aqueles antioxidativos, estejam sejam desencadeados pelos flavonóides da *Ip*, como a produção de NO e seu posterior benefício via vasodilatação, melhora da perfusão miocárdica e da função cardíaca durante a I/R.

Palavras chaves: *Ilex paraguariensis – isquemia – reperfusão – estado oxidativo – área de infarto*

Introdução Geral

As transformações econômicas, políticas, sociais e culturais produzidas pelas sociedades ao longo do tempo modificam o estilo de vida da população. Tais modificações repercutem diretamente no padrão de adoecimento das populações. Consideradas como epidemias na atualidade, as doenças crônicas não-transmissíveis constituem um sério problema de saúde pública mundial (Brasil, 2008).

A Organização Mundial da Saúde (OMS, 2005) define como doenças crônicas as doenças cardiovasculares (cerebrovasculares e isquêmicas), as neoplasias, as doenças respiratórias crônicas, diabetes *mellitus* (todas apresentando fatores de risco em comum) além das desordens mentais e neurológicas, doenças bucais, ósseas e articulares, desordens genéticas e as patologias oculares e auditivas (OMS, 2005)

Os fatores de risco ao surgimento das doenças crônicas podem ser classificados em "não modificáveis" (sexo, idade e herança genética) e "comportamentais" (tabagismo, alimentação, sedentarismo, consumo de álcool e outras drogas e estresse). Os fatores de risco comportamentais são potencializados pelos fatores condicionantes socioeconômicos, culturais e ambientais. O cenário contemporâneo potencializa os fatores de risco associados ao sedentarismo, à alimentação com excesso de gorduras, açúcares e sal, ao consumo de tabaco, ao uso abusivo de álcool e outras drogas e às atitudes violentas na mediação de conflitos. Ao mesmo tempo, reduz-se a ação dos fatores protetores como o acesso a alimentos *in natura* e de melhor qualidade nutricional, a existência de redes de suporte social e de espaços públicos seguros e facilitadores de interação social por meio de práticas esportivas e culturais entre outros (Brasil, 2008; Hoffman et al., 1996).

A transição epidemiológica caracteriza-se pela mudança do perfil de morbidade e mortalidade da população, com diminuição progressiva das mortes por doenças infecto-contagiosas e elevação das mortes por doenças crônicas. No Brasil, nas últimas décadas, a mortalidade por doenças infecto-contagiosas e parasitárias caiu de 46% (em 1930) para 5,3% (em 2005), enquanto as mortes por doenças e agravos não transmissíveis chegaram em

2005 a representar dois terços da totalidade das causas conhecidas. As doenças que afetam o aparelho circulatório passaram de 10% para cerca de 32% no mesmo período (Brasil, 2008).

A doença cardiovascular é a causa líder de morte no mundo e há expectativa de que ela seja a principal causa de morte na América Latina nas próximas décadas. O crescimento econômico da região e a melhora de indicadores sociais têm elevado a expectativa de vida da população, e, embora estudos prévios venham descrevendo os fatores de risco cardiovascular, o conhecimento sobre o impacto de cada um deles sobre a doença arterial coronariana ainda é limitado (Lanas, 2007).

Dentre as cardiopatias, a isquemia e posterior reperfusão (I/R) são dois fenômenos sabidamente lesivos à integridade funcional do miocárdio, que podem levar ao infarto do miocárdio e à morte celular. A isquemia é definida como sendo o fluxo arterial insuficiente para manter as funções normais teciduais, isto é, a diminuição de nutrientes para os tecidos e o retardo na retirada dos metabólitos. A isquemia pode ser total quando o fluxo arterial for insuficiente para manter a vida celular ou tecidual, ou parcial, a qual mantém a viabilidade celular, porém com risco de evoluir para a morte celular, dependendo da nobreza do tecido e do tempo da isquemia (D'alecy and Zelenock, 1990). A reperfusão, processo de reestabelcimento da perfusão tecidual após a isquemia, é um evento que ativa monócitos, macrófagos e neutrófilos levando a um aumento da produção e liberação dos radicais livres do oxigênio no tecido cardíaco (Evora et al., 1996).

Durante a isquemia a deficiência de oxigênio leva a mudanças no metabolismo e na função cardíaca, incluindo depressão na atividade contrátil e mesmo necrose tecidual (Ek et al., 2008). Evidências indicam que vários fatores inter-relacionados, incluindo a diminuição nos níveis de ATP celular, a produção de espécies reativas de oxigênio (ERO), o acúmulo de íons hidrogênio, a geração de espécies reativas de nitrogênio (ERN), o aumento da concentração de cálcio intracelular, e a ativação de leucócitos, contribuem para os danos causados pela I/R-induzida, podendo esta determinar diversos graus de injúria conforme sua duração (Powers et al., 2008).

De acordo com Powers et al. (2008) três níveis de danos são descritos para I/R-induzida. No primeiro nível, surgem as arritmias cardíacas. Em geral, a

reperfusão após 1-5 minutos de isquemia pode resultar em taquicardia ou fibrilação. O segundo nível de danos é observado quando a reperfusão decorre após 5-20 minutos de isquemia, resultando em paralisia do miocárdio (do inglês *heart stunning*), caracterizado por um déficit na contractibilidade função da morte celular. O terceiro e maior nível de dano ocorre quando a isquemia é superior a 20 minutos, quando o dano irreversível resulta na morte celular por necrose ou por apoptose. Estes autores reforçam a idéia de que os danos mitocondriais possuem papel fundamental para a evolução da morte celular.

Os problemas causados pela I/R apresentam origem no desequilíbrio no status oxidativo. Já é conhecido que muitos produtos naturais apresentam potencial para reduzir os danos causados pela I/R. Tendo em vista o alto custo dos medicamentos, os produtos naturais podem contribuir como adjuvantes no tratamento de doenças.

Segundo Akerele (1993) cerca de 65 a 80% da população mundial, principalmente moradores do interior, depende essencialmente de produtos naturais para os primeiros cuidados de saúde e muitos estudos demonstram a existência nestes produtos naturais de componentes protetores às doenças cardiovasculares. No Brasil, apenas 20% da população é responsável por 63% do consumo dos medicamentos sintéticos disponíveis, sendo que o restante encontra nos produtos de origem natural, especialmente plantas medicinais, a principal ou a única fonte de recursos terapêuticos (Reis et al., 2004 *apud* Rates, 2004). Apontando positivamente para este cenário está o fato de que o Brasil é o país com a maior diversidade genética vegetal do mundo, contando com mais de 55.000 espécies catalogadas de um total estimado entre 350.000 e 550.000 (Guerra and Nodari, 2004).

Os efeitos cardioprotetivos de muitos produtos naturais tem sido atribuídos aos compostos flavonóides (Rice-Evans et al., 1995), que são polifenóis antioxidantes encontrados em vegetais, frutas e bebidas como chás e vinhos (Hertog et al., 1993). Como toda molécula com propriedades antioxidantes, estes flavonóides são capazes de atrasar o processo oxidativo, inibindo a cadeia de polimerização pelos radiais livres e as reações oxidativas subseqüentes (Halliwell and Aruoma, 1991).

Muitos estudos experimentais têm conseguido demonstrar os efeitos protetivos de produtos naturais e flavonóides durante o processo de isquemia e

reperfusão. Danos causados pela isquemia e reperfusão renal induzida foram minimizados em coelhos quando tratados com extrato bruto de chá verde (Rah et al., 2007). Também foi observado que substâncias encontradas em ervas muito utilizadas na medicina chinesa como, *Pueraria lobata* e *Salvia miltiorrhiza*, são capazes de diminuir a área lesada pela I/R, a peroxidação lipídica, e podem aumentar os níveis da enzima superóxido dismutase (Wu et al., 2007). Substâncias biologicamente ativas extraídas de *Camellia oleifera*, diminuem os níveis de espécies reativas de oxigênio e de lipídeos peroxidados e aumentam significativamente os níveis de superóxido dismutase, catalase e glutationa proxidase (Chen et al., 2007).

Além da capacidade antioxidante, também tem sido demonstrado que extratos naturais são capazes de induzir a vasodilatação através da ativação da enzima óxido nítrico sintase, responsável pela produção de óxido nítrico pelas células endoteliais. Segundo Baron-Menguy e colaboradores (2007), também o vinho tinto apresenta compostos fenólicos com poder vasodilatador. Extrato aquoso de bulbos de *Fritillaria sp*, uma planta muito utilizada na medicina chinesa, também possui ação vasodilatadora NO-induzida (Kang et al., 2004).

Dentre os produtos naturais que apresentam propriedades benéficas está a erva mate. Esta planta é muito utilizada na região Sul do Brasil devido a costumes da região.

A erva-mate, *Ilex paraguariensis,* é um arbusto característico de plantas de sub-bosque, pertencente a um agrupamento vegetal típico do sul do Brasil, conhecido como "formação de araucária", sendo característica de regiões com altitude acima de 400 metros (Costa, 2002). Pertencente à família Aquifoliaceae o gênero *Ilex* possui mais de 550 espécies, das quais 60 ocorrem no Brasil, mas apenas cinco delas se prestam ao beneficiamento para consumo (Anuário Brasileiro da Erva-Mate, 1999).

As plantas são dióicas, sendo que cada árvore produz somente flores masculinas ou somente flores femininas e a floração ocorre de setembro a dezembro, predominando em outubro. O amadurecimento dos frutos se dá de janeiro a março (Anuário Brasileiro da Erva-Mate, 1999). As melhores condições de desenvolvimento, bem como de longevidade, sanidade e produtividade da erva-mate estão intimamente ligados à fertilidade do solo e à

sua exploração racional (Costa, 2002). Ocorrem naturalmente em solos profundos; bem drenados; ácidos ou ligeiramente ácidos; argilosos, argilo-silicosos ou sílico-argilosos, ou parcialmente arenosos (Anuário Brasileiro da Erva-Mate, 1999; Costa, 2002).

A área de distribuição natural da erva-mate abrange aproximadamente 540.000 Km^2, compreendendo territórios do Brasil, da Argentina e do Paraguai, onde têm grande importância econômica e cultural (Grigoletti Jr. et al., 1997). Só no Brasil, os ervais nativos se estendem por 450.000Km^2, abrangendo a região centro-norte do Rio Grande do Sul, quase todo o estado de Santa Catarina, centro-sul e sudoeste do Paraná, sul do Mato Grosso do Sul e reduzidas áreas em Minas Gerais e São Paulo (Costa, 2002; Grigoletti Jr. et al., 1997).

As folhas estabilizadas (sapecadas) e rasuradas são utilizadas para preparação de bebidas alimentícias e estimulantes, como o chá, chimarrão, e tererê, as quais são típicas de cada região. Além disso, também vem sendo consumida no mercado europeu como matéria-prima para produtos fitoterápicos indicados como auxiliares em regimes hipocalóricos e como diuréticos (Rates, 2004). A produção e comercialização do produto no Brasil é regulamentada pelo Ministério da Saúde, Divisão de Alimentos (Portaria 464/97) e os critérios de qualidade encontram-se fixados nas portarias 233/98 e 234/98 da Secretaria de Vigilância Sanitária.

A erva-mate apresenta em sua constituição química vitaminas, aminoácidos, saponinas triterpênicas (Schenkel et al., 1997 *apud* Rates, 2004), açúcares, compostos fenólicos, principalmente ácido clorogênico (ac. 3-cafeoilquímico) e seus produtos de oxidação, metilxantinas (0,7 a 2,3% de cafeína, 0,3% de teobromina e traços de teofilina) (Graham, 1984), flavonóides como: quercetina e rutina (Furlong et al., 2003).

A *Ilex paraguariensis* é capaz de interferir no sistema circulatório, por apresentar efeitos antioxidantes que auxiliariam na prevenção de doenças cardiovasculares (Gugliucci and Stahl, 1995; Gugliucci, 1996). A erva-mate também é um protetor contra a oxidação de DNA e de LDL e tem se mostrado um grande aliado na prevenção de câncer (Heck and Mejia, 2007).

Outros trabalhos já observaram seu efeito vasodilatador e hipotensor dependente do endotélio no mesentério isolado de ratos (Muccillo-Baisch et al.,

1998; Stein et al., 2005). Neste último, foi constatada ainda a redução dos níveis de colesterol e triglicerídeos em ratos tratados cronicamente com o extrato bruto de *Ilex paraguariensis*. Estes efeitos são mediados através da via de produção do óxido nítrico. O mesmo efeito vasodilatador foi observado quando os animais foram tratados com extrato ácido e em menor proporção naqueles que receberam extrato aquoso. Recentemente, a ação hipotensora e hipocolesterolêmica do extrato aquoso bruto de *I. paraguariensis* foi demonstrada para ratos machos hipertensos e hipercolesterolêmicos, durante treinamento de natação de baixa intensidade (Wally, 2007).

Considerando que os problemas cardiovasculares, sobretudo relativos a morbidade e mortalidade advindos da isquemia e reperfusão, estão relacionados a eventos oxidativos exacerbados pela I/R, e que existem produtos naturais com potencial etnofarmacológico para a redução dos danos induzidos pela I/R, capazes de serem utilizados como adjuvantes na terapia tradicional dada sua aceitação entre certas populações, este trabalho pretende avaliar os efeitos cardioprotetivos de uma fração ácida rica em flavonóides do extrato de erva-mate *Ilex paraguariensis*, no modelo de isquemia e reperfusão induzidas *in vivo* em *Rattus norvegicus*.

Objetivo Geral

Investigar as propriedades cardioprotetivas e antioxidantes da fração ácida rica em flavonóides do extrato de *Ilex paraguariensis* (EIp) no sistema cardiovascular de ratos machos Wistar durante a indução de isquemia e reperfusão (I/R) *in vivo*.

Objetivos específicos

Os objetivos específicos deste projeto são:

- Determinar a área de lesão miocárdica na I/R-induzida;
- Quantificar a produção de ERO pelo tecido miocárdico após a I/R-induzida;
- Testar a capacidade antioxidante total do tecido miocárdico após I/R-induzida;
- Quantificar a formação de lipídios peroxidados pelo tecido miocárdico após a I/R-induzida;
- Avaliar a influência do óxido nítrico, após I/R-induzida, na presença ou ausência do inibidor da óxido nítrico sintase, L-NAME;

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Cardioprotetive effect of the rich flavonoid extract from *Ilex paraguariensis* during experimental *in vivo* ischemia and reperfusion

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Abstract

Aim of the study. Cardiovascular disease is the leading cause of death worldwide. Ischemia and myocardial infarction leads to changes in metabolism and cardiac function, including depression in contractile activity and even tissue necrosis. It is known that the inhabitants of the interior of the Brazilian states rely on natural products for the first health care. Mate *Ilex paraguariensis* is commonly used in south, southeast and central west of Brazil for the preparation of food beverages and stimulants, such as "mate". As many natural products have potential to reduce myocardial damage, and can be used as adjuvant to traditional therapy given its acceptance among populations, this study aimed to evaluate the effects of an acidic fraction, rich flavonoids extract from *I. paraguariensis (Ip)*, in the model of experimental I/R *in vivo* in *Rattus norvegicus*.

Materials and Methods. Rats were divided into four groups: control, I /R (15 min I/ 15 min R); I /R + Ip (I/R and *iv* injection of acidic fraction, rich flavonoid extract of *Ip*) and I/R + *Ip* + L-NAME (I /R + *Ip*, and treatment with nitric oxide synthase inhibitor L-NAME). The risk and infarcted areas, by Evans Blue and Triphenyl Tetrazolium chloride were determinate. Generation of reactive oxygen species (assayed by 2´,7´-dichlorodihydrofluorecein diacetate indicator), lipid peroxidation (reaction of malondialdehyde) and antioxidant competence (against peroxyl radicals assayed by the generator 2,2´-azobis 2 methylproprionamidine dihydrochloride) of the myocardium were evaluated.

Results. Animals treated with the acidic fraction of flavonoids *Ip* showed reduction at risk and infarcted areas when compared to I/R rats. Animals

subjected to I/R and treated with the extract did not show the classic antioxidant effects of flavonoids to reduce oxidative stress.
Conclusions. The cardioprotective effect observed in the reduction of lesion area, leads us to suggest that flavonoids in the preparation have been able to directly or indirectly stimulate NO production and may have led to improved myocardium perfusion and cardiac function during ischemia by stimulating the repair of myocardial damage and consequent reduction of risk and infarcted areas.

Key-words: *Ilex paraguariensis – ischemia – reperfusion – oxidative status – infarcted area*

1. Introduction

When myocardial ischemia develops coronary blood supply is difficult and becomes insufficient in relation to heart energetic demands (Weber and Janicki, 1979). It promotes tissue necrosis, changes in metabolic patterns and cardiac dysfunction as contractility depression (Hearse, 1990; Harden et al., 1979). Myocardial ischemia–reperfusion (IR) injury is a major contributor to the morbidity and mortality associated with coronary artery disease (Reeve et al., 2005). The level of IR-induced myocardial injury may range from a small insult resulting in limited tissue damage to a large injury leading to cellular death.

Cardiac injury induced by I/R is a duration depending process. First detectable level of injury is the generation of reperfusion-induced cardiac arrhythmias. In general, reperfusion after 1–5 min of ischemia may result in ventricular tachycardia or fibrillation without cell death or a deficit in ventricular contractile performance (Downey, 1990). Reperfusion after 5-20 min ischemic event results in the second level of myocardial injury, known as “myocardial stunning” (Downey, 1990; Bolli and Marban, 1999). Myocardial stunning is characterized by a deficit in myocardial contractility that occurs without myocardial cell death. Typically, I/R-induced myocardial stunning results in ventricular contractile deficits lasting 24–72 h after the IR event (Bolli and Marban, 1999). The third and harvest level of IR injury occurs with 20 min or more ischemia. In these circumstances, cardiac myocytes become irreversibly damaged, resulting in cell death (Downey, 1990). It is now clear that I/R-induced cardiac myocyte death occurs due to both apoptosis and necrosis and that mitochondrial injury plays a major role in both forms of cellular death (Gottlieb, 2001; Honda et al., 2005; Nakagawa et al., 2005).

Several interrelated factors, including a decrease in cellular ATP levels, production of ROS, accumulation of hydrogen ions, generation of reactive nitrogen species (RNS), calcium overload, calpain activation, and leukocyte activation, contribute to I/R injury (Bolli and Marban, 1999; Gottlieb, 2003; Green and Kroemer, 2004; French et al., 2006; Hoffman et al., 2004; Solaini and Harris, 2005; Zweier et al., 2001; Zweier and Talukder, 2006). Experimental evidence for the involvement of ROS/RNS in myocardial I/R injury includes detection of lipid peroxides, protein oxidation, and protein nitration products in reperfused hearts (Adderley and Fitzgerald, 1999; Demirel et al., 1998;

Hamilton et al., 2003; Powers et al., 1998; Zweier et al., 2001). The importance of ROS-mediated damage to the heart after an I/R insult has been confirmed by studies indicating that antioxidants can provide myocardial protection against IR-induced injury (Adlam et al., 2005; Angelos et al., 2006; Coombes et al., 2000; Hamilton et al., 2003). It is also pointed out that ROS are produced in both ischemic and reperfused myocytes from a variety of sources, including NADPH oxidase, xanthine oxidase, and mitochondria (Adlam et al., 2005; Angelos et al., 2006), and mitochondrial ROS production is a dominant step to I/R-mediated oxidative injury in the heart (Adlam et al., 2005; Angelos et al., 2006; Downey, 1990; Li and Jackson, 2002). It is noteworthy that calcium overload in the cell plays an important role in mediating the production of ROS from each of these sources. Moreover, neutrophil infiltration during reperfusion can contribute to ROS production in the heart (Li and Jackson, 2002).

According to Halliwell and Aruoma, (1991) antioxidants are substances that delay the oxidation process, inhibiting the polymerization chain initiated by free radicals and other subsequent oxidizing reactions.The use of traditional medicine is widespread and plants are still present as a large source of novel active biological compounds with different activities, including anti-inflammatory, anti-cancer, anti-viral, anti-bacterial and cardioprotective activities (Yan et al, 2002). The numerous beneficial effects attributed to phenolic products (Hertog et al., 1995; Stoner and Mukhtar, 1995) have given rise to a new interest searching for botanical species with high phenolic content and relevant biological activity. Epidemiological studies report an inverse association between polyphenol consumption such as fruits and vegetables, tea, and red wine and mortality from cardiovascular diseases and cancers (Baron-Menguy et al., 2007; Hertog et al., 1993; Kang et al., 2004; Rah et al., 2007; Schinella et al., 2005; Schinella et al., 2009).

Several studies have been demonstrated that many edible plants may have compounds that could be protective against cardiovascular diseases (Baron-Menguy et al., 2007; Chen et al., 2007; Hertog et al., 1993; Kang et al., 2004; Rah et al., 2007; Schinella et al., 2005; Schinella et al., 2009; Wu et al., 2007). Mate tea or simply ‘‘mate” is a traditional beverage from Brazil, Argentine, Uruguay and Paraguay, with a ethnical significance, as a intrinsic costume from these populations, specially for that of the countryside (Bracesco

et al., 2010). It is prepared as infusions or decoctions of the dried and minced leaves and twigs of the native South America *Ilex paraguariensis* (St. Hil), Aquifoliaceae. Its effects upon the circulatory system are appreciated by those who drink the mate to reduce hypertension as a diuretic agent (Mazzafera, 1994). In the last decades, the admiration for mate properties and history has spread to many areas including the Middle East, particularly Syria, Lebanon, Israel, countries which approximately import a 65% of total Argentinian yerba mate exportation. Recently, researchers had demonstrated a high antioxidant activity of aqueous extracts of *I. paraguariensis* (Filip et al., 2000; Gugliucci, 1996; Schinella et al., 2000, Schinella et al., 2005, Schinella et al., 2009). This extract also produced a significant attenuation of myocardial stunning and lipid peroxidation in an global *in vitro* ischemia and reperfusion model (Schinella et al. 2005).

Considering the relevance of cardiovascular problems elicited by ischemia and reperfusion insults, plus the importance of to know the proprieties and to incentives the uses of natural compounds derived from ethnoproducts with high acceptance by local populations to complement a tradicional therapy, the aim of this work is evaluate the cardioprotective effects of the rich flavonoid fraction of the extract of *Ilex paraguariensis* during ischemia and reperfusion induced *in vivo* in the *Rattus norvegicus* model.

2. Materials and Methods

2.1. Animal model and care

Rattus norvegicus Wistar (sixty males), ageing average of nine-months-old, were obtained from the Central Animal House of the Universidade Federal do Rio Grande – FURG, Brazil, weighting 312±10g. Animals were housed in plastic cages (five animals/ cage) and maintained in a temperature controlled room (24±1°C), with a 12h light-dark cycle and were feed commercial rodent food (15g/animal/day) according AIN-93 (Reeves, 1997) and water "*ad libitum*". Animal care followed the official governmental guidelines, in compliance with the Federation of Brazilian Societies from Experimental Biology and the study

was approved by the Ethic Committee Research of Health (CEPAS No. 92/2008) from the Universidade Federal do Rio Grande – FURG.

2.2. Experimental groups

The rats were divided into four groups: Control (which suffer the surgical procedures only); I/R (submitted to fifty minutes of ischemia followed by fifty minutes of reperfusion); I/R+Ip (a I/R group treated with an acid fraction flavonoid rich extract of *Ilex paraguariensis*); and I/R+Ip+L-NAME (like I/R+Ip group with oxide nitric inhibitor L-NAME injected ten minutes before ischemia). The acid fraction of Ip (900µg/50 µL) and L-NAME (10mM/50 µL) were administered by intravenous bolus injection (Muccillo-Baisch et al. 1998). The final doses administered considering the mean weight of rats (312g) and a mean blood volume of 64 mL/kg was 45 µg/mL of acid fraction Ip and 6,75 µg/mL of L-NAME per animal. The acid fraction Ip was administered thirty seconds before ischemia and L-NAME before tracheotomy.

2.3. Preparation of acid rich flavonoids fraction from the Ilex paraguariensis extract

Acid rich flavonids fraction from *I. paraguariensis* extract was prepared based on the methodology of Stein et al. (2005). Commercial rich leaves handcraft triturate was extracted by boiling (55 g) in distilled water-ethanol solution (4:6) for 15 min. The strained extract was evaporated with a rotatory evaporator at 78°C to remove the ethanol (Fisaton, Brazil). The aqueous residue was extracted with chloroform to remove lipids, carotenoids, sterols, and chlorophyll. The aqueous suspension was then evaporated to remove the solvent and extracted with ethyl acetate to remove saponins. The aqueous suspension was then dissolved in n-butanol (n-BuOH) in order to obtain a rich flavonoid fraction. The solvent was evaporated and the residue was taken up in 1% NaOH to remove phenolic compounds. The pH was adjusted for 4 – 5 and extractable in n-Butanol. The n-Butanol fraction was evaporated with a rotator evaporator. It was stored at -18ºC and diluted immediately before use with Krebs Ringer bicarbonate solution.

2.4. Ischemia-Reperfusion Protocols

Ischemia/Reperfusion was conducted following the methodology of Kobayashi et al. (2008). Briefly, rats were anaesthetized with 40 mg Kg^{-1} and 20 mg Kg^{-1} of the sodium thiopental and xylazine intraperitoneal (i.p.), respectively. Trachea was cannulated for artificial respiration, using an adapted mechanical ventilator pump ventilated with room air. Femoral vein was cannulated for drugs administration. Thoracotomy was performed horizontally in the fourth intercostal space, about 2 mm to the left of sternum. After pericardium incision, the heart was exteriorized. A nylon thread (6-0) was passed around the left anterior descending coronary artery between the pulmonary artery and the left atrial appendage. The ends of the thread were passed through a small bead to form a snare.

A small plastic bead was threaded through the ligature and placed in contact with the heart. The coronary artery could then be occluded by applying strength to the ligature, and reperfusion was achieved by releasing tension.

2.5. Tissue samples

By the end of the reperfusion period the heart was rapidly excised and washed inside and outside by physiological solution (0,9% NaCl) for blood clean up. Auricles from ventricules were separated and ventricules were divided into two samples for measured lipid peroxidation (frozen at -70ºC) and for generation of oxidative species (ROS) and total capacity against peroxyl radicals (ACAP) processed immediately.

2.6. Infarct size measurement

Measure infarct size induced by I/R in 28 rats (n= 7/group) followed Kobayashi et al. (2008) methodology. At the end of the reperfusion period, 0,5mL of 10% Evans Blue was injected via femoral vein to denote the risk area (the area affected by the ischemic event) still with coronary occlusion. The hearts were then excised and frozen -15ºC. Once frozen, the hearts were small sliced transversely. Evans blue solution stained the perfused myocardium, while the occluded vascular bed remained unstained. To distinguish between risk area and infarcted area (the myocardium area when the ischemic event promote cellular death), the slices were incubated in 1% triphenyl tetrazolium

chloride (TTC) at 37ºC for 20 minutes, and fixed in 10% formalin solution to enhance the contrast between the viable and non-viable tissue. When the myocardium was functional (no cellular death, with intact dehydrogenase enzyme system) the tissue stained in dark red, whereas the infarcted tissue did not react with TTC and remained pale. Each slice was photographed by stereo microscope (Leica® model DMLS), coupled to a computer, after Evans Blue (risk area) and TCC (infarcted area) dyings. To quantify the area at risk and infarcted images were analyzed by the software Image Tool (mm^2). By means of comparison the values from risk area and infarcted area of the I/R group was considerate a hundred percent and values from I/R+Ip and I/R+Ip+L-NAME groups was expressed as a percentage of it.

2.7. Generation of reactive oxygen species (ROS)

Myocardium tissue was homogenized according to Bello-Klein et al. (2006) methodology and generation of ROS followed Amado et al., (2009). Briefly, left ventricle tissue (approximately 100 mg) was homogenized in KCl 1.15% and PMSF (10 mmol/L) for 30s, followed by 3000 ×g centrifugation for 10 min, at for 4 ℃. Total protein content was determined by colorimetric assay (Biuret method LABTEST kits), in triplicate, using a microplate reader (BioTek LX 800) at 550 nm. Each sample was diluted to 2 mg of protein/ml, in homogenization buffer. ROS generation was assayed by 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) indicator (Invitrogen, Carlsbad, CA, USA). Non-fluorescent compound H_2DCF-DA is first de-acetylated and then oxidized by ROS to the fluorescent compound DCF, which is detected at 488 and 525 nm, for excitation and emission, respectively, into a fluorescence microplate reader (Victor 2, Perkin Elmer). Readings were performed every five min during 90 min. Fluorescence data were adjusted to a second order polynomial function and integrated in order to calculate the area that expressed ROS concentration (Amado et al. 2009).

2.8. ***Antioxidant competence against peroxyl radicals (ACAP)***

Total antioxidant competence against peroxyl radicals is a new method described by Amado et al. (2009) which determine the tissue capacity to confront peroxyl radicals. According to these authors, peroxyl radicals are

produced by thermal decomposition of the generator 2, 2´-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM; Aldrich) at 35°C. Our assays were accomplished at 37°C because this temperature is indicated for mammalian models. This methodology is conducted as an ROS assay, but in this case DCF production is increased by ABAP-peroxyl radicals generation. ROS concentration in the presence of ABAP is also expressed by the area calculated from the second order polynomial function, resulted from the adjustment of fluorescence units along the measurement time. Total antioxidant competence was expressed as relative area calculated by the rate of the difference between [ROS] area with ABAP and without ABAP divided by [ROS] area without ABAP, as a standardization factor of ROS background production.

ACAP= ROS area ABAP – ROSareaBrackground / ROSareaBrackground

2.9. Determination of Lipid Peroxidation (LPO)

Myocardium samples (50mg) were homogenized with KCL 1.15% plus 35 mM of butylated hydroxytoluene (BHT) according Oakes and Van Der Kraak (2003). This method involves the reaction of malondialdehyde (MDA), a degradation product of lipid peroxidation, with 2-thiobarbituric acid (TBA) under conditions of high temperature and acidity to generate a fluorescent adduct that was measured spectrofluorometrically (Victor 2, Perkin Elmer) after excitation at 515 nm and emission of 553 nm. The concentration of TBARS (nmols/mg of proteins) was calculated employing tetramethoxypropane (TMP, Across Organics).

2.10. Statistical Analysis

Data were expressed as mean ± S.D. ANOVA assumptions of normality and homocedasticicity were tested by Kolmogorv-Smirnof and Levene´s Tests and when was not reached, data was log-10 transformed. Data were performed by one-way ANOVA, followed by *a posteriori* Tukey HSD test. Statistical significance was accepted if $p \leq 0.05$.

3. Results

3.1. Infarct Size Measurements

Surgical procedures did not affect the myocardium of the control rats, where no lesion was observed (Figure 1A). Risk and Infarcted areas was significantly reduced by Ip extract (Figure 1C) ($p<0,05$) L-NAME independently, when related to I/R group (Figure 1B). Risk area values from I/R+Ip (1,51±31,8 mm^2) and I/R+Ip+L-NAME (1,55±14,7mm^2) decreased 37% and 34%, respectively, considering I/R (2,37±10,7mm^2) rats (Figure 2). A similar pattern was verifyed to infarcted area. I/R+Ip (1,39±35,3 mm^2) and I/R+Ip+L-NAME (1,29±20,9mm^2) showed 20% and 22% lesion reduction in relation to I/R group (2,12±10,5 mm^2).

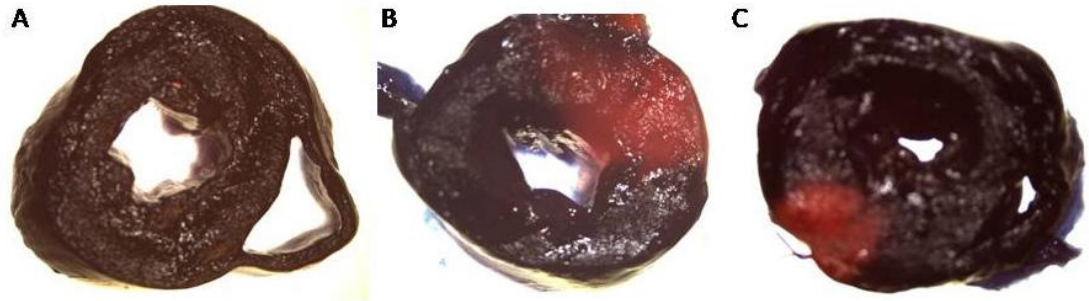

Figure 1 - Area of risk in different groups undergoing I/R and treated with acid fraction of rich flavonoids of *Ilex paraguariensis (Ip)* colored by Evans Blue and Triphenyl Tetrazolium Chloride, TTC. A - control group without ischemic lesion, B - I/R group: rats that underwent induction of ischemia and reperfusion, with ischemic area uncolored by Evans Azul (red tissue portion) and C - I/R+Ip: the group treated with 900µg of the acid fraction of rich flavonoids of *Ilex paraguariensis* (Ip) before induction of I/R, showing a reduction in the ischemic area, denoted by a smaller uncolored area.

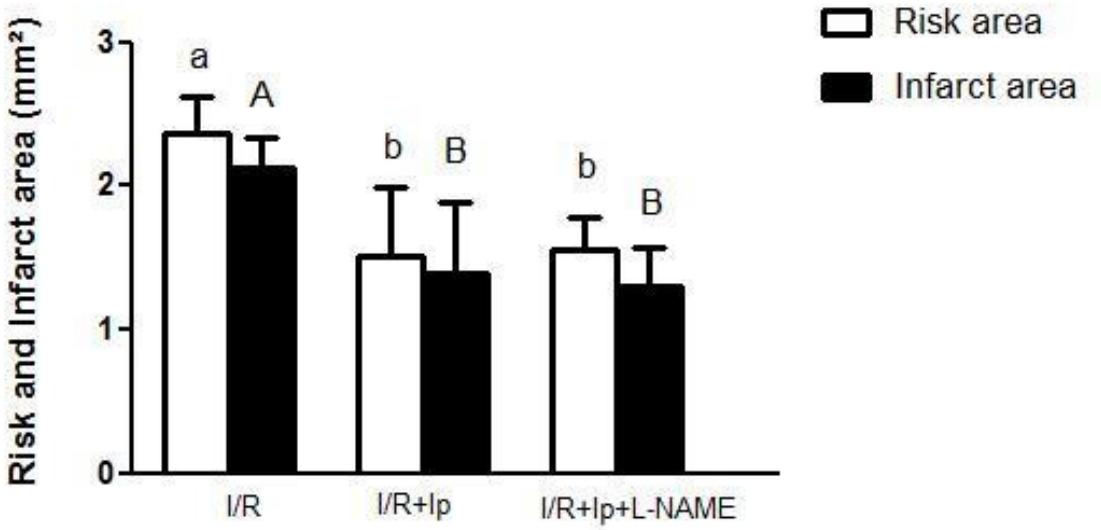

Figure 2 - Risk and infarcted areas in experimental I/R myocardial rats. Bars represent mean (mm^2) areas of risk and infarction. I/R - animals that underwent experimental ischemia and reperfusion *in vivo*; I/R+Ip – I/R animals treated with 900µg of the acid fraction rich of flavonoids of *Ilex paraguariensis* (Ip); I/R+Ip+L-NAME – I/R animals treated with *Ip* and 10 mM L-NAME (inhibitor of nitric oxide synthase). Different lowercase letters mark significant differences to the area of risk and different capital letters mark the significant differences in infacted area ($p<0.05$).

3.2. Oxidative status

ROS generation was significantly higher in the three experimental groups than control ones ($p<0,05$), without differences between then (Figure 3). A highest ACAP against peroxyl radicals was performed by I/R group (Figure 4), while ACAP from I/R+Ip and I/R+Ip+L-NAME was similar to that of control animals. LPO concentration was increased to I/R+Ip and I/R+Ip+L-NAME in relation to control group (Figure 5). I/R rats were not significantly different from any other group.

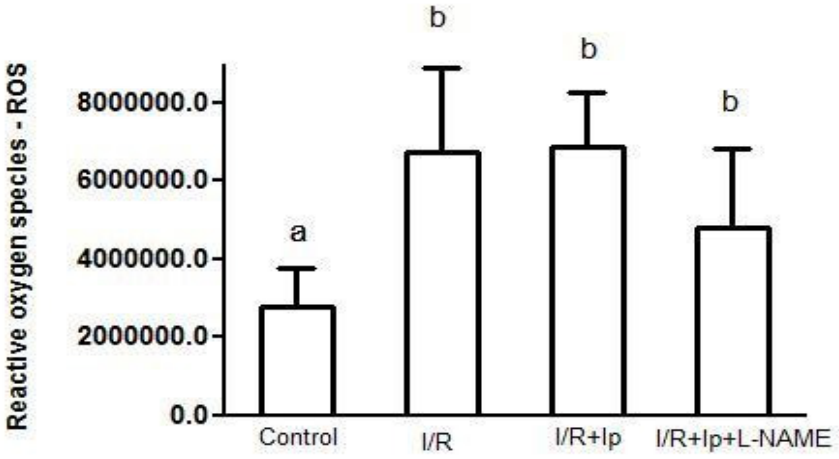

Figure 3 - Generation of reactive oxygen species (ROS) in cardiac tissue induced I/R (Arbitrary units). I/R - animals that underwent induction of ischemia and reperfusion, I/R+Ip - the group treated with 900µg of the acid fraction of rich flavonoids of *Ilex paraguariensis* (Ip) before induction of I/R, I/R+Ip+L-NAME - treated group with 900µg of Ip and 10 mM L-NAME (inhibitor of nitric oxide synthase). Different lowercase letters mark significant differences ($p<0.05$).

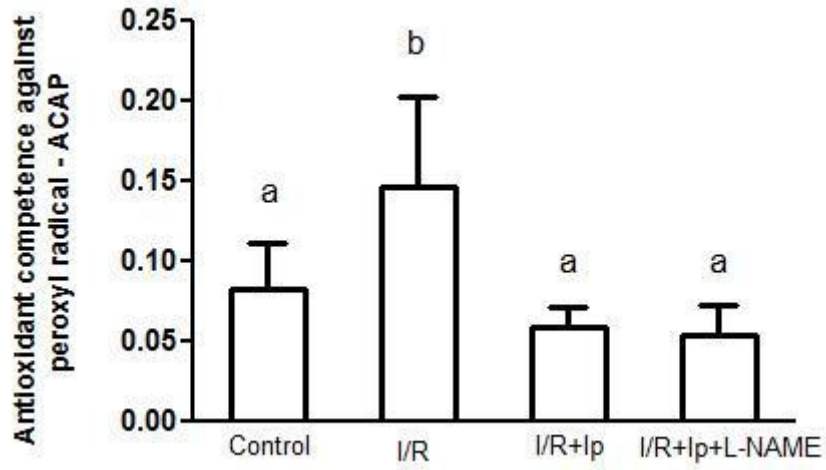

Figure 4 - Antioxidant competence peroxyl against radicals (ACAP) in cardiac tissue induced I/R (Arbitrary units). I/R - animals that underwent induction of ischemia and reperfusion, I/R+Ip - the group treated with 900µg of the acid fraction of rich flavonoids of *Ilex paraguariensis* (Ip) before induction of I/R, I/R+Ip+L-NAME - treated group with 900µg of Ip and 10 mM L-NAME (inhibitor of nitric oxide synthase). Different lowercase letters mark significant differences ($p<0.05$).

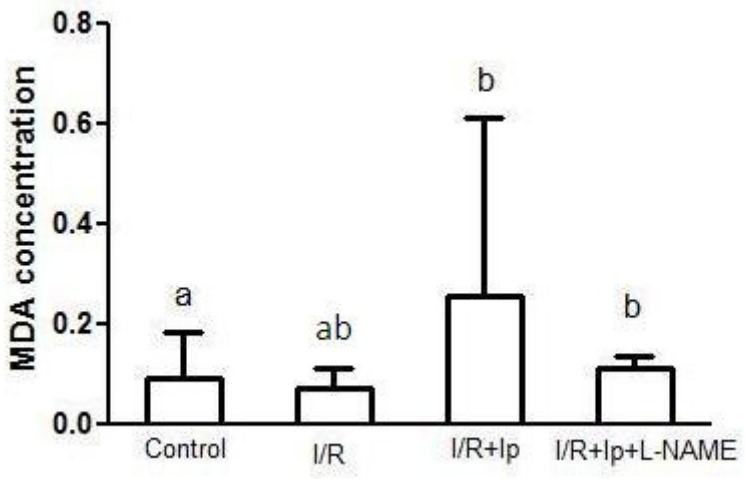

Figure 5 - Determination of lipid peroxidation by measurement of malondyaldeid MDA concentration in the heart of rats subjected to I/R. I/R - animals that underwent induction of ischemia and reperfusion, I/R+Ip - the group treated with 900µg of the acid fraction of rich flavonoids of *Ilex paraguariensis* (Ip) before induction of I/R, I/R+Ip+L-NAME - treated group with 900µg of Ip and 10 mM L-NAME (inhibitor of nitric oxide synthase). Different lowercase letters mark significant differences ($p<0.05$).

4. Discussion

Several studies have shown protective effects against cardiac damage produced by I/R, attributed to extracts of natural products. In their work, Rao et al. (2005) observed infactedion extension reduction in myocardium of rats, induced by 30 minutes of ischemia and 4 hours of reperfusion *in vivo*, after chronic treatment (7 days) with alcoholic extract of *Tinospora cordifolia*. Using similar protocol of I/R, it was possible to reduce the area of infactedion in a dose-dependent manner, in rats subjected to 30 minutes of ischemia and 120 minutes of reperfusion, treated with *propofol* (an anesthetic with molecular structure similar to α-tocopherol) (Kobayashi et al., 2008). However, some studies, focus on the effects of natural products and drugs in models of I/R in isolated hearts. According to Thuc et al. (2010) *pravastatin* (a drug class of statins - a cholesterol blood lower) administered immediately before global ischemia (20 min of global ischemia and 30 minutes of reperfusion) in isolated heart of rats is able to reduce significantly the infactedion area. The cardioprotective effect of this drug is diminished in the presence of L-NAME (blocker of nitric oxide synthase), N-acetylcysteine (NAC, a ROS interceptor ROS) and 5-Hidoxydecanoic acid sodium salt (5HD, a channel of mitochondrial

K + ATP-sensitive inhibitor) (Thuc et al., 2010). Our data demonstrate that acute administration of 900µg of a rich flavonoid fraction of yerba mate *I. paraguariensis* is able to lower both the infactedion area, agreeing with other articles, as well as the risk area, not commonly observed in the literature up to this moment. It is known that the damage caused by reperfusion (reperfusion injury) is proportional to the damage suffered during ischemia (ischemic injury) (Pasqualin and Auler Jr, 2008) and involves the production of ROS, increased intracellular calcium and hydrogen, activation of proteases and the reduction of ATP (Powers et al., 2008). We could considering that the risk area is a mark of the total commitment of tissue after I/R damage, whereas the infacteded area, represents the final damage, after the intrinsic capacity of the tissue revert at least, partially, the I/R lesion. By the way, we can suggest that the rich flavonoid acid fraction of *I. paraguariensis* could be promoting the reduction of both infacteded and risk areas by means of vasodilatation and than producing a less severe ischemic event, and a proportionally minor reperfusion injury.

Unlike other studies, our results could not demonstrate the involvement of NO in reducing the risk area and lesion area, since the group treated with extract and L-NAME, showed the same results of the group that received only the extract. With the same dose of L-NAME, in the study by Stein et al. (2005) it was possible to abolish the vasodilatation effect of the rich flavonoid acid fraction of *I. paraguariensis* in isolated rat mesentery. However, most studies that were able in demonstrating the influence of NO using a continuous dose of L-NAME before ischemia that was maintained during all or part of the reperfusion period (Schinella et al., 2005; Thuc et al., 2010). We believe that the influence of NO cannot be observed, because the administration of L-NAME via bolus 10 minutes before ischemia was not sufficient to maintain the inhibition of nitric oxide synthase in the entire period of *in vivo* I/R.

The extract of yerba mate *I. paraguariensis* (raw or fractions) is known for having high antioxidant activity (Anesini, 2006; Bastos et al., 2006; Bixby et al., 2005; Schinella et al., 2009), decreased levels of lipid peroxidation (Schinella et al., 2000 ; Schinella et al., 2005; Schinella et al., 2009) and decreased production of ROS (Schinella et al., 2000). Contrary to this, in this study, ROS production was increased not only in the ischemic group, but also in the groups treated with the extract (Ip and Ip + L-NAME). Given that our extract

has a rich fraction of flavonoids and that these molecules possess antioxidant activity and are recognized as scavengers of superoxide anion (Chun and Lee, 2003; Jovanivic and Simic, 2000), peroxyl radicals (Boadi et al., 2005; Nakao et al., 1998) and peroxynitrites (Pollard et al., 2006), we suggest that the lack of attenuation in ROS production, may be a dose-dependent effect. By injecting the extract at a concentration of 900µg *in vivo*, this dose suffering a dilution to 45 µg/ml of blood was not capable to promote the expected reduction of ROS. However, some studies showed an antioxidant effect of raw *I. paraguariensis* extract with a final dose of 30 µg/ml, but in a model of I/R in isolated hearts, with continuous administration of extract before the ischemic event and keeping the administration during reperfusion (Schinella et al., 2005). The complexity of the model *in vivo* in the presence of all functional physiological mechanisms of the animal may explain why a final dose of 45 µg/ml of the flavonoid preparation administered in *bolus* is not able to promote the lowering effect of ROS observed by other studies, even with smaller doses in the isolated organ model.

Many studies have assessed the total antioxidant activity of natural products. Schinella et al. (2009) showed that the raw *I. paraguariensis* and *I. brasiliensis* has a high antioxidant capacity *in vitro*. Also through *in vitro* tests, Bixby et al. (2005) observed that infusion of *I. paraguariensis* has 70% more antioxidant capacity than green tea, *Camellia sinensis*. There is a positive correlation between antioxidant capacity and phenolic concentration of the extract, and *I. paraguariensis* is pointed out to have the highest concentrations of these, when compared to several types of red wine and green tea (Bixby et al., 2005).

Unexpectedly, the antioxidant competence against peroxyl radicals was higher in the ischemic group. This increase in myocardial competence could be expected since the I/R induces increased production of ROS (Akhlaghi and Bandy, 2009, Powers et al. 2008). In a recent study on the effect of *pravastatin* during I/R in isolated hearts and rat cardiomyocytes, Thuc et al. (2010) concluded that the generation of ROS may serve as intracellular signaling for phosphorylation pathways and activation of antioxidant enzymes such as catalase. It is therefore possible that the increase of ACAP in ischemic rats, observed here, is a process stimulated by I/R damage. However, and surprisingly, the ischemic animals treated with Ip extract, had ACAP values

similar to control ones. In a recent review on the protection of flavonoids against myocardial I/R, Akhlaghi and Bandy (2009) point out that although the principal known effect of flavonoids is their antioxidant activity (direct chelation of ROS, inhibition of ROS formation, inhibition of lipid oxidation, and induction of antioxidant enzymes) there are other mechanisms of direct action of the flavonoid molecule modulating the action of enzymes and inducing genes that may have cardioprotective responses. The lower antioxidant power of the hearts of rats treated here with the rich flavonoid acid fraction of *I. paraguariensis*, leads us to suggest that it promotes other intracellular signaling, capable of bringing the benefits already described for the injured myocardium, but without stimulating the intrinsic tissue antioxidant competence. A critical look should be put to the technique that measures ACAP, because only the competence against peroxyl radicals is being evaluated and may other defense systems could be stimulated by I/R or by the flavonoid extract Ip.

Some studies have shown that natural extracts and substances with antioxidant properties, significantly reduced the levels of lipid peroxidation during I/R *in vivo* (plant extract of *Tinospora codifolia* - Rao et al. 2005; fruit extract of *Aristotelia chilensis* Céspedes et al. 2008; administration of *propofol* - Kobayashi et al. 2008; administration of *tamoxifen* - Ek et al. 2008), however these studies used either chronic or continuous administration during I/R, of the test substance. In isolated heart models administration of 30 µg of raw extract of *I. paraguariensis* and *I. brasiliensis* continuously before induction of ischemia and during the early period of reperfusion (Schinella et al. 2005; Schinela et al. 2009) was also able to reduce lipid peroxidation. In our experiment the production of LPO was higher in animals treated with Ip extract, which is consistent with the levels of ROS observed and no increase in ACAP. The ischemic untreated hearts had intermediate values of LPO not significantly different from controls or groups Ip, which may result from its higher antioxidant power. As in the case of the generation of ROS, extract blood dilution via *bolus* without dose maintenance may be the cause of no reduction in lipid peroxidation of treated groups.

Given that the dose used in this study was not able to reduce oxidative damage in I/R, the mechanism of cardioprotection which provided the effective

reduction of the risk area and area of myocardial infactedion may have occurred by a different route from that of classic antioxidant action of flavonoids.

The endothelium-dependent vasodilator properties of the extract (raw and fractions) of *I. paraguariensis* is already known (Muccillo-Baisch et al., 1998; Stein et al., 2005). It is also known that the increase of NO reduces the incidence and variety of ventricular arrhythmias, decreasing the fibrillation even at doses subvasodilatadoras (Thuc et al., 2010) and also improving the capacity of ventricular contraction by decreasing the myocardial stunning induced by I/R. ROS has been recognized as pre-conditioning ischemia, ie preparing the tissue for injury derived from I/R, through the activation of mitogen-activated protein kinase (MAPK) (Das et al., 2006), involved in stimulation of the antioxidant defense system. Also the cardioprotective effect against damage induced by I/R have been attributed to activation of potassium channels mitochondrial ATP-sensitive (O'Rourke 2004), and NO may be involved in the signaling pathway that activates these channels and causes an increase in phosphorylation MAPK and the consequent increase in antioxidant enzymes such as catalase (Thuc et al. 2010).

Therefore, in this study, the myocardium may be defended through vasodilation induced by NO production at the beginning of the ischemic event, preventing the establishment of an ischemia as severe as that which occurs in animals that do not receive the extract. This could be the reason due to I/R rats treated with Ip does not respond by increasing its ACAP. The benefits of treatment with the rich of flavonoid acid fraction could be dependent on NO production to avoid disturbances of myocardial function during ischemia. Considering that vasodilation may be leading to a lower intensity of ischemia, which would explain lower risk and infacted areas, the oxidative damage observed during reperfusion, even greater than in untreated I/R animals, could be reversed, since that the tissue is suffering minor injury during the ischemic phase. Thus the cardioprotective action of the rich flavonoid acid fraction of Ip extract may reduce the risk area and myocardial infactedion by an indirect antioxidant effect of flavonoids via NO production, improving perfusion during ischemia and even during post-ischemic myocardial recovery.

5. Conclusion

The cardioprotection induced by rich flavonoids acid fraction of *Ilex paraguariensis* in the model of ischemia and reperfusion in rats *in vivo* detected by the decrease in the risk and infacteded areas did not seem to be directly linked to the classic antioxidant activities of flavonoids. It is suggested that flavonoids in the preparation have been able to directly or indirectly stimulate NO production and may have led to improved myocardium perfusion and cardiac function during ischemia by stimulating the repair of myocardial damage and consequent reduction of infacteded area. Future studies with continuous infusion of the extract of *I. paraguariensis*, L-NAME and other blockers in comparison to model *in vivo* and *in vitro* I/R may show these aspects of the involvement of NO in reducing injury-induced myocardial ischemia and reperfusion.

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Conclusão Geral

A cardioproteção induzida pela fração ácida rica em flavonóides do extrato de *Ilex paraguariensis* no modelo de isquemia e reperfusão em ratos *in vivo,* é evidenciada pela redução da área de risco e de infarto.

O mecanismo de cardioproteção não está relacionado a redução da produção de espécies reativas de oxigênio ou dano lipídico.

O efeito cardioprotetivo pode ser resultado de uma ação direta ou indireta do extrato, estimulando o aumento da produção de NO e levando a vasodilatação. Por sua vez estes fatores podem ter melhorado a perfusão tecidual, com conseqüente diminuição da lesão isquêmica e de reperfusão.

Estudos futuros com infusão contínua do extrato de *I. paraguariensis*, L-NAME e outros bloqueadores comparando os modelos *in vivo* e *in vitro* de I/R poderão demonstrar estes aspectos do envolvimento do NO na redução da injúria miocárdica induzida por isquemia e reperfusão.

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