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EFEITOS DO RESVERATROL, DO SUCO DE UVA E DO VINHO TINTO NOS BIOMARCADORES DE ESTRESSE OXIDATIVO E NA ATIVIDADE DE ECTOENZIMAS EM RATOS DIABÉTICOS

TESE DE DOUTORADO

Roberta Schmatz

Santa Maria, RS, Brasil

2011

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Roberta Schmatz

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Biológica: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de
Doutor em Bioquímica Toxicológica

Orientadora: Prof[a]. Dra. Vera Maria Melchiors Morsch
Co - orientadora: Prof[a]. Dra. Maria Rosa Chitolina Schetinger

Santa Maria, RS, Brasil.

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Universidade Federal de Santa Maria
Centro de Ciências Naturais e Exatas
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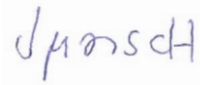
A Comissão Examinadora, abaixo assinada, aprova a Tese de Doutorado

EFEITOS DO RESVERATROL, DO SUCO DE UVA E DO VINHO TINTO NOS BIOMARCADORES DE ESTRESSE OXIDATIVO E NA ATIVIDADE DE ECTOENZIMAS EM RATOS DIABÉTICOS

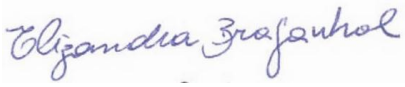
elaborada por
Roberta Schmatz

como requisito parcial para a obtenção do grau de
Doutor em Bioquímica Toxicológica

COMISSÃO EXAMINADORA



Prof[a]. Dra. Vera Maria Melchiors Morsch (Presidente/Orientador)



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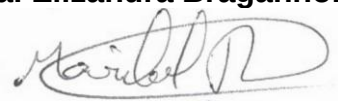

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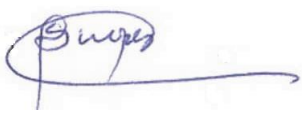

Prof[a]. Dra. Sonia Terezinha dos Anjos Lopes (UFSM)

Santa Maria, 28 de novembro de 2011.

"Não faz mal que seja pouco,
O que importa é que o avanço de hoje
Seja maior que o de ontem
Que nossos passos de amanhã
Sejam mais largos que os de hoje
Atuem agora e vivam o presente
Com a certeza de que neste exato instante
Está se erguendo o futuro
A dificuldade no momento presente
Será a glória em seu futuro!"
Daisaku Ikeda

Aos meus pais Irineu e Jacinta

Ao meu esposo Eduardo

À vocês, amores da minha vida, que em muitos momentos acreditaram mais em mim do que eu mesma, que sustentaram comigo cada minuto de dificuldade e alegria, dedico este trabalho e todo o meu amor!

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À minha querida orientadora “Profe” Vera, pela oportunidade, pela paciência, dedicação e amizade durante todos estes anos. Obrigada pelos ensinamentos valiosos sobre a vida e sobre pesquisa. Serás eternamente a minha Profe. Obrigada por tudo.

A minha co-orientadora, Prof[a] Maria Rosa, pelos ensinamentos, pelo apoio, amizade, preocupação e incentivo em todos os momentos.

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Rafael, Diéssica, Luana, Marília, Cláudio e Eduardo, pelos momentos compartilhados.

Aos membros da banca examinadora desta tese, professoras Elizandra, Sonia, Maribel e Marina, pela disponibilidade em avaliar este trabalho e pelas contribuições que foram fundamentais para o aprimoramento do mesmo.

A UFSM e ao curso de Doutorado em Bioquímica Toxicológica, pela oportunidade. A CAPES pela bolsa concedida.

E a todas as pessoas que, de uma forma ou de outra, contribuíram para a realização deste trabalho, gostaria de expressar minha profunda gratidão.

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

EFEITOS DO RESVERATROL, DO SUCO DE UVA E DO VINHO TINTO NOS BIOMARCADORES DE ESTRESSE OXIDATIVO E NA ATIVIDADE DE ECTOENZIMAS EM RATOS DIABÉTICOS

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Data e local de Defesa: Santa Maria, 28 de Novembro de 2011.

O diabetes mellitus (DM) é um dos maiores problemas de saúde pública no mundo. A hiperglicemia crônica presente no estado diabético está associada com um aumento do estresse oxidativo, o qual tem sido apontado por ter um papel central no desenvolvimento e na progressão de complicações diabéticas. Alterações na morfologia e função das plaquetas observadas no diabetes têm sido consideradas agravantes importantes para a patogênese das complicações vasculares desta endocrinopatia. Os nucleotídeos extracelulares ATP, ADP e o nucleosídeo adenosina regulam diversos processos fisiológicos no sistema vascular, incluindo a agregação plaquetária, o tônus vascular e as funções cardíacas. O controle dos níveis extracelulares destas moléculas e a conseqüente sinalização purinérgica por elas induzida é realizada por uma variedade de enzimas como as NTPDases (Nucleotídeo Trifosfato Difosfoidrolase), E-NPPs (Ecto-Nucleotídeo Pirofosfatase/Fosfodiesterases), 5'-nucleotidase e a adenosina deaminase (ADA). Estudos têm revelado uma série de ações benéficas do vinho tinto e do suco de uva, que podem ser atribuídas às altas concentrações de polifenóis que estes possuem, principalmente de resveratrol o qual apresenta propriedades antioxidantes e antiplaquetárias. Neste contexto, o objetivo do presente estudo foi verificar o efeito do resveratrol, do vinho tinto e do suco de uva no perfil oxidativo em fígado e rim, na atividade de ectoenzimas em plaquetas e na agregação plaquetária de ratos diabéticos induzidos com estreptozotocina. Além disso, foram realizados testes in vitro com os polifenóis resveratrol, quercetina, rutina, ácido caféico e ácido gálico na atividade das ectonucleotidases, ADA e agregação plaquetária de ratos controles e diabéticos. Os resultados obtidos demonstraram um aumento nos níveis de peroxidação lipídica em fígado e rim de ratos diabéticos e o tratamento com resveratrol (10 e 20 mg/kg) preveniu este aumento. A atividade da superóxido dismutase (SOD), catalase (CAT) e δ-aminolevulinato ácido desidratase (δ-ALA-D) e os níveis de tióis não protéicos (NPSH) e vitamina C foram significativamente decrescidos no fígado e rim de ratos diabéticos. Contudo o tratamento com resveratrol (10 e 20 mg/kg) preveniu o decréscimo nas defesas antioxidantes de ratos diabéticos. Em relação à NTPDase, E-NPP ,5'-Nucleotidase e ADA, observou-se um aumento na atividade destas ectoenzimas em plaquetas de ratos diabéticos acompanhado de um aumento na agregação plaquetária neste animais. O tratamento com resveratrol (10 e 20 mg/kg), suco de uva e vinho tinto aumentou a atividade da NTPDase, E-NPP and 5′-nucleotidase e preveniu o aumento na atividade da ADA em ratos diabéticos. A agregação plaquetária decresceu significativamente em ratos diabéticos tratados com suco de uva e vinho tinto. Nos testes *in vitro*, resveratrol, ácido caféico e ácido gálico aumentaram a hidrólise do ATP, ADP e AMP, enquanto a rutina e a quercetina decresceram a hidrólise destes nucleotídeos em plaquetas de ratos diabéticos. A atividade da ADA e agregação plaquetária foram reduzidas na presença de todos os polifenóis testados *in vitro.* Assim, os resultados descritos aqui sugerem que o resveratrol tem efeito protetor contra danos hepáticos e renais induzidos pelo estresse oxidativo no estado diabético, o qual foi evidenciado pela capacidade deste polifenol de modular as defesas antioxidantes e decrescer a peroxidação lipídica nestes tecidos. Além disso, o resveratrol, o vinho tinto e o suco de uva, bem como o ácido cafeíco, o ácido gálico, a quercetina e a rutina são capazes de modular a atividade das ectoenzimas e decrescer a agregação plaquetária, podendo prevenir e reduzir anormalidades plaquetárias e consequentemente complicações vasculares no estado diabético.

Palavras-chave: Diabetes. Estresse oxidativo. Ectoenzimas. Plaquetas. Resveratrol. Suco de uva. Vinho tinto.

ABSTRACT

Thesis of Doctor's Degree
Post-Graduate Program in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

EFFECTS OF RESVERATROL, GRAPE JUICE AND RED WINE ON BIOMARKERS OF OXIDATIVE STRESS AND ON ECTOENZYMES ACTIVITY IN DIABETIC RATS

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Date and place of the defense: Santa Maria, November, 28 th, 2011.

Diabetes mellitus is a major public health problem throughout the world. The chronic hyperglycemia present in the diabetic state is associated with increase of oxidative stress, which has been appointed to play a central role in the development and progression of diabetic complications. Changes in the platelet morphology and function observed in diabetes have been considered as important aggravating to the pathogenesis of vascular complications in this endocrinopathia. The extracellular nucleotides ATP, ADP and nucleoside adenosine are known to regulate various physiological processes in the vascular system, including platelet aggregation, vascular tone and cardiac functions. The control of extracellular levels of these molecules and consequent purinergic signaling induced by them is controlled by a variety of enzymes such as NTPDases (Nucleotide Triphosphates Diphosphohydrolase), E-NPPs (Ecto-Nucleotide Pyrophosphatase Phosphodiesterases), 5'-nucleotidase and adenosine deaminase (ADA). On the other hand, studies have revealed a number of beneficial actions of red wine and grape juice, which can be attributed to high concentrations of polyphenols presents in these beverages, mainly of resveratrol, which presented antioxidant and antiplatelet properties. In this context, the objective of this study was to investigate the effect of resveratrol, of red wine and of grape juice in the biomarkers of oxidative stress in liver and kidney, in the ectoenzimas activities in platelets and platelet aggregation of streptozotocin-induced diabetic rats. In addition, tests were performed *in vitro* with the polyphenols resveratrol, quercetin, rutin, caffeic acid and gallic acid on the activity of ectonucleotidases, ADA and platelet aggregation in diabetic and control rats. The results showed an increase in the levels of lipid peroxidation in liver and kidney of diabetic rats and treatment with resveratrol (10 and 20 mg/kg) prevented this increase. The activities of catalase (CAT), superoxide dismutase (SOD) and aminolevulinic acid dehydratase (δ-ALA-D) and the levels of non protein thiols (NPSH) and vitamin C presented a significant decrease in diabetic rats. However, the treatment with resveratrol (10 and 20 mg/kg) was able to prevent the decrease in the antioxidant defenses in diabetic rats. In relation to NTPDase, E-NPP, 5'-nucleotidase and ADA, there was an increase in activity of these ectoenzimas in platelets of diabetic rats accompanied by an increase in platelet aggregation in these animals. Treatment with resveratrol (10 and 20 mg/kg), grape juice and red wine increased the activity of NTPDase, E-NPP and 5'-nucleotidase and prevented the increase in ADA activity in diabetic rats. Platelet aggregation significantly decreased in diabetic rats treated with grape juice and red wine. In the *in vitro* tests, resveratrol, caffeic acid, and gallic acid increased ATP, ADP and AMP hydrolysis, while quercetin and rutin decreased the hydrolysis of these nucleotides in platelets of diabetic rats. The ADA activity and platelet aggregation were reduced in platelets of diabetic rats in the presence of all polyphenols tested *in vitro*. The results described here suggest that resveratrol could have a protector effect against hepatic and renal damage induced by oxidative stress in the diabetic state, which was evidenced by the capacity of this polyphenol to modulate the antioxidant defense and to decrease the lipid peroxidation in these tissues. In addition, resveratrol, red wine and grape juice as well as caffeic acid, gallic acid, quercetin and rutin were able to modulate the ectoenzimas activities and decrease platelet aggregation could contribute to the prevention and reduction of platelet abnormality and consequently vascular complications in diabetic state.

Key words: Diabetes. Oxidative stress. Ectoenzymes. Platelets. Resveratrol. Grape juice. Red wine.

LISTA DE ABREVIAÇÕES

ADA – Adenosina deaminase

ADP – Adenosina difosfato

δ-ALA-D – Delta-aminolevulinato ácido desidratase

δ-ALA – Ácido delta-aminolevulínico

AMP – Adenosina monofosfato

ATP – Adenosina trifosfato

CAT – Catalase

DM – Diabetes mellitus

E-NPP – Ecto -nucleotídeo pirofosfatase / fosfodiesterases

EROs – Espécies reativas de oxigênio

GPx – Glutationa Peroxidase

GSH – Glutationa Reduzida

MDA – Malondialdeído

NPSH – Tióis não protéicos

NTPDases – Nucleosídeo trifosfato difosfoidrolases

SOD – Superóxido Dismutase

STZ – Estreptozotocina

TBARS – Substâncias Reativas ao Ácido Tiobarbitúrico

SUMÁRIO

APRESENTAÇÃO

Este estudo consiste na tese de doutorado intitulada “Efeitos do resveratrol, do suco de uva e do vinho tinto nos biomarcadores de estresse oxidativo e na atividade de ecto-enzimas em ratos diabéticos”.

O estudo apresenta-se estruturado em três partes como se segue: resumo, introdução e objetivos (parte I); manuscrito e artigos (parte II); discussão, conclusões, referências (parte III).

Os resultados deste trabalho estão apresentados na forma de artigos e manuscrito e os itens discussão e conclusão apresentam interpretações e comentários gerais sobre os artigos e o manuscrito contidos neste trabalho. As referências bibliográficas referem-se somente as citações que aparecem nos itens introdução e discussão dessa tese.

1. INTRODUÇÃO

O diabetes mellitus (DM) é considerado uma das doenças mais importantes que afetam a humanidade, situando-se entre as dez principais causas de morte nos países ocidentais e, apesar dos progressos em seu controle clínico, ainda não foi possível controlar de fato suas conseqüências letais (NORTHAM et al., 2006; KING et al., 2008).

O DM é uma doença metabólica de etiologia múltipla, decorrente de defeitos na produção de insulina e/ou da incapacidade deste hormônio de exercer adequadamente os seus efeitos. Esta doença caracteriza-se por hiperglicemia crônica acompanhada por distúrbios no metabolismo de carboidratos, lipídios e proteínas (AMERICAN DIABETES ASSOCIATION, 2010).

Como conseqüência destes distúrbios a maioria dos pacientes com DM manifesta a curto-prazo um quadro de glicosúria, polifagia, polidipsia, poliúria e perda de peso. Estes sintomas freqüentes na população diabética são conhecidos como clássicos na história da doença. No entanto, a ausência dos mesmos é comum em muitos pacientes e não descarta a possibilidade de que exista um grau de hiperglicemia suficiente para causar alterações funcionais ou patológicas antes que o diagnóstico seja estabelecido (ATKINSON & EISENBARTH, 2001).

As conseqüências do DM em longo prazo ocorrem devido a alterações micro e macrovasculares que levam a disfunção, dano ou falência de vários órgãos. As complicações microvasculares crônicas compreendem a nefropatia, com possível evolução para insuficiência renal, a retinopatia, com possibilidade de cegueira, neuropatia periférica, com risco de úlceras nos pés e amputações. Além disso, pacientes diabéticos apresentam alterações macrovasculares com elevado risco de doença vascular aterosclerótica, como as doenças coronariana, arterial periférica e vascular cerebral, sendo estas consideradas a principal causa da redução da sobrevida e da mortalidade destes pacientes (AMERICAN DIABETES ASSOCIATION, 2010).

A classificação atual do DM toma como referência a etiologia dos distúrbios glicêmicos e inclui o diabetes tipo 1 (DM tipo 1), diabetes tipo 2 (DM tipo 2), diabetes gestacional e diabetes associado a desordens em mecanismos específicos ou

relacionados à doenças. A grande maioria dos pacientes diabéticos pertence a uma das duas classes etiopatogênicas: DM tipo 1 e DM tipo 2 (AMERICAN DIABETES ASSOCIATION, 2010).

O DM tipo 1 que antigamente fora denominado de diabetes mellitus insulino dependente ou diabetes juvenil compreende aproximadamente 10 % de todos os casos de diabetes. Manifesta-se geralmente em crianças, adolescentes e adultos jovens e caracteriza-se pela ausência da secreção de insulina devido a uma grave ou total destruição das células β pancreáticas. Quanto a origem etiológica o DM tipo 1 pode ser dividido em diabetes imunomediado e diabetes idiopático. No diabetes imunomediado há uma forte associação com a região HLA (antígenos leucocitários humanos) no cromossomo 6 e uma relação com a destruição autoimune das células β pancreáticas. (GANNON, 2001; CNOP et al., 2005). Além da predisposição genética a destruição das células β pode ainda decorrer de fatores ambientais que afetem as características imunogênicas das células pancreáticas. Estudos epidemiológicos sugerem que infecções virais também podem desencadear o processo autoimune característico do DM tipo 1 (AMERICAM DIABETES ASSOCIATION, 2010).

Por outro lado, o diabetes idiopático é a forma de DM tipo 1 que não têm nenhuma etiologia conhecida. Alguns destes pacientes têm insulinopenia permanente e são propensos a cetoacidose, outros exibem graus variados de deficiência de insulina entre episódios. Esta forma de diabetes tem fatores hereditários, mas não existem evidências de autoimunidade das células β pancreáticas, além de não haver associação com o HLA (AMERICAM DIABETES ASSOCIATION, 2010).

O DM tipo 2 que antigamente fora denominado de DM não-insulino-dependente ou diabetes senil é considerado a forma mais comum da doença a qual afeta aproximadamente 90% da população diabética. Manifesta-se geralmente em pacientes com idade superior a 40 anos e tem elevado componente hereditário. Além disso, fatores genéticos, ambientais e quadros de obesidade contribuem de forma significativa para o surgimento do DM tipo 2. Esta forma de diabetes geralmente resulta de graus variáveis de resistência tecidual à insulina e/ou de uma deficiência relativa na secreção do hormônio pelas células beta pancreáticas. Como

conseqüência os pacientes apresentam complicações no sistema micro e macrovascular e ao contrário do DM tipo 1 não necessitam da reposição de insulina.

Adicionalmente o DM tipo 2 tem demonstrado ser a manifestação de uma doença muito mais ampla conhecida atualmente como “síndrome metabólica”. Esta representa um grupo de fatores de risco para doenças cardiovasculares que predispõem a manifestação de DM tipo 2 (HANSEN, 1999). Estes fatores incluem hipertensão arterial, lipidemias, hiperinsulinemia, intolerância à glicose e obesidade visceral.

De acordo com estimativas da OMS, o número de portadores de DM em todo o mundo é de cerca de 177 milhões, com expectativa de alcançar 370 milhões de pessoas em 2030 (WHO Database, 2011). No Brasil existem aproximadamente 11 milhões de diabéticos correspondendo a 8,9% da população adulta entre 30 e 69 anos, sendo que aproximadamente 50% destes pacientes desconhecem o diagnóstico (MINISTÉRIO DA SAÚDE, 2009).

As conseqüências humanas, sociais e econômicas do diabetes são devastadoras: são quatro milhões de óbitos por ano relativos ao diabetes e suas complicações, o que representa 9% da mortalidade mundial. As doenças cardiovasculares são responsáveis por mais de 80% das mortes de pacientes diabéticos e também por 30% das internações em centros de tratamento intensivo. Já a nefropatia diabética acomete mais de um terço dos pacientes e é a causa mais comum da doença renal terminal e por ingressos em programas de hemodiálise. Mais de 70% de todas as amputações de pés e membros inferiores estão relacionados ao diabetes e em algumas regiões, estes níveis podem chegar a 90%. Esta síndrome além de contribuir de forma significativa para o desenvolvimento de acidente vascular cerebral e de hipertensão arterial, também constitui a principal causa de cegueira adquirida (MINISTÉRIO DA SAÚDE, 2009; AMERICAN DIABETES ASSOCIATION, 2010).

Tendo em vista a crescente incidência do diabetes, nas últimas décadas modelos experimentais têm sido amplamente utilizados a fim de contribuir para melhorar o entendimento das causas, conseqüências e tratamento desta desordem metabólica. Agentes diabetogênicos como a estreptozotocina (STZ) e o aloxano, reproduzem nos animais o quadro de alterações metabólicas e sinais clínicos semelhantes aos que ocorrem na enfermidade naturalmente adquirida (MENDES &

RAMOS, 1994). Tais drogas caracterizam-se por seu efeito tóxico seletivo às células β das ilhotas de Langerhans do pâncreas (ISLAS-ANDRADE et al., 2000; HU & HUAN, 2008).

A STZ (2-deoxi-2-(3-metil-3- nitrosoureído)-D-glicopiranose) é uma antibiótico de amplo espectro de ação, produzida a partir de microorganismos *Streptomyces achromogenes* (HERR, 1960). Quando utilizada em animais, determina uma deficiência grave de insulina, verificando-se uma instalação imediata do quadro de hiperglicemia. Experiências demonstram que doses de STZ na faixa de 50 a 100 mg/kg de peso corporal causam necrose das células β com desenvolvimento do DM tipo I, um ou dois dias após a aplicação da droga (HOUNSOM, 1998). SCHNEDL et al (1994) demonstraram que a toxicidade dirigida para as células β é devida à similaridade da molécula da STZ com a glicose, o que permite que a mesma seja internalizada via transportadores GLUT-2. De fato, a estrutura química da STZ compreende uma molécula de glicose com uma cadeia lateral nitrosurea altamente reativa, que inicia a ação citotóxica deste composto (ISLAS-ANDRADE et al., 2000; HU & HUAN, 2008).

O mecanismo de ação da STZ está relacionado tanto à metilação do DNA quanto à produção excessiva de espécies reativas de oxigênio (EROs) (HU & HUAN, 2008). Após sua administração ocorre a destruição das membranas celulares e indução da quebra no DNA pelas EROs, levando à ativação da enzima poli (ADP-ribose) sintase e à depleção da nicotinamida adenina dinucleotídeo (NAD). Esta enzima está localizada no núcleo das células β do pâncreas e necessita de NAD para realizar o reparo do DNA nuclear. Um aumento na sua atividade pode levar à depleção do NAD intracelular, sendo impossível produzir insulina nas células β pancreáticas (TAKAMA et al., 1995) (Figura 1).

Similarmente à pacientes diabéticos, os animais com diabetes induzida por STZ desenvolvem danos nos olhos, rins, fígado, cérebro e no sistema vascular, constituindo dessa forma um importante modelo para o estudo de complicações agudas e crônicas do DM (SCHAAN et al., 2004).

Neste contexto, extensivas pesquisas tanto em humanos quanto em modelos experimentais, têm demonstrado que o estresse oxidativo possui um papel central no desenvolvimento de complicações crônicas características do estado diabético (MARITIMIM et al., 2003; GIACCO & BROWNLEE, 2010). A hiperglicemia de longa

duração parece ser o principal fator envolvido na gênese e no aumento do estresse oxidativo evidenciado no diabetes (STEVENS et al., 2005). De fato, eventos conseqüentes da hiperglicemia como auto-oxidação da glicose; glicação não-enzimática de proteínas e a consequente formação de PTGAs (produtos terminais de glicação avançada) culminam em um aumento na produção de EROs (RAINS & JAIN, 2011).

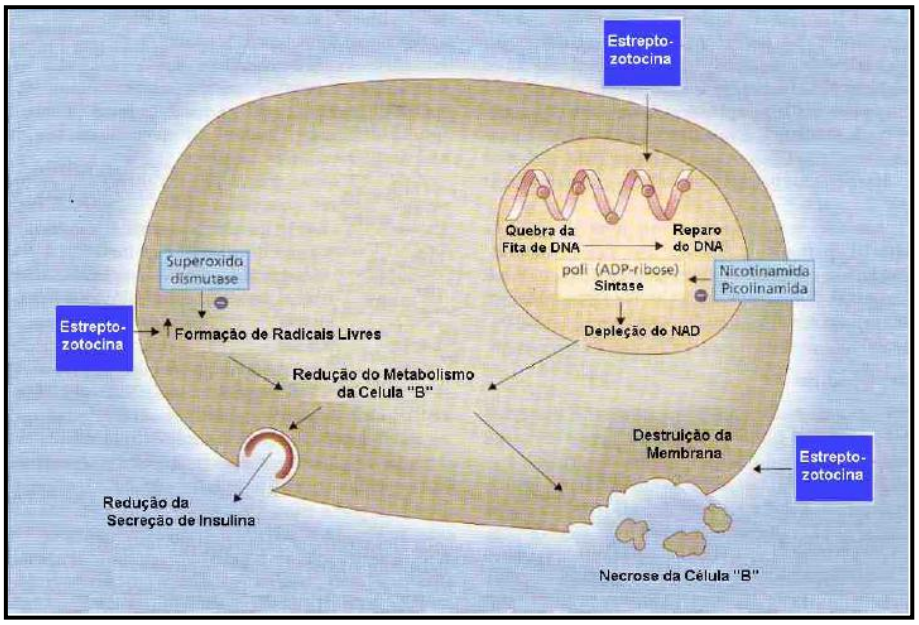


Figura 1. Mecanismo de ação da estreptozotocina no desenvolvimento do diabetes (Adaptado de PICKUP & WILLIAMS, 1997).

As EROs como o ânion superóxido ($O_2^{\bullet -}$), peróxido de hidrogênio (H_2O_2), radical hidroxila ($OH^{\bullet}$) e outros oxidantes se formam em condições fisiológicas em proporções controladas por mecanismos de defesa celular (DRÖGE, 2002). Porém, em condições patológicas, como no diabetes, essa produção de EROs pode aumentar substancialmente resultando em estresse oxidativo (GIACCO & BROWNLEE, 2010). Denomina-se estresse oxidativo situações em que há um desequilíbrio entre os níveis de antioxidantes e pró-oxidantes, com o predomínio destes últimos Assim, a diminuição dos sistemas de defesa antioxidante, ou, o aumento da geração de espécies oxidantes, radicalares ou não, pode resultar em lesões oxidativas em macromoléculas como proteínas, lipídios e DNA e que se não

forem reparadas, alterarão a funcionalidade de células, tecidos e órgãos (BARREIROS & DAVID, 2006) (Figura 2).

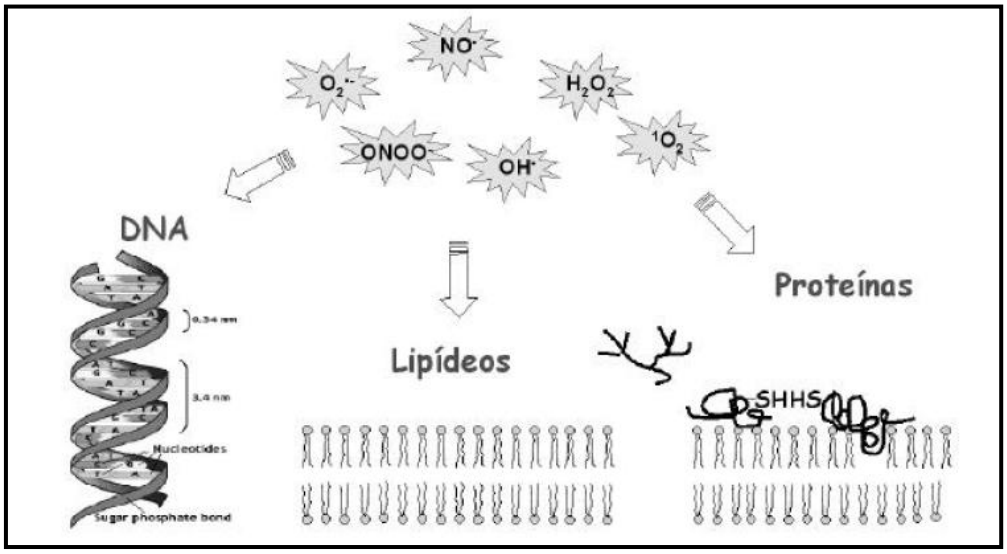


Figura 2. Dano oxidativo a macromoléculas biológicas (Adaptado de TORRES, 2003).

Todos os componentes celulares são suscetíveis à ação das espécies reativas, porém a membrana plasmática é um dos mais atingidos em decorrência da peroxidação lipídica (STARK, 2005). A peroxidação lipídica é o processo através do qual as EROs agridem os ácidos graxos polinsaturados dos fosfolipídeos das membranas das células, desintegrando-as e permitindo, deste modo, a entrada dessas espécies nas estruturas intracelulares. Este processo promove grave alteração da membrana celular, causando perda da fluidez e alteração da função secretora. Além disso, tem sido observada perda da seletividade na troca iônica, com liberação do conteúdo de organelas, levando à formação de produtos citotóxicos e até a morte celular (DMITRIEV & TITOV, 2010). Um dos produtos da lipoperoxidação bem conhecidos é o malondialdeído (MDA), que é o produto final da degradação não enzimática de ácidos graxos poliinsaturados (CHERUBINI et al., 2005). Altos níveis de MDA elevam a formação de lipoperóxidos e indicam um aumento da lipoperoxidação (KASHYAP et al., 2005).

Além dos lipídios, as proteínas também são moléculas biológicas alvos para a ação das EROs. A exposição das proteínas ao ataque dos radicais livres resulta em múltiplas modificações nestas moléculas. Estas modificações incluem a oxidação

dos grupos das cadeias laterais de aminoácidos, fragmentação, modificações na hidrofobicidade e na conformação de proteínas e formação de novos grupos reativos, como os grupos carbonil. Os grupos carbonil são produzidos pela oxidação das cadeias laterais das proteínas. A proteína carbonil também pode ser introduzida nas proteínas por uma reação secundária das cadeias laterais nucleofílicas dos aminoácidos cisteína, histidina e lisina com aldeídos produzidos durante a peroxidação lipídica como o MDA (DONNE et al., 2003; STADTMAN, 2004)

Os níveis de proteína carbonil e de peroxidação lipídica são frequentemente utilizados como marcadores de dano oxidativo no estado diabético. Diversos estudos relatam o aumento desses biomarcadores tanto em pacientes quanto em animais diabéticos, indicando um aumento na produção de EROs e conseqüente estresse oxidativo nessa endocrinopatia (FIDAN & DÜNDAR, 2008; LIKIDLILID et al., 2010; PALSAMY et al.,2010)

Por outro lado, o estresse oxidativo tem seus danos minimizados pelo sistema de defesa antioxidante enzimático e/ou não enzimático (VALKO et al., 2007). Entre as principais enzimas responsáveis pela defesa antioxidante do organismo destacam-se a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx), que constituem a primeira linha de defesa endógena de neutralização das EROs agindo em um mecanismo de elevada sincronia e de forma altamente cooperativa (NORDBERG et al., 2001; BARREIROS & DAVID, 2006) (Figura 3).

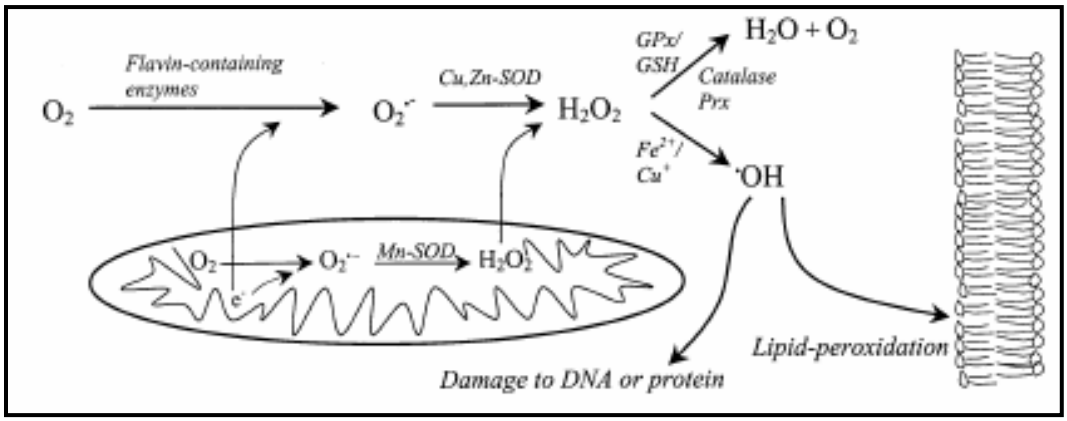


Figura 3. Mecanismo enzimático antioxidante (NORDBERG & ARNER, 2001).

A enzima SOD (EC 1.15.1.1) foi isolada em 1939, mas somente em 1969 Mc Cord e Fridovich comprovaram a atividade antioxidante desta enzima, o que propiciou um grande avanço das pesquisas na área de toxicidade do oxigênio (MC CORD & FRIDOVICH, 1969). A SOD constitui-se de uma metaloenzima abundante nas células aeróbias e uma das defesas antioxidantes enzimáticas mais efetivas. Esta metaloenzima é capaz de aumentar em 104 vezes a velocidade da reação de dismutação do radical $O_2^{\bullet-}$ em H_2O_2 e O_2 em pH fisiológico (VALKO et al., 2007).

Já a CAT (EC 1.11.1.6) é uma enzima tetramérica, consistindo de quatro subunidades idênticas de 60 KDa, que contém um único grupo ferroprotoporfirina por unidade, e tem uma massa molecular de aproximadamente 240 KDa. É encontrada em praticamente todos os órgãos, estando particularmente concentrada nos hepatócitos, nos rins e nos eritrócitos. Dentro das células a CAT encontra-se no interior dos peroxissomos e sua atividade é dependente de NADPH (VALKO et al., 2006).

A CAT promove a conversão do H_2O_2 à H_2O e O_2. Esta enzima possui uma das mais altas taxas de renovação, sendo que uma molécula de CAT pode converter cerca de 6 milhões de moléculas de H_2O_2 em H_2O e O_2 a cada minuto (HALLIWELL, 2000). O H_2O_2 tem vida longa, é capaz de atravessar camadas lipídicas, podendo reagir com a membrana eritrocitária e com proteínas ligadas ao Fe^{2+}. Apesar de não ser um radical livre, pela ausência de elétrons desemparelhados na última camada, o H_2O_2 é um metabólito do oxigênio extremamente deletério, porque participa da reação que produz o radical OH• (HALLIWELL & GUTTERIDGE, 2000, WIERNSPERGER, 2003). Assim o balanço entre a atividade da SOD e da CAT é de fundamental importância na determinação do equilíbrio dos níveis de radical O2•- e do H_2O_2 nas células a fim de evitar a formação do radical OH• altamente reativo (SANTINI et al., 1997; BRIONES & TOUYZ, 2010).

Além das defesas antioxidantes enzimáticas as defesas antioxidantes não-enzimáticas desempenham um papel de fundamental importância para a célula. Nesse grupo destacamos o papel dos tióis não-protéicos e da vitamina C (VALKO et al., 2007).

Os tióis não-protéicos têm uma importante função na defesa contra EROs (HALLIWELL & GUTTERIDGE, 2000; MASSELA et al., 2005). A glutationa reduzida (GSH) é o tiol não-protéico mais abundante presente nas células animais (LI et al., 2001). Esta é formada por cisteína, glicina e resíduos de ácido glutâmico e sua

capacidade redutora é determinada pelo grupamento -SH, presente na cisteína. Dentre as funções celulares desempenhadas pela GSH podemos destacar: é capaz de transformar as vitaminas E e C oxidadas, a suas formas originais; tem um papel importante na síntese e também no reparo da molécula do DNA; é usada pelo fígado na desintoxicação de compostos tóxicos; e desempenha um papel protetor frente às proteínas (TOWNSEND et al., 2003).

A GSH participa na decomposição do H_2O_2, potencialmente tóxico, que é convertido em água na reação catalisada pela GPx, às custas da GSH; a glutationa oxidada resultante é reciclada à forma reduzida pela glutationa redutase e NADPH (Figura 4). O NADPH é regenerado pela via das pentoses fosfato, em reação catalisada pela glicose 6-fosfato desidrogenase, a qual é particularmente importante nos eritrócitos. Dessa forma, este processo de reciclagem e consequente manutenção de níveis adequados de GSH podem prevenir o dano celular causado pelo estresse oxidativo (GIUSTARINI et al., 2011). Em situações de estresse oxidativo muito intenso a GSH pode ser perdida de maneira irreversível, permanecendo na forma oxidada e não sendo novamente reduzida (GUL et al, 2000).

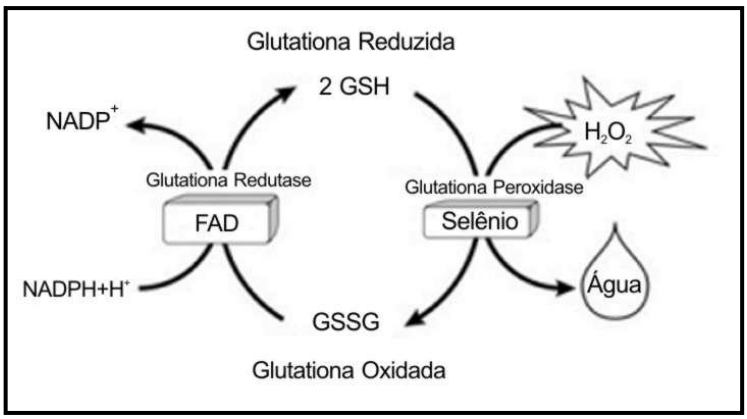


Figura 4. Papel da GPx e GSH na decomposição do peróxido de hidrogênio.

Outro antioxidante não-enzimático de grande relevância é a vitamina C (ácido ascórbico), que é um nutriente hidrossolúvel e termolábil encontrado primariamente em frutas e vegetais e exerce ação protetora sobre componentes hidrossolúveis do organismo (MAYNE, 2003). Esta vitamina age diretamente sobre os radicais $O_2^{\bullet-}$ e $OH^{\bullet}$ e está envolvida na regeneração de α-tocoferil em α-tocoferol (Vitamina E)

(VILLACORTA et al., 2007). A vitamina C interage com as EROs na fase aquosa do plasma antes que eles possam agir oxidativamente sobre lipídios e lipoproteínas (STAHL & SIES, 1997; MAYNE, 2003).

Dessa forma, o equilíbrio entre a geração e a neutralização de oxidantes pelos antioxidantes enzimáticos e não-enzimáticos é responsável pela manutenção dos componentes celulares vitais e consequentemente pela prevenção de diversas patologias (WIERNSPERGER et al., 2003). Por outro lado, situações de desequilíbrio no estado redox das células, como observado no diabetes, contribuem para o desenvolvimento e para a progressão de complicações em diversos órgãos e tecidos, destacando-se o fígado e os rins como importantes alvos para os danos causados pelas EROs (SHARMA et al., 2000; YLMAZ et al., 2004; JIN et al., 2008; PALSAMY et al., 2010). O fígado é o órgão central de processos oxidativos e de desintoxicação, e as reações dos radicais livres e os biomarcadores de estresse oxidativo estão elevados neste órgão em um estágio inicial de muitas doenças, incluindo o DM (SHAO et al., 2005; GILL et al., 2006). A alta prevalência de diversas alterações hepáticas no DM, como a esteatose, a esteatohepatite não alcoólica e a cirrose sugere que seus portadores possuem um risco aumentado para desenvolver doenças hepáticas (PAROLIN, 2006). A microangiopatia associada ao estresse oxidativo parece ser o principal mecanismo fisiopatológico envolvido nos danos hepáticos no estado diabético (SHAO et al., 2005; PALSAMY et al., 2010).

Além do fígado, os rins também se encontram lesados de várias maneiras no diabetes (PALSAMY et al., 2011). Há certas lesões renais que estão particularmente associadas ao diabetes, como o espessamento da membrana basal glomerular, a glomeruloesclerose diabética, lesões exsudativas glomerulares e necrose papilar. Estas lesões quando progressivas causam morte dos néfrons e podem levar ao longo do tempo à insuficiência renal crônica (CRITCHLEY et al., 2002; MARIC-BILKAN et al., 2011; FU et al., 2011). Dados da literatura demonstram um aumento significativo na peroxidação lipídica e alterações nas defesas antioxidantes no tecido renal de animais diabéticos, indicando um importante papel dos danos oxidativos no desenvolvimento e progressão de complicações renais no diabetes. Assim, o uso de terapias antioxidantes ou compostos que aumentam a atividade de sistemas enzimáticos de defesa no fígado e nos rins tem se tornado importantes opções terapêuticas no estudo da hepatotoxicidade e nefrotoxicidade do diabetes

(MANSOURI et al., 2011; KARUNA et al., 2011; PALSAMY et al., 201; RIBEIRO et al., 2011).

Por outro lado, estudos têm demonstrado que a atividade da enzima delta-aminolevulinato ácido desidratase (δ-ALA-D, E.C. 4.2.1.24) utilizada juntamente com parâmetros de estresse oxidativo pode ter um importante papel como marcador de danos oxidativos e de alterações nos processos metabólicos em diversas patologias, incluindo o diabetes (NOGUEIRA et al., 2003; FERNANDEZ-CUARTERO et al., 1999).

A enzima citoplasmática δ-ALA-D, também conhecida como porfobilinogênio sintetase ou 5-aminolevulinato hidroliase foi isolada na década de 50. Esta enzima é uma metaloproteína que catalisa a formação do composto monopirrólico, porfobilinogênio (PBG), através da condensação e ciclização assimétrica de duas moléculas de ácido delta-aminolevulínico (δ-ALA) com perda de 2 moléculas de água (GIBSON et al., 1955) (Figura 5). Assim, por participar da biossíntese de moléculas tetrapirrólicas, a δ-ALA-D tem ação de constituição de grupos prostéticos de importantes proteínas fisiológicas como a hemoglobina e os citocromos (SASSA, 1989).

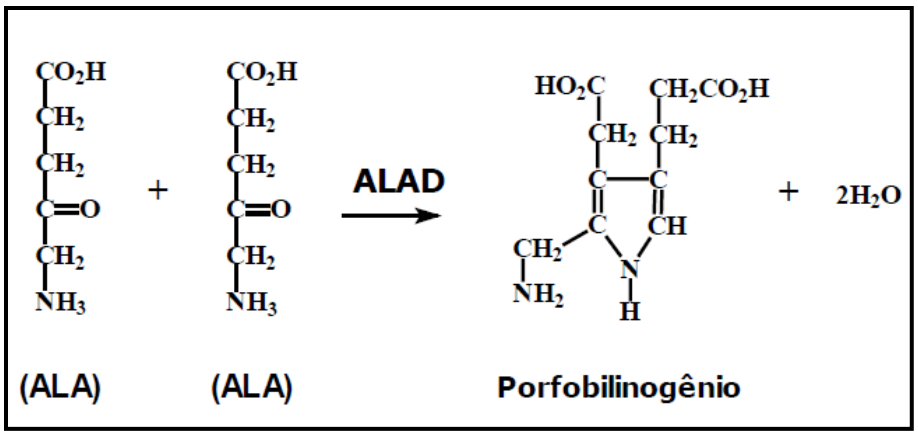


Figura 5. Condensação assimétrica de duas moléculas do ácido 5-aminolevulínico catalisada pela enzima δ-ALA-D.

A δ-ALA-D é uma enzima sulfidrílica formada por oito subunidades idênticas apresentando o zinco como cofator (GIBSON et al., 1955; SHEMIM, 1976). Os resíduos de cisteína são sensíveis a metais pesados, moléculas de oxigênio e outros

agentes oxidantes que induzem a formação de ligações dissulfeto e levam a inibição enzimática (NOGUEIRA et al., 2003; KADE et al., 2009).

Além da insuficiente produção do heme, a inibição da δ-ALA-D pode resultar no acúmulo do seu substrato δ-ALA no sangue, o qual está relacionado com a superprodução de EROS. O δ-ALA pode sofrer enolização e oxidação na presença de metais em pH fisiológico, produzindo radical superóxido, peróxido de hidrogênio e radical hidroxila (BECHARA et al., 1996; ROCHA et al., 2003).

Trabalhos recentes têm indicado que em patologias associadas com estresse oxidativo, tais como câncer (GONÇALVES et al., 2005), insuficiência renal crônica (SILVA et al., 2007) e diabetes (KADE et al., 2009) ocorre uma inibição da atividade da δ-ALA-D, ao mesmo tempo em que ocorrem danos oxidativos.

Experimentos com ratos diabéticos demonstraram que há uma inversa correlação entre o nível de glicose sanguínea e a atividade da δ-ALA-D, nos eritrócitos e no tecido hepático (GARRO et al., 1990; BITAR et al., 1994; POLO et al., 2000). O mesmo acontece em pacientes diabéticos, onde o dano na atividade da ALA-D de eritrócitos é paralelo ao aumento da hemoglobina glicosilada, provavelmente como resultado da glicosilação desta enzima (POLO et al., 1993). De fato, em suas pesquisas FOLMER et al. (2003) observaram que, no tecido renal, hepático e cerebral, houve uma correlação negativa entre a atividade da δ-ALA-D e a porcentagem de hemoglobina glicosilada, e da mesma forma entre a atividade da δ-ALA-D e os níveis de TBARS.

A glicação não-enzimática de proteínas e o estresse oxidativo têm sido propostos como os principais mecanismos para a inibição da atividade da δ-ALA-D no diabetes A auto-oxidação da glicose que ocorre no estado de hiperglicemia gera EROs, as quais promovem a oxidação dos resíduos de cisteína no sítio ativo da δ-ALA-D inibindo a enzima (FOLMER et al., 2003; KADE et al., 2009). Outro mecanismo proposto para explicar a referida inibição seria a glicação do resíduo de lisina, o qual compõe o sítio ativo da enzima e forma uma base de Schiff com a primeira molécula do substrato δ-ALA (BRITTO et al., 2007; SOUZA et al., 2007). Assim, a inibição da atividade da δ-ALA-D no diabetes, pode ser um importante parâmetro para avaliação de danos oxidativos e também para avaliação de alterações no metabolismo dos carboidratos (FERNANDES-CUARTERO et al., 1999).

Por outro lado, embora o diabetes seja um distúrbio metabólico, atualmente também tem sido considerado um distúrbio vascular (CERBONE et al., 2009). Indivíduos com DM tipo 1 ou 2 têm risco aumentado, de duas a quatro vezes, para doença arterial coronariana (DAC), acidente vascular cerebral (AVC) e doença arterial periférica (DAP) (NADEAU & REUSCH, 2011). Em especial, a DAC é uma importante causa de óbito e o diabetes ocupa lugar de destaque, visto que em 25 % dos pacientes diabéticos, a primeira manifestação da DAC é o infarto agudo do miocárdio (IM) ou a morte súbita (LONG & DAGOGO, 2011)

Estudos têm reconhecido que o estado metabólico anormal que acompanha o diabetes é responsável pelas disfunções vasculares observadas nesta patologia. Anormalidades relevantes incluem a hiperglicemia crônica, dislipidemia e resistência a insulina (GRANT, 2007). Todos estes fatores favorecem um acentuado estado pró-trombótico sendo capazes de alterar propriedades funcionais de múltiplos tipos de células, incluindo o endotélio e as plaquetas, com consequente aumento da susceptibilidade para o desenvolvimento de aterotrombose (MUDALIAR, 2004; CERBONE et al., 2009).

De fato, alterações na morfologia e função das plaquetas no estado diabético têm sido relatadas em vários estudos (COLWELL & NESTO, 2003; ANGIOLILLO et al., 2007; HAOUARI & ROSADO, 2008). Em geral, plaquetas de pacientes diabéticos exibem hiperatividade com exagerada adesão, agregação e geração de trombos. Além disso, as plaquetas destes pacientes exibem grande tendência para agregação espontânea e são altamente hipersensíveis a agonistas tais como trombina, colágeno e ADP (HAOUARI & ROSADO, 2008). Estas anormalidades podem resultar em um decréscimo da produção endotelial de antiagregantes como o óxido nítrico e a prostaciclina, aumentando a produção de fibrinogênio e de ativadores plaquetários, tais como a trombina, o ADP e os fatores de Von-Willebrand (MATSUNO et al., 2005; MICHNO et al., 2007).

Vários mecanismos têm sido propostos para explicar o aumento da reatividade plaquetária em pacientes com diabetes. Estudos demonstram que a glicação não-enzimática de proteínas é um dos mecanismos chave na patogênese das complicações vasculares observadas no diabetes. No estado hiperglicêmico, a glicose se liga não-enzimaticamente ás proteínas da membrana das plaquetas, causando anormalidades na função e danos na estrutura e conformação, bem como uma redução da fluidez da membrana lipídica das plaquetas, contribuindo para a

hiperfunção plaquetária no DM (HASEGAWA et al.,2002; YAMAGISHI et al., 2005). Além disso, vários estudos têm indicado que o estresse oxidativo que acompanha o diabetes pode induzir disfunção endotelial e aumentar a reatividade plaquetária através de efeitos diretos sobre as plaquetas, tais como um aumento da peroxidação lipídica e depleção de sistemas de defesa antioxidantes (FREEDMAN, 2008). Tomados juntos, estes mecanismos podem contribuir para o aumento do potencial trombótico característico do estado diabético (COLWELL & NESTO, 2003; GRANT, 2007).

Atualmente, a sinalização purinérgica constitui-se um importante alvo de estudos devido ao seu papel em modular uma variedade de processos biológicos incluindo a tromboregulação, a inflamação e a neurotransmissão (BURNSTOCK, 2006; ROBSON et al., 2006; DI VIRGILIO, 2007; SCHETINGER et al., 2007). No sistema vascular a sinalização purinérgica tem o potencial de influenciar funções cardíacas, participar de respostas vasomotoras e controlar as funções plaquetárias e os processos inflamatórios (BURNSTOCK, 2002).

O sistema purinérgico envolve três componentes principais: os nucleotídeos e nucleosídeos extracelulares, os receptores através dos quais estes nucleotídeos e nucleosídeos exercem seus efeitos e as ectoenzimas responsáveis pelo controle dos níveis extracelulares destas moléculas (YEGUTKIN, 2008).

Os nucleosídeos são moléculas resultantes da união de uma base púrica ou pirimídica a uma pentose. Exemplos destas moléculas incluem a citidina, a uridina, a guanosina, a timina, a inosina e a adenosina. Quando estes nucleosídeos são fosforilados por quinases específicas formam moléculas denominadas de nucleotídeos (ATKINSON et al., 2006). Os nucleotídeos de adenina ATP, ADP e AMP e seu correspondente nucleosídeo adenosina são considerados importantes moléculas sinalizadoras em vários tecidos (YEGUTKIN, 2008) mediando seus efeitos através de receptores purinérgicos localizados na superfície celular (ILLES & RIBEIRO, 2004).

Os nucleotídeos de adenina constituem 90% dos nucleotídeos plaquetários e encontram-se distribuídos em dois pools distintos: o pool metabólico, utilizado na manutenção das funções celulares, constituído principalmente por ATP, e o pool de armazenamento, que contém ATP e ADP para a liberação durante a secreção plaquetária (LEE et al., 1998).

O ADP constitui-se no principal agonista envolvido no recrutamento e agregação das plaquetas em locais de injúria vascular, sendo o controle dos níveis extracelulares deste nucleotídeo fundamental para a regulação dos processos trombóticos e/ou hemorrágicos (REMIJIN et al., 2002). O ATP possui um papel complexo nos mecanismos de regulação da agregação plaquetária sendo considerado um inibidor competitivo das ações mediadas pelo ADP. Altas concentrações de ATP inibem a agregação induzida pelo ADP, mas baixas concentrações podem contribuir para aumentar a agregação induzida pelo colágeno, trombina e tromboxano A_2 (BIRK et al., 2002; ROZALSKI et al., 2005).

A adenosina, resultante da hidrólise dos nucleotídeos da adenina, é um potente inibidor da agregação plaquetária e também atua como modulador do tônus vascular (ANFONSSI et al., 2002). Este nucleosídeo apresenta uma ação cardioprotetora em episódios de isquemia ou hipóxia e também na insuficiência cardíaca. A função protetora da adenosina se manifesta pela vasodilatação coronariana e de vasos colaterais, aumentando o suprimento de oxigênio para os tecidos (KINUGAWA et al., 2006). Além disso, a adenosina é um ativador endógeno do sistema de enzimas antioxidantes durante os processos de injúria e isquemia celular (RAMKUMAR et al., 1995).

O controle dos níveis extracelulares dos nucleotídeos e nucleosídeos de adenina e a conseqüente sinalização purinérgica por eles induzida através dos receptores, é realizada por uma variedade de enzimas ancoradas na membrana celular ou localizadas no meio intersticial de forma solúvel (ZIMMERMANN et al., 2007). Dentre estas enzimas podem-se destacar as E-NTPDases (Ecto-nucleotídeo trifosfato difosfoidrolase, EC 3.6.1.5), a família das E-NPPs (Ecto-nucleotídeo pirofosfatases/fosfodiesterases, EC 3.6.1.5), a ecto-5’-nucleotidase (E.C. 3.1.3.5) e ecto-adenosina deaminase (ADA, EC 3.5.4.4) (ROBSON et al., 2006; YEGUTKIN, 2008). Estas enzimas atuam em conjunto, formando uma cadeia enzimática que tem início com a ação da E-NTPDase e da E-NPP, as quais catalisam a hidrólise de ATP e do ADP formando AMP (ZIMMERMANN et al., 2007). A seguir a enzima 5’-nucleotidase hidrolisa a molécula de AMP formando adenosina, a qual posteriormente é degradada pela ação da ADA gerando inosina (Figura 6) (YEGUTKIN, 2008).

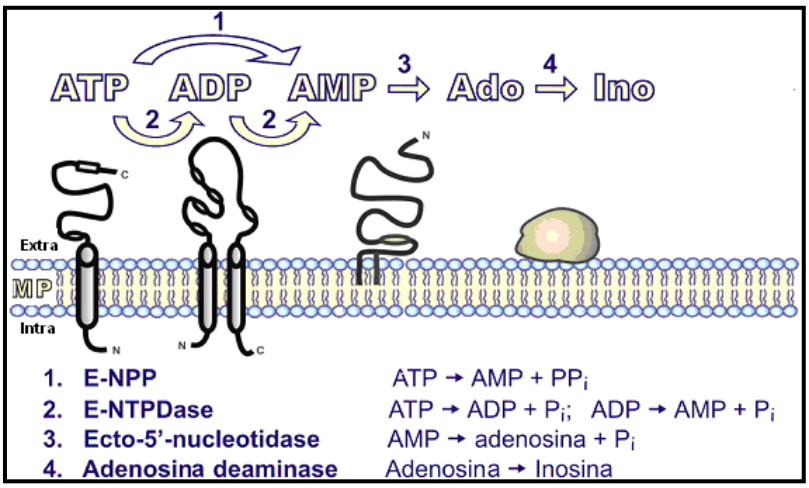


Figure 6. Cascata de ectoenzimas responsáveis pela hidrólise de nucleotídeos de adenina e adenosina. (Adaptado de YEGUTKIN, 2008)

As NTPDases são uma família de enzimas responsáveis pela hidrólise de nucleotídeos tri e difosfatados (ZIMMERMANN et al., 2007) . Oito membros desta família já foram identificados e diferem quanto à especificidade ao substrato, distribuição tecidual e localização celular. Quatro das NTPDases são enzimas tipicamente localizadas na membrana celular com um sítio catalítico na face extracelular (NTPDase 1, 2, 3 e 8) e quatro delas exibem localização intracelular (NTPDases 4, 5, 6 e 7) (Figura 7) (ROBSON et al., 2006).

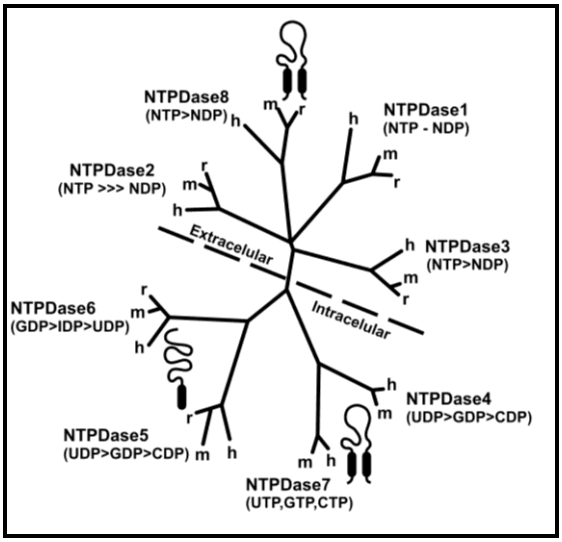


Figura 7. Membros da família da NTPDase (Adaptado de ROBSON et al., 2006).

Estas enzimas são amplamente distribuídas nos tecidos animais e representam as principais ectonucleotidases expressas pelo endotélio e células musculares lisas do sistema circulatório (ZIMMERMANN et al., 1999; SCHETINGER et al., 2001; VIERA et al., 2001; RÜCKER et al., 2008).

A NTPDase-1 (ATP difosfoidrolase, Apirase, Ecto/CD 39) foi a primeira enzima da família NTPDase a ser descrita, e está ancorada na superfície celular através de duas regiões transmembranas próximas ao grupamento amino e carboxi terminal, com o seu sítio catalítico voltado para o meio extracelular (ZIMMERMANN, 2001). Esta enzima hidrolisa tanto ATP como ADP formando AMP na presença de íons Ca^{2+} e Mg^{2+} (ROBSON et al., 2006).

A NTPDase-1 de plaquetas intactas de humanos está envolvida na regulação da concentração dos nucleotídeos, na circulação e no tônus vascular. Alguns estudos indicam que o uso de NTPDase-1 solúvel constitui-se num potencial agente terapêutico para inibição de processos trombóticos mediados por plaquetas. A solução purificada da CD39 bloqueou *in vitro* a agregação plaquetária induzida por ADP e inibiu a reatividade plaquetária induzida por colágeno, demonstrando uma importante função na tromboregulação (GAYLE III et al., 1998; ENJYOJI et al., 1999; MARCUS et al., 2005). As respostas tromboregulatórias da NTPDase podem ser observadas em estudos *in vivo* e *in vitro* que demonstraram sua participação na hemostasia através de um potente efeito anti-trombótico (MARCUS et al., 2003; MARCUS et al., 2005;).

A família das E-NPPs é constituída por sete enzimas nomeadas de NPP1 até NPP7, sendo numeradas de acordo com sua ordem de descoberta (YEGUTKIN, 2008). Com exceção a NPP2, que é secretada no meio extracelular, todos os demais membros são ligados à membrana por um único domínio transmembrana. As NPP1 e 3 têm uma orientação transmembrana do tipo II, com sua porção aminoterminal voltada para o meio intracelular, enquanto que as NPPs 4-7 têm uma orientação do tipo I com sua porção aminoterminal voltada para o meio extracelular (STEFAN et al., 2006).

As E-NPPs possuem uma ampla especificidade por substratos e são capazes de hidrolisar ligações pirofosfato e fosfodiéster de nucleotídeos, ácidos nucléicos, nucleotídeos de açúcar bem como de fosfodiésteres de colina (STEFAN et al., 2005). Entretanto somente as NPP1-3 são capazes de hidrolisar nucleotídeos e são,

portanto relevantes no contexto da cascata de sinalização purinérgica (STEFAN et al., 2006; YEGUTKIN, 2008).

As E-NPPs possuem uma ampla distribuição tecidual o que lhe confere múltiplos papéis biológicos, incluindo reciclagem de nucleotídeos, modulação da sinalização purinérgica, regulação dos níveis de pirofosfato extracelular, proliferação e motilidade celular (STEFAN et al., 2005; 2006). Tem sido reportado que alterações em fosfodiesterases ocorrem em doenças cardiovasculares associadas ao diabetes (GODING et al., 2003). Nagaoka e colaboradores (1998) mostraram um aumento na atividade da NPP3 em aorta de ratos resistentes à insulina propensos à aterosclerose e esse aumento se relacionou positivamente ao aumento dos níveis de mRNA da NPP3. Outros estudos têm demonstrado a correlação entre o aumento da expressão da NPP1 e a resistência à insulina. Foi visto que pacientes portadores de DM tipo 2 tiveram elevação tanto nos níveis de proteína, quanto de atividade enzimática para a NPP1 (MADDUX et al., 1995; MADDUX et al., 2000). Contudo, os mecanismos envolvidos nestas alterações ainda não estão completamente elucidados (STEFAN et al., 2005; TERKELTAUB, 2006).

A ecto-5’-nucleotidase também participa do metabolismo dos nucleotídeos da adenina, atuando em conjunto com as E-NTPDases e E-NPPs. A ecto-5’-nucleotidase é uma glicoproteína ligada a membrana via um glicosil fosfatidilinositol (GPI) com seu sítio catalítico voltado para o meio extracelular que catalisa a hidrólise éster fosfórica do 5’-ribonucleotídeo para o correspondente ribonucleosídeo e fosfato. Esta enzima é amplamente encontrada em uma variedade de tecidos como rins, fígado, encéfalo, pulmão, endotélio vascular, plaquetas e células do sistema imune (COLGAN et al., 2006).

A ecto-5’-nucleotidase pode exercer uma ampla variedade de funções dependendo de sua expressão tecidual e celular. O principal papel fisiológico atribuído a ecto-5’-nucleotidase, é a formação de adenosina a partir do AMP extracelular e a subsequente. No sistema nervoso, ativação dos receptores P1 resulta principalmente na inibição da liberação de neurotransmissores excitatórios (BRUNDEGE & DUNWIDIE, 1997), enquanto que em sistema vascular, resulta em vasodilatação e na inibição da agregação plaquetária (KAWASHIMA et al., 2000).

A enzima ADA também faz parte do conjunto de enzimas responsáveis pela degradação seqüencial dos nucleotídeos e nucleosídeos de adenina (YEGUTKIN, 2008). A ADA é responsável pela deaminação da adenosina com a conseqüente

produção de inosina regulando assim, as concentrações extracelulares deste nucleosídeo (FRANCO et al., 1997).

A atividade da ADA encontra-se aumentada em várias patologias, tais como doenças hepáticas, tuberculose, pneumonia, artrite reumatóide e também no DM (PRAKASH et al.,2006; MALDONADO et al., 2008; BECKER et al., 2010) . Estudos têm sugerido que a insulina está envolvida na regulação da atividade da ADA no diabetes, sendo que a administração de insulina é capaz de diminuir a elevada atividade desta enzima (KURTUL et al., 2004; PRAKASH et al.,2006).

Nos últimos anos, diversos estudos desenvolvidos por nosso grupo de pesquisa têm evidenciado a importância das ecto-enzimas NTPDase, E-NPP, 5'-nucleotidase e ADA no processo de tromborregulação em várias condições patológicas, como o câncer (ARAÚJO et al., 2005, MALDONADO et al., 2010), insuficiência renal (SILVA et al., 2005), esclerose múltipla (SPANEVELLO et al., 2010), artrite reumatóide (BECKER et al., 2010), infarto agudo do miocárdio (BAGATINI et al., 2011) e diabetes (LUNKES et al., 2003, 2004, 2008). De particular interesse, em plaquetas de pacientes com DM tipo 2 e em ratos diabéticos induzidos com aloxano observou-se um aumento na atividade da NTPDase e da 5'-nucleotidase em relação ao grupo saudável, possivelmente funcionando como um mecanismo compensatório para gerar adenosina e assim prevenir a formação de trombos no estado diabético (LUNKES et al., 2003, LUNKES et al., 2004). Recentemente foi demonstrado que o aumento na hidrólise do ATP e ADP encontrado em plaquetas de pacientes diabéticos seria em função de uma maior expressão da CD39 (NTPDase1) (LUNKES et al., 2008; SCHETINGER et al., 2007). Além disso, Lunkes et al. (2008) demonstraram que o aumento na atividade da NTPDase e 5'-nucleotidase de plaquetas é diretamente proporcional ao aumento da concentração de glicose e frutose *in vitro,* sugerindo a participação de altos níveis de glicose como um provável fator de interferência na atividade das ectonucleotidases.

Tendo em vista esses trabalhos que sugerem uma possível participação do sistema purinérgico na patofisiologia do diabetes, torna-se extremamente interessante elucidar as possíveis relações entre o sistema purinérgico e as patologias associadas ao diabetes, como as decorrentes de alterações plaquetárias, tão freqüentes nos pacientes portadores desta doença.

Por outro lado, o interesse pela prevenção e cura de doenças através da alimentação vem aumentando a cada dia, e cada vez com mais embasamento

científico. Neste contexto, um crescente número de estudos epidemiológicos tem associado o consumo de uvas e seus produtos derivados, como o vinho e suco com uma grande variedade de efeitos benéficos para a saúde, como à prevenção de doenças cardiovasculares, hepáticas, neurodegenerativas e vários tipos de câncer (BRADAMANTE & VILLA, 2004; LEIFERT & ABEYWARDENA, 2008; YADAV et al., 2009; IRITI & FAORO, 2009).

Embora os primeiros sinais de consumo do vinho ocorreram há 7000 anos, no Mediterrâneo, as referências sobre o efeitos benéficos do consumo moderado e regular surgiram somente em 1992 com a publicação do paradoxo francês (RENAUD & LORGERIL, 1992). Esse paradoxo despertou atenção da comunidade científica para os compostos do vinho, principalmente o resveratrol, em relação aos benefícios para a saúde humana, destacando-se principalmente a prevenção de complicações cardiovasculares (BERTELLI, 2007).

Durante os anos de 1985 a 1993, a OMS desenvolveu o Projeto MONICA ("MONItoring system for CArdiovascular disease"), um sistema organizado de dados sobre doenças átero-coronarianas, com o objetivo de estudar as características populacionais, regionais e temporais de 37 países, incluindo o Canadá, Itália, França, Grã-Bretanha e os Estados Unidos (KEYS et al., 1986; RENAUD & LORGERIL, 1992). Os dados do projeto MONICA levaram ao surgimento do famoso "Paradoxo Francês", o qual afirmou que os franceses, quando comparados com outros povos do mesmo nível socioeconômico e cultural, apresentavam 2,5 vezes menos mortes por doenças coronarianas, apesar de serem mais sedentários, fumarem mais e consumirem mais gorduras saturadas (TUNSTALL-PEDOE et al., 1999; IMHOF et al., 2004). A principal explicação para tal paradoxo estaria no consumo regular e moderado do vinho tinto pela população francesa, o qual estaria associado à redução do risco de morbidade e mortalidade cardiovascular em 40 a 60% (RENAUD & LORGERIL, 1992; TRICHOPOULOU et al., 2003; LIPI et al., 2010). Vários estudos surgiram a partir desta descoberta exaltando o vinho como uma bebida com atividades antioxidante, antiinflamatória, antimicrobiana, anticarcinogênica e vasodilatadora (SOLEAS et al., 1997; HUANG et al., 2010; RODRIGO et al., 2011; TOZZI CIANCARELLI et al., 2011).

Inicialmente os efeitos benéficos do vinho tinto foram atribuídos ao seu conteúdo de etanol, já que vários estudos populacionais demonstraram que o consumo moderado de bebidas alcoólicas (10-30 g/dia de álcool puro) reduzia o

risco de doenças cardiovasculares, principalmente através da inibição da agregação plaquetária e do aumento dos níveis de colesterol HDL (RENAUD & LORGERIL, 1992; VENKOV et al.,1999). Contudo, uma metanálise de 13 estudos envolvendo mais de 200 mil indivíduos comparou a relação entre o consumo de diferentes bebidas alcoólicas como vinho, cerveja e destilados e o risco de morte por doença cardiovascular Os resultados demonstraram que o consumo diário e moderado de vinho tinto, cerca de 250 mL por dia, reduziu significativamente o risco de morte por tais doenças, enquanto o consumo de cerveja e destilados acarretou pouca ou nenhuma alteração (DI CASTELNUOVO et al., 2002; KLATSKY et al., 2003). DI CASTELNUOVO et al. (2002) ainda relataram que, a partir de certo patamar de consumo diário, o vinho exerce efeito contrário, isto é, aumentando o risco de morte por cirrose e outras causas. Assim, os resultados obtidos nesta metanálise e confirmados posteriormente por outros estudos indicam que o vinho tinto confere benefícios adicionais para a saúde quando comparado com outras bebidas alcoólicas, demonstrando que outros compostos além do álcool, podem estar envolvidos nos efeitos protetores do vinho (DI CASTELNUOVO et al., 2002; KLATSKY et al., 2003).

Numerosos estudos têm atribuído os efeitos benéficos do vinho a alta concentração de compostos fenólicos presentes nesta bebida, os quais também são amplamente encontrados em outros produtos derivados da uva (COOPER et al., 2004; LEIFERT & ABEYWARDENA et al., 2008) . De fato, as uvas constituem uma das principais fontes de polifenóis, entre as frutas (BREKSA et al., 2010). Os compostos fenólicos são produzidos pelos vegetais com a função de protegê-los de estresses causados por fatores bióticos como fungos patogênicos, vírus e bactérias ou por fatores abióticos como a radiação ultravioleta. Assim, os níveis de polifenóis nas uvas variam de acordo com a quantidade de infecção por fungos, exposição ao sol, composição do solo, além do processo de fabricação e conservação dos vinhos (SOLEAS et al., 1997; DIXON, 2001). A composição fenólica do vinho também depende do tipo de uva usada para vinificação. Estudos demonstram que variedades como Sangiovese, Merlot e Tannat apresentam as maiores concentrações de polifenóis, principalmente de resveratrol (SOUTO et al., 2001; HE et al., 2006).

Nos vinhos, já foram identificados cerca de 200 polifenóis, os quais estão distribuídos nas folhas da videira, nas sementes e principalmente na casca das uvas

(BREKSA et al., 2010). Dessa forma os vinhos tintos, que são fermentados na presença das cascas e sementes, têm cerca de 10 vezes mais polifenóis (1000-4000 mg/L) que os vinhos brancos (200-300 mg/L), fermentados na ausência delas, atribuindo ao vinho tinto mais benefícios para a saúde quando comparado com o branco (DUDLEY et al., 2008; OLALLA et al., 2008).

Os compostos fenólicos presentes na uva e seus derivados apresentam uma grande diversidade e são subdivididos em dois grandes grupos em razão da similaridade de suas cadeias de átomos de carbono: flavonóides e não-flavonóides. Do ponto de vista químico, os compostos fenólicos são caracterizados por apresentar um núcleo benzênico, com um ou mais substituintes hidroxílicos, incluindo seus grupos funcionais (WATERHOUSE, 2002; ABE et al., 2007)

Os flavonóides são caracterizados por um esqueleto base contendo 15 átomos de carbono (C6-C3-C6), do tipo 2-fenil benzopirona. Esta grande família é dividida em inúmeras subclasses, as quais se distinguem entre si através do grau de oxidação do seu grupo pirano (BRAVO, 1998) (Figura 8). Fazem parte deste grupo os flavanóis (catequina, epicatequina e epigalocatequina), flavonóis (caempferol, quercetina, rutina e miricetina) e as antocianinas (malvidina e cianidina) (MAMEDE & PASTORE, 2004). Também fazem parte do grupo dos flavonóides, a classe dos dihidroflavonóis e as flavonas das folhas da parreira (GONZALEZ-PARAMAS et al., 2004).

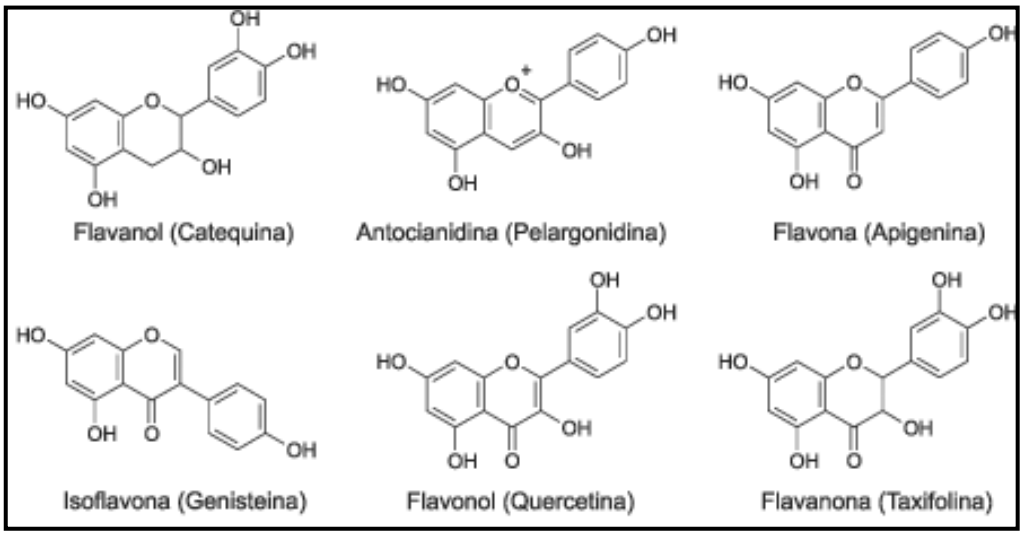


Figura 8. Estrutura das principais classes de flavonóides (CERQUEIRA et al., 2007).

Os não-flavonóides correspondem aos compostos fenólicos mais simples, tais como os ácidos benzóicos (C6-C1) p-hidroxibenzóico, protocatéico, vanílico, gálico e siríngico (Figura 9A); os ácidos cinâmicos, p-cumárico, caféico e ferúlico, portadores de cadeia lateral insaturada (C6 -C3) (Figura 9B) e outros derivados fenólicos de grande importância como os estilbenos, destacando-se o resveratrol (BRAVO, 1998; FLANZY, 2000). Os taninos também são polifenóis abundantes na uva, contudo, não há consenso na literatura sobre sua classificação. Estudos apontam que os taninos estão inseridos na classe de flavonóides (MAMEDE & PASTORE, 2004), outros mostram que eles pertencem à classe de não-flavonóides (HE et al., 2006)

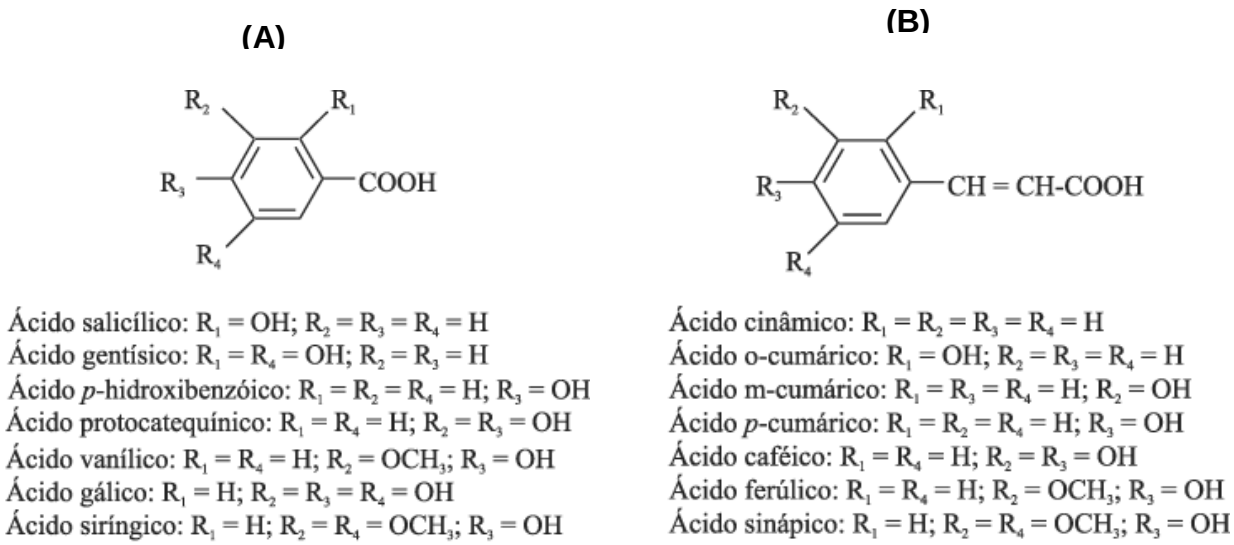


Figura 9. A) Estrutura química dos ácidos benzóicos. B) Estrutura química dos principais ácidos cinâmicos (Adaptado de RAMALHO & JORGE, 2006).

Os compostos fenólicos são componentes importantes nos vinhos, contribuindo para as características sensoriais como cor, sabor, adstringência e dureza (ABE et al., 2007; GIEHL et al., 2007). Além das características organolépticas, todos os flavonóides e não-flavonóides são responsáveis pela potente atividade antioxidante atribuída ao vinho tinto e ao suco de uva (RODRIGO et al., 2011). Tem sido mostrado que uma hora após a ingestão de 300 mL de vinho tinto a capacidade antioxidante do soro aumentou 28%, o que é comparável com aumento de 22% após a ingestão de 1 g de vitamina C (MODUN et al., 2008). De fato, os polifenóis agem como antioxidantes ativos, doando hidrogênio dos seus grupos hidroxila aos radicais livres como $O_2^{\bullet-}$, peroxila e $OH^{\bullet}$, inativando-os

(COOPER et al. 2004; RODRIGO et al., 2011). Além disso, os compostos fenólicos reduzem a formação de hidroperóxidos, impedem a peroxidação lipídica e a oxidação de proteínas e inibem as enzimas oxidativas como a fosfolipase A2, cicloxigenase e a lipoxigenase (QUIDEAU et al., 2011). Os fenóis também podem atuar como protetores e regeneradores dos antioxidantes primários do organismo como o ácido ascórbico, o α-tocoferol e o β-caroteno (FABRIS et al., 2008). Em adição, alguns estudos demonstram que os polifenóis do vinho previnem lesões oxidativas pela modulação da expressão de enzimas antioxidantes (CAO et al., 2004; SPANIER et al., 2009).

Além da proteção antioxidante a ingestão moderada de vinho tinto apresenta vários efeitos antiaterogênicos, destacando-se a inibição da oxidação do colesterol LDL e da agregação plaquetária, a melhora da função endotelial, a indução da liberação de óxido nítrico, a promoção da vasodilatação e o decréscimo da pressão arterial (DELL'AGLI et al., 2004; BERTELLI et al., 2007; SCHMITT & DIRSCH, 2009; GRESELE et al., 2011). Alguns autores ainda relatam efeitos positivos na sensibilidade à insulina e diminuição da progressão da nefropatia diabética, colocando essas substâncias como possíveis coadjuvantes no tratamento de pacientes diabéticos (NAPOLI et al., 2005; ABRAHAM, 2010).

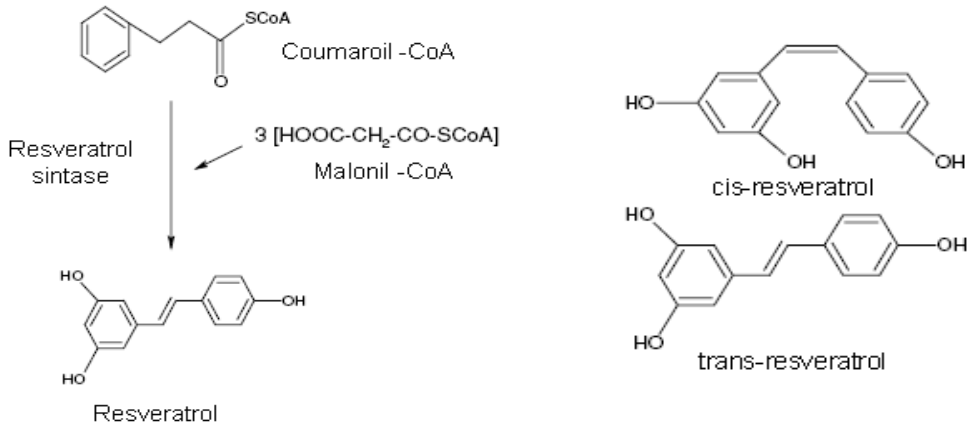
Assim como o vinho, o suco de uva melhora os fatores de risco relacionados ao desenvolvimento da aterosclerose, contudo não evita a oxidação do colesterol LDL (GIEHL et al., 2007). O conteúdo de compostos fenólicos totais encontrados no suco de uva é semelhante ao encontrado no vinho tinto, contudo a concentração de resveratrol e antocianinas são maiores no vinho (MACHADO et al., 2011). Diante disso, o suco de uva pode ser considerado uma boa fonte de compostos fenólicos para a população abstêmia (DOHAWALA & VITA, 2009). No entanto, muitos estudos têm demonstrado que o vinho tinto apresenta efeitos benéficos maiores do que aqueles observados para o suco de uva, os quais tem sido atribuídos a maior absorção pelo organismo dos polifenóis provenientes do vinho (PACE-ASCIAK et al., 1996). De fato, tem sido demonstrado que o etanol aumenta a absorção intestinal e consequentemente a biodisponibilidade dos polifenóis do vinho no organismo. Além disso, o etanol também previne a precipitação dos polifenóis do vinho no trato digestivo, sendo considerado um estabilizante natural destes compostos (SERAfiNI et al., 1996; DRAGONI et al., 2006).

Dentre todas as substâncias polifenólicas presentes na uva e seus derivados destaca-se uma em especial: o resveratrol. Estudos recentes mostraram que os efeitos benéficos do vinho provêm principalmente desta substância, mesmo considerando que o vinho contém diversos outros polifenóis, como a quercetina, as catequinas e os taninos, que também poderiam contribuir para este efeito (BORRIELLO et al., 2010; GRESELE et al., 2011)

Dezenas de trabalhos são publicados anualmente mostrando que o resveratrol pode prevenir ou diminuir a progressão de diversas doenças, incluindo o câncer (RASHID et al., 2011), o diabetes (SZKUDELSKA & SZKUDELSKI, 2010) as doenças cardiovasculares (GRESELE et al., 2011) e as neurodegenerativas (FOTI et al., 2011). Foi demonstrado também que o resveratrol pode mimetizar os efeitos da restrição calórica e prevenir diversos processos do envelhecimento, aumentando a longevidade (BAUR et al., 2006; ABE et al., 2007; AGARWAL & BAUR, 2011)

O resveratrol (3,5,4'-triidroxiestilbeno) é um polifenol encontrado em cerca de 72 espécies de plantas e também em produtos alimentares tais como amendoim, amora, framboesa e principalmente em uvas e no vinho tinto, onde sua presença é mais conhecida e estudada (SAIKO et al., 2008). Na uva o resveratrol é sintetizado quase que exclusivamente na casca, onde é formado a partir da condensação de três moléculas de malonil-CoA e uma molécula de p-coumaroil-CoA, em uma reação catalisada pela enzima estilbeno sintase (KING et al., 2006) (Figura 10A).

O resveratrol é uma fitoalexina, que atua no controle da infecção por fungos. Logo, os níveis de resveratrol são dependentes de fatores que podem gerar mais ou menos estresse, tais como: ambiente de cultivo, clima, exposições a patógenos e, no caso dos vinhos, pelo método de produção (SIGNORELLI & GHIDONI 2005). Por essas razões, os níveis de resveratrol encontrados nos vinhos tintos variam muito, situando-se em média entre 0,82 e 5,75 mg/L podendo chegar a 9 mg/L, enquanto o suco de uva comercial contém aproximadamente 0,07 a 1,59 mg/L de resveratrol (SOUTO et al., 2001; DONG, 2003). SOUTO et al. (2001), verificaram que a média de resveratrol nos vinhos brasileiros fica em torno de 3,57 mg/L, sendo uma das mais altas do mundo. Esses valores, provavelmente devem ser resultantes da alta precipitação pluviométrica na região da Serra Gaúcha, o que favorece a proliferação de doenças fúngicas na parte aérea das videiras (SOUTO et al., 2001; MACHADO et al., 2011).



(A) **(B)**

Figura 10. A) Síntese do resveratrol. B) Isômeros cis- e trans-resveratrol (Adaptado de King et al., 2006).

O resveratrol é um polifenol da classe dos estilbenos com uma estrutura química constituída por dois anéis aromáticos unidos por uma ponte metila e ligações de três grupos hidroxila reativos inseridos nos anéis (KING et al., 2006). Esta estrutura é sintetizada naturalmente sob duas formas isômeras: *trans* e *cis* (Figura 10B). O isômero trans é encontrado em maiores quantidades e é responsável pelos efeitos biológicos do resveratrol. Na forma *cis* e *trans* o resveratrol também pode apresentar-se ligado a uma molécula de açúcar formando seus respectivos glicosídeos. A forma trans é fotossensível, podendo ser convertida em presença de luz visível, na forma cis, perdendo assim a sua função biológica (VITRAC et al., 2005; BAUR & SINCLAIR, 2006).

Os efeitos benéficos do resveratrol a saúde dependem em parte da sua biodisponibilidade e do seu metabolismo. Recentemente foi demonstrado que a administração oral de resveratrol em humanos resulta em alta absorção, em torno de 70%, mas com baixa biodisponibilidade (WALLE et al., 2004). A partir da absorção, o resveratrol é metabolizado sofrendo principalmente glicuronidação e sulfatação e pode interagir com proteínas hepáticas de fase I e II (YU et al., 2002). Vitaglione e colaboradores (2005) demonstraram que o metabólito mais abundante no soro humano é o resveratrol glicoronídeo. As concentrações de resveratrol não conjugado na corrente circulatória e tecidos após a sua ingestão são relativamente baixas, menor do que 2μM no plasma (VITAGLIONE et al., 2005; PIGNATELLI et al., 2006). Nos tecidos o resveratrol foi encontrado principalmente no fígado e nos rins, mas

também no cérebro, coração e pulmões (EL-MOHSEN et al., 2006). Porém, a concentração de resveratrol nos tecidos é baixa: em ratos tratados via oral com 20 mg/kg de resveratrol, a concentração encontrada no cérebro após 10 minutos foi de 0.11 nmol/g (ASENCI et al., 2002). Por outro lado, administração via intraperitoneal e intravenosa gera uma concentração maior de resveratrol tecidual, tendo sido a via utilizada em diversos modelos animais recentes.

Outro aspecto importante da farmacocinética do resveratrol é o fato de o composto se ligar em proteínas do plasma (1:1), como a albumina sérica e a hemoglobina, mantendo o resveratrol no organismo por um período maior de tempo e contribuindo para a sua distribuição nos tecidos (JANNIN et al., 2004). Além disso, estudos *in vivo* demonstram que muitos dos efeitos benéficos do resveratrol podem ser atribuídos a uma associação com outros componentes do vinho, como a quercitina, que impediria a sua modificação metabólica (DE SANTI et al., 2000).

O resveratrol possui uma variedade de propriedades biológicas e farmacológicas estabelecidas que contribuem para seus efeitos benéficos na prevenção e tratamento de diversas patologias (SOLEAS et al, 1997; FREMONT et al, 2000; KING et al., 2006; SAIKO et al., 2007).

Uma das mais conhecidas e importantes propriedades do resveratrol é a sua potente capacidade antioxidante atenuando os efeitos das EROs no organismo (FABRIS et al. 2008). Tal propriedade está associada principalmente com a estrutura química deste composto (KING et al., 2006). Os grupos hidroxilas dos anéis fenólicos do resveratrol agem como doadores de elétrons, sendo responsáveis pela capacidade de neutralizar e seqüestrar o radical hidroxil e o ânion superóxido prevenindo dessa forma a peroxidação lipídica das membranas, oxidação protéica e danos ao DNA (LÓPEZ-VÉLEZ et al., 2003; LEONARD et al., 2003; MOKNI et al., 2007). O resveratrol pode atuar também como um agente quelante de metais como o ferro, que exerce um papel crucial na geração de EROs através de reações como a de Fenton. Além disso, dados da literatura demonstram que os efeitos antioxidantes do resveratrol podem ser mediados através da capacidade deste induzir o aumento na expressão e na atividade de enzimas antioxidantes como CAT, SOD e GSHPx (CAO & LI, 2004; MOKNI et al, 2007). Em células endoteliais, o resveratrol induz um aumento na atividade da gama glutamilcisteinil sintetase, enzima envolvida na síntese da GSH, provocando um aumento nos níveis

intracelulares deste tripeptídeo e consequentemente um decréscimo do estresse oxidativo (RODRIGO et al., 2011).

Outra propriedade bastante estudada e de grande importância do resveratrol é seu potente efeito na proteção e redução da incidência de doenças coronárias (BRADAMANTE et al., 2004; MOKNI et al., 2006). Estudos têm demonstrado que o resveratrol inibe os diferentes passos da ativação plaquetária, tais como adesão das plaquetas ao colágeno e ao fibrinogênio, redução da secreção granular e inibição da agregação plaquetária (SZEWCZUK et al., 2004; OLAS & WACHOWICZ, 2005). Além disso, o resveratrol também aumenta a vasodilatação através de mecanismos envolvendo o óxido nitrico (CRUZ et al., 2006), previne a oxidação de lipoproteínas de baixa densidade (LDL) e a formação de placas ateroscleróticas (LI et al., 2006), protegendo o coração contra a injúria da isquemia e da reperfusão (WANG et al., 2004).

Estudos sugerem também que o resveratrol tem um importante papel neuroprotetor em condições de injuria cerebral e nos processos neurodegenerativos (DORE, 2005; ANEKONDA, 2006). Além disso, o resveratrol possui propriedades anti-inflamatórias, devido a sua capacidade de inibir a transcrição e a atividade da ciclooxigenase (COX), em especial a COX-2, prejudicando dessa forma o metabolismo do ácido araquidônico e interrompendo a produção de prostaglandinas que são substâncias pró-inflamatórias (KIM et al., 2006; ZHU et al., 2008). Este polifenol também exerce atividade antitumoral, inibindo os três estágios da carcinogênese: iniciação, promoção e progressão (SAIKO et al., 2006; RASHID et al., 2011). Ele também está envolvido em várias vias de sinalização que regulam o ciclo celular e apoptose (SIGNORELLI & GHIDONI, 2005), e recentemente foi associado ao aumento da expectativa de vida em mamíferos, envolvendo uma família de proteínas denominadas sirtuínas (BAUR & SINCLAIR, 2006).

Em relação ao diabetes, o resveratrol tem mostrado um papel benéfico na prevenção e também no alívio de algumas complicações. Em ratos diabéticos induzidos com STZ este composto tem diminuído eficientemente os danos causados pelo estresse oxidativo, tanto no soro quanto em tecidos como fígado, rim (CHEN, 2005) e cérebro (ATES et al., 2005). Além disso, o resveratrol mostrou um papel benéfico na disfunção renal (SHARMA et al., 2006) e disfunção cardíaca em animais diabéticos (THIRUNAVUKKARASU et al., 2007).

Neste contexto, tendo em vista os inúmeros efeitos benéficos relacionados aos polifenóis presentes na uva e nos seus subprodutos torna-se relevante investigar os efeitos do tratamento com resveratrol, com vinho tinto e com suco de uva, na atividade das enzimas que hidrolisam nucleotídeos de adenina em plaquetas, na agregação plaquetária e no perfil oxidativo de ratos diabéticos induzidos com STZ, a fim de contribuir para a busca de novas terapias que possam beneficiar pacientes com esta endocrinopatia.

2. OBJETIVOS

2.1. Objetivo geral

Verificar o efeito do tratamento com resveratrol, vinho tinto e suco de uva nos biomarcadores de estresse oxidativo em fígado e rim, na atividade da NTPDase, E-NPP, 5'-nucleotidase e ADA em plaquetas e na agregação plaquetária de ratos diabéticos induzidos por estreptozotocina.

2.2. Objetivos específicos

- Verificar o efeito do tratamento com resveratrol nos níveis de peroxidação lipídica, na atividade das enzimas CAT, SOD e nos níveis de tióis não-protéicos e ácido ascórbico em fígado e rim de ratos diabéticos.

- Determinar o efeito do tratamento com resveratrol na atividade da enzima delta-aminolevulínico ácido desidratase (δ-ALA-D) em fígado e rim de ratos diabéticos.

- Determinar o efeito do tratamento com resveratrol, suco de uva e vinho tinto na atividade das enzimas NTPDase, 5'-nucleotidase, E-NPP, ADA em plaquetas de ratos diabéticos.

- Determinar o efeito do tratamento com suco de uva e vinho tinto na agregação plaquetária de ratos diabéticos.

- Avaliar, *in vitro*, os efeitos dos polifenóis resveratrol, quercetina, rutina, ácido gálico e ácido cafeíco na agregação plaquetária e na atividade das enzimas NTPDase, 5'-nucleotidase e ADA de plaquetas de ratos diabéticos.

3. METODOLOGIA E RESULTADOS

Os resultados desta tese estão apresentados na forma de dois artigos científicos e um manuscrito. Os itens materiais e métodos, resultados, discussão e referências bibliográficas encontram-se nos próprios artigos e manuscrito e representam a íntegra deste estudo. Os artigos estão estruturados de acordo com as versões publicadas nas revistas Biochimie (Artigo I), Life Sciences (Artigo II) e o manuscrito esta nas normas da revista científica Journal of Nutritional Biochemistry para o qual foi submetido.

Artigo I: Effects of resveratrol on biomarkers of oxidative stress and on the activity of delta aminolevulinic acid dehydratase in liver and kidney of streptozotocin-induced diabetic rats

Artigo II: Effects of resveratrol on nucleotide degrading enzymes in streptozotocin-induced diabetic rats

Manuscrito I: Moderate red wine and grape juice consumption modulate the hydrolysis of the adenine nucleotides and decrease platelet aggregation in streptozotocin-induced diabetic rats

3.1. ARTIGO I

Effects of resveratrol on biomarkers of oxidative stress and on the activity of delta aminolevulinic acid dehydratase in liver and kidney of streptozotocin-induced diabetic rats

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Effects of resveratrol on biomarkers of oxidative stress and on the activity of delta aminolevulinic acid dehydratase in liver and kidney of streptozotocin-induced diabetic rats

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ABSTRACT

The present study investigated the effects of resveratrol (RV), a polyphenol with potent antioxidant properties, on oxidative stress parameters in liver and kidney, as well as on serum biochemical parameters of streptozotocin (STZ)-induced diabetic rats. Animals were divided into six groups ($n = 8$): control/saline; control/RV 10 mg/kg; control/RV 20 mg/kg; diabetic/saline; diabetic/RV10 mg/kg; diabetic/RV 20 mg/kg. After 30 days of treatment with resveratrol the animals were sacrificed and the liver, kidney and serum were used for experimental determinations. Results showed that TBARS levels were significantly increased in the diabetic/saline group and the administration of resveratrol prevented this increase in the diabetic/RV10 and diabetic/RV20 groups ($P < 0.05$). The activities of catalase (CAT), superoxide dismutase (SOD) and aminolevulinic acid dehydratase (δ-ALA-D) and the levels of non protein thiols (NPSH) and vitamin C presented a significant decrease in the diabetic/saline group when compared with the control/saline group ($P < 0.05$). The treatment with resveratrol was able to prevent these decrease improving the antioxidant defense of the diabetic/RV10 and diabetic/RV20 groups ($P < 0.05$). In addition, the elevation in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamiltransferase (γ-GT) activities as well as in levels of urea, creatinine, cholesterol and triglycerides observed in the diabetic/saline group were reverted to levels close to normal by the administration of resveratrol in the diabetic/RV10 and diabetic/RV20 groups ($P < 0.05$). These findings suggest that resveratrol could have a protector effect against hepatic and renal damage induced by oxidative stress in the diabetic state, which was evidenced by the capacity of this polyphenol to modulate the antioxidant defense and to decrease the lipid peroxidation in these tissues.

1. Introduction

Diabetes mellitus is a serious, complex chronic condition which is a major source of ill health all over the world [1]. This metabolic disorder is characterized by hyperglycemia and is associated with disturbances in carbohydrate, protein and fat metabolism which occur secondary to an absolute or relative lack of insulin [1].

The precise cellular and molecular mechanism which underlies the etiology and progression of diabetes is still not fully understood. However, increasing evidence suggests that oxidative stress plays a crucial role in the pathogenesis of diabetes and its complications [2,3]. Elevated levels of blood glucose can induce non-enzymatic and auto-oxidative glycosylation, increase polyol and hexosamine pathway, promote protein kinase-C activation and lead to alterations levels of inflammatory mediators, as well as in the status of antioxidant defense [2–5]. These pathways are involved in the

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generation of reactive oxygen species (ROS) in diabetic state, which directly contribute to the increase of oxidative stress in various organs and tissues [2,5]. In addition to the pancreatic β-cells, supraphysiological glucose levels are notorious to provoke oxidative damage in hepatic and renal cells and consequently has been associated with functional and morphological alterations in liver and kidney of diabetic rats [6–8]. In fact, the liver is the focal organ of oxidative and detoxifying processes as well as free radical reactions and the biomarkers of oxidative stress are elevated in the liver at an early stage in many diseases, including diabetes mellitus [8].

The most commonly recognized effect of oxidative stress is the oxidation and damage of macromolecules such as proteins, lipids, DNA, and enzymes involved in energy production, thereby contributing to cellular injury, energetic deficit, and the acceleration of cell death through apoptosis and necrosis [9,10]. In both human and animal diabetics, significant increases in lipoperoxidation products and/or decreases of some antioxidants have been reported, and the presence of oxidative stress has been judged by these indices [10,11].

The effect of ROS is balanced by the antioxidant action of non-enzymatic antioxidants as well as by antioxidant enzymes. The most efficient enzymatic antioxidants involve superoxide dismutase (SOD) and catalase (CAT) while non-enzymatic antioxidants include non-proteics thiol, vitamin C and vitamin E [2,9,12]. These antioxidant defenses are extremely important since they represent the direct removal of free radicals, providing maximal protection for biological sites [12].

Of particular interest, aminolevulinate dehydratase (δ-ALA-D), also known as porphobilinogen synthase, is the second enzyme of the heme pathway. This enzyme catalyzes the condensation of two molecules of δ-aminolevulinic acid (δ-ALA), yielding to the monopyrrole product porphobilinogen [13]. It is a metalloenzyme, containing sulfhydryl (-SH) groups and zinc, which are essential for its activity [14]. Notably, its cysteinyl residues are highly sensitive to heavy metals [15], molecular oxygen, and other pro-oxidant conditions such as hyperglycemia [16] that induces disulfide bond formation and enzyme inhibition. Therefore, the sulfhydryl enzyme δ-ALA-D, used together with other parameters, can play an important role as a marker of oxidative stress and impairment of metabolic processes [15,16].

In addition, the use of compounds with antioxidant properties has demonstrated a series of beneficial effects in the prevention and treatment of diabetic complications caused by oxidative stress [17]. Resveratrol (3, 4′, 5- trihydroxy-trans-stilbene), a natural polyphenol found mainly in grapes and red wine, has been reported to have a wide range of biological properties [18]. The potent antioxidant activity of resveratrol is well established in the literature [19], however, evidence has demonstrated that this compound also possesses anti-inflammatory [20], antiaggregant and neuroprotective properties [21,22]. Based on these findings, resveratrol has become attractive as a therapeutic agent in the treatment of a variety of pathologies including neurodegeneration, cancer, cardiovascular disease and diabetes mellitus [20,22,23]. The mechanisms underlying the benefic effects of resveratrol are not totally elucidated, but have been related mainly to its antioxidant activity that has demonstrated to protect tissues, such as liver, kidney and brain against a variety of damage caused by oxidative stress [19,24].

Therefore, considering that resveratrol has important antioxidant actions and that the oxidative stress has been associated with the pathogenesis of different diabetic complications the aim of this study was to investigate the effects of resveratrol in the oxidative stress parameters in liver and kidney, and in the blood biochemical parameters in streptozotocin (STZ)-induced diabetic rats, a well-characterized animal model of type 1 diabetes, in order to verify the potential therapeutic this compound in hepatic and renal oxidative damage in diabetic state in rats with STZ-induced type 1 diabetes.

2. Materials and methods

2.1. Chemicals

Streptozotocin (STZ), resveratrol (3, 5, 4′-trihydroxy-trans-stilbene, approximately 99% purity), δ- aminolevulinic acid (δ–ALA), reduced glutathione (GSH), 5,5′- dithio-bis-2-nitrobenzoic acid (DTNB), tris (hydroxymethyl)-aminomethane GR, thiobarbituric acid (TBA) and Coomassie brilliant blue G were purchased from Sigma Chemical Co (St. Louis, MO, USA). All other reagents used in the experiments were of analytical grade and of the highest purity.

2.2. Animals

Adult male Wistar rats (70–90 days; 180–280 g) from the Central Animal House of the University Federal of Santa Maria (UFSM) were used in this experiment. The animals were maintained at a constant temperature (23 ± 1 °C) on a 12 h light/dark cycle with free access to food and water. All animal procedures were approved by the Animal Ethics Committee from the Federal University of Santa Maria (protocol under number: 21/2007).

2.3. Experimental induction of diabetes

Type 1 diabetes was induced by a single intraperitoneal injection of 55 mg/kg streptozotocin (STZ), diluted in 0.1 M sodium-citrate buffer (pH 4.5). The age-matched control rats received an equivalent amount of the sodium-citrate buffer. STZ-treated rats received 5% of glucose instead of water for 24 h after diabetes induction in order to reduce death due to hypoglycemic shock. Blood samples were taken from the tail vein 48 h after STZ or vehicle injection to measure glucose levels [25]. Glucose levels were measured with a portable glucometer (ADVANTAGE, Boehringer Mannheim, MO, USA). Only animals with fasting glycemia over 16 mmol/L were considered diabetic and used for the present study. During the experiment the levels of blood glucose was verified four times (2, 10, 20 and 30 days after the beginning of treatment). The animals that maintained fasting glycemia higher than 16 mmol/L were considered diabetic and selected for the assays.

2.4. Treatment with resveratrol

The animals were randomly divided into six groups (8 rats per group): Control/saline; Control/RV 10 mg/kg; Control/RV 20 mg/kg; Diabetic/saline; Diabetic/RV 10 mg/kg; and Diabetic/RV 20 mg/kg. Two week after diabetes induction, the animals belonging to group control/RV10 and diabetic/RV10 received 10 mg/kg of resveratrol and the animals from control/RV20 and diabetic/RV20 groups received 20 mg/kg of resveratrol, while the animals from control/saline and diabetic/saline groups received saline solution. Resveratrol was freshly prepared in 25% ethanol and was administered intraperitoneally at between 10 and 11 a.m. once a day during 30 days, at a volume not exceeding 0.1 ml/100 g rat weight.

The choice of the doses of 10 and 20 mg/kg of resveratrol was made based in previous works of our research group, in which diabetic rats were treated with resveratrol in the concentrations of 10 and 20 mg/Kg (i.p.) [25–27,53,54] and also in several studies that used theses same concentrations of resveratrol and obtained beneficial results [28–30]. Furthermore, the choice of these concentrations take into account that red wine is the main

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dietary source of resveratrol [31]. Assuming that the average concentration of trans-resveratrol in wine is 5 mg/L [32] and moderate daily consumption of wine is 250 mL, the mean daily intake of resveratrol under these conditions is 0.02 mg/kg. Thus, we selected a dose that was 500 (10 mg/Kg) and 1000 (20 mg/kg) times higher than this estimate to provide a sufficiently large safety margin [28].

2.5. Tissue preparation

Twenty-four hours after the treatment, the animals were previously anesthetized with halothane and submitted to euthanasia. The samples of liver and kidney were quickly removed, placed on ice and homogenized within 10 min in cold 50 mM Tris–HCl pH 7.4 (1/10, w/v). The homogenate was centrifuged at 2000 g, at 4 °C, for 10 min to yield the low speed supernatant (S1) that was used immediately for TBARS, vitamin C, non-protein thiol group (NPSH) and δ ALA-D assay. Furthermore, during all procedures the S1 was maintained on ice. It is important to note that the immediate use of sample can prevent a possible alteration in the sample caused by storage time and by freezing process. Moreover, it is important point out that if any oxidation occurs spontaneously in samples from diabetic and diabetic rats treated with resveratrol it will be deleted, since all the procedures for the test samples and control sample are carried out together. In order to perform SOD and CAT assay, liver and kidney were diluted and homogenized as described in the respective (Sections 2.7).

2.6. Determination of lipid peroxidation

Lipid peroxidation in liver and kidney was estimated colorimetrically by measuring thiobarbituric acid reactive substances (TBARS) using the method described previously by Ohkawa et al. [33]. In short, the reaction mixture contained 200 μL of samples of S1 from liver and kidney or standard (MDA-malondialdehyde 0.03 mM), 200 μL of 8.1% sodium dodecylsulfate (SDS), 750 μL of acetic acid solution (2.5 M HCl, pH 3.5) and 750 μL of 0.8% TBA. The mixtures were heated at 95 °C for 90 min. TBARS tissue levels were expressed as nmol MDA/mg protein.

2.7. Catalase (CAT) and superoxide dismutase (SOD) activities

For the CAT assay, liver and kidney were homogenized in 50 mM potassium phosphate buffer, pH 7.5, at a proportion of 1:9 (w/v) and 1:5 (w/v), respectively. The homogenate was centrifuged at 2000 g for 10 min to yield a supernatant that was used for the enzyme assay. CAT activity was measured by the method of Nelson and Kiesow [34]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7), 10 mM H_2O_2 and 20 μL of the supernatant. The rate of H_2O_2 reaction was monitored at 240 nm for 2 min at room temperature. The enzymatic activity was expressed in units mg^{-1} protein (One unit of the enzyme is considered as the amount of CAT which decomposes 1 μmol of H_2O_2 per min at pH 7 at 25 °C.)

With the purpose of performing the SOD assay [35] kidney and liver was adequately diluted with Tris–HCl pH 7.4 at a proportion of 1:40 (w/v) and 1:60(w/v) respectively. Briefly, epinephrine undergoes auto-oxidation at pH 10.2 to produce adrenochrome, a colored product that was detected at 480 nm. The addition of samples (10, 20, 30 μL) containing SOD inhibits the auto-oxidation of epinephrine. The rate of inhibition was monitored during 180 s. The amount of enzyme required to produce 50% inhibition was defined as 1 unit of enzyme activity.

2.8. Vitamin C and non-protein thiol group (NPSH) content

Hepatic and renal vitamin C levels were determined by the method of Jacques-Silva et al. [36]. Proteins of liver and kidney were precipitated in a cold 10% trichloroacetic acid (TCA) solution at a proportion of 1:1 (v/v) and submitted to centrifugation again. This supernatant was then used for analysis. A 300 μL aliquot of sample in a final volume of 575 μL of solution was incubated for 3 h at 37 °C then 500 μL H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using a color reagent containing 4.5 mg/mL dinitrophenyl hydrazine (DNPH) and $CuSO_4$ (0.075 mg/mL). Vitamin C levels are expressed as μg ascorbic acid/g tissue.

NPSH was measured spectrophotometrically with Ellman's reagent [37]. An aliquot of 100 μL for liver and 200 μL for kidney in a final volume of 900 μL of solution was used for the reaction. The reaction product was measured at 412 nm after the addition of 10 mM 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB) (0.05 mL). A standard curve using cysteine was added to calculate the content of thiol groups in samples, and was expressed as μmol SH/g tissue.

2.9. δ-Aminolevulinic acid dehydratase activity (δ-ALA-D)

Hepatic and renal δ-ALA-D activity was assayed according to the method of Sassa [38] by measuring the rate of porphobilinogen (PBG) formation, except that in all enzyme assays the final concentration of ALA was 2.2 mM. An aliquot of 200 μL of sample S1 was incubated for 0.5 h (liver) and 1 h (kidney) at 37 °C. The reaction was stopped by addition of 250 μL of trichloroacetic acid (TCA). The reaction product was determined using modified Ehrlich's reagent at 555 nm. ALA-D activity was expressed as nmol porphobilinogen (PBG) mg^{-1} protein h^{-1}.

2.10. Biochemical analysis

The activities of the enzymes of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and γ-glutamyltransferase (γ-GT) and the levels of urea and creatinine were determined using standard methods on Cobas MIRA® (Roche Diagnostics, Basel, Switzerland) automated analyzer. In addition, serum total cholesterol and triglycerides concentrations were measured using standard enzymatic methods using Ortho-Clinical Diagnostics® reagents on the fully automated analyzer (Vitros 950® dry chemistry system; Johnson & Johnson, Rochester, NY, USA).

2.11. Protein determination

Protein was measured by the method of Bradford [39] using bovine serum albumin as standard.

2.12. Statistical analysis

Statistical analysis was done by the commercial SPSS package for Windows©. All data were expressed as mean ± S.D. Data were analyzed statistically by one-way ANOVA followed by the Duncan's multiple tests. Differences were considered significant when the probability was $P < 0.05$.

3. Results

3.1. Blood glucose and body weight

The body weight and blood glucose levels determined at the onset and at the end of the experiment are presented in Table 1. As can be observed, the blood glucose levels and body weight at the onset of the study showed no significant differences among the

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Table 1
The effect of different doses of resveratrol (RV) on body weight and fasting blood glucose levels in control and diabetic rats at the onset and the end of the experiment (30 days after resveratrol treatment).

Groups	Blood glucose levels (mmol/L)		Body weight (g)	
	Onset	End	Onset	End
Control/saline	6.30 ± 1.10	6.20 ± 1.19	256 ± 4.50	284 ± 8.15
Control/RV 10 mg/kg	5.60 ± 0.86	6.10 ± 2.87	250 ± 5.19	269 ± 9.40
Control/RV 20 mg/kg	6.20 ± 0.90	5.80 ± 1.27	240 ± 3.10	261 ± 4.68
Diabetic/saline	6.10 ± 1.28	25.10 ± 1.26*	250 ± 5.09	189 ± 15.44*
Diabetic/RV10 mg/kg	5.80 ± 2.13	25.60 ± 2.24*	265 ± 4.17	202 ± 7.47*
Diabetic/RV20 mg/kg	5.90 ± 1.19	23.20 ± 1.52*	270 ± 5.24	202 ± 11.41*

Values are expressed as mean ± S.D. *Significant difference when compared to the control/saline group (*$P<0.05$, n=6–8). ANOVA-Duncan's Test.

groups. In the end of experiment, the blood glucose levels were significantly increased, while the body weight was significantly decreased in diabetic/saline, diabetic/RV 10 and diabetic/RV20 groups ($P < 0.05$) when compared to the control/saline group. However, the treatment with resveratrol had no effects on glucose levels or on body weight in the diabetic/RV10 and diabetic/RV20 groups, when compared to the control/saline group. Similarly, no significant differences in glucose levels and body weight were observed when resveratrol was administered per se in the control/RV10 and control/RV20 groups at the end of the study when compared to the control/saline group.

3.2. Lipid peroxidation

Hepatic and renal lipid peroxidation estimated by TBARS levels is shown in Fig. 1A and B, respectively. As can be observed, TBARS levels in liver and kidney were significantly increased in the diabetic/saline group when compared to the control/saline group ($P < 0.05$). However, treatment with resveratrol prevented an increase of lipid peroxidation in both tissues in the diabetic/RV10 and diabetic/RV20 groups, when compared to the diabetic/saline group ($P < 0.05$). No significant difference on TBARS level in kidney was observed in the control/RV10 and control/RV20 groups when compared to the control/saline group.

3.3. Catalase (CAT) and superoxide dismutase (SOD) activities

The effect of resveratrol on the activities of enzymatic antioxidants CAT and SOD in hepatic and renal tissues are presented in Figs. 2 and 3, respectively. Liver (Fig. 2A) and kidney (Fig. 2B) CAT activity was significantly decreased in the diabetic/saline group when compared to the control/saline group ($P < 0.05$). The treatment with resveratrol prevented this decrease in the enzyme activity in liver and kidney in the diabetic/RV10 and diabetic/RV20 groups when compared to the diabetic/saline group ($P < 0.05$). In

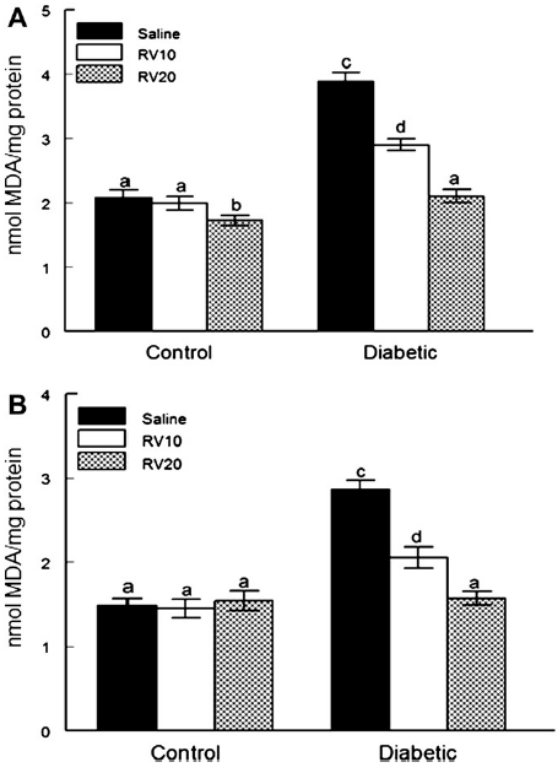

Fig. 1. Levels of thiobarbituric acid reactive substances (TBARS) in liver (A) and kidney (B) from STZ-induced diabetic rats and those treated with resveratrol. Bars represent means ± S.D. Groups with different letters are statistically different ($P < 0.05$; $n = 8$). ANOVA-Duncan's Test.

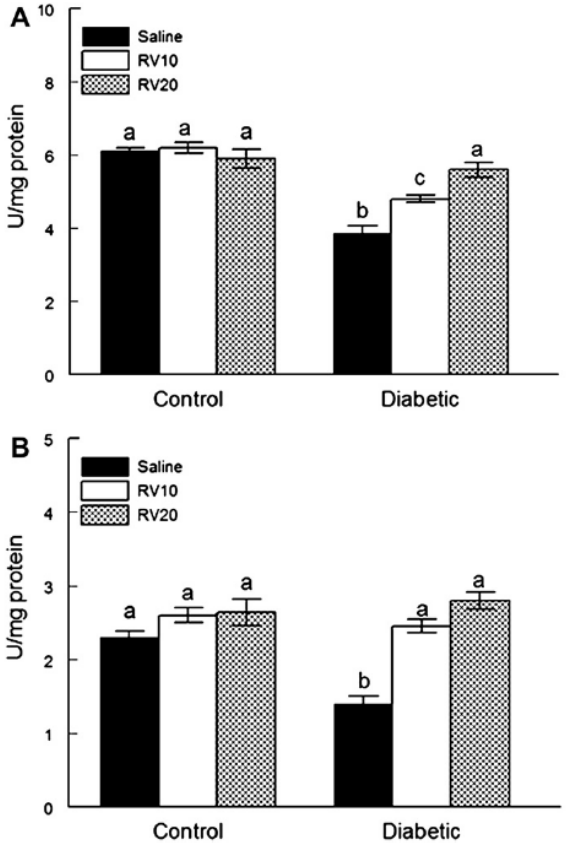

Fig. 2. CAT activity in liver (A) and kidney (B) from STZ-induced diabetic rats and those treated with resveratrol. Bars represent means ± S.D. Groups with different letters are statistically different ($P < 0.05$; $n = 8$). ANOVA-Duncan's Test.

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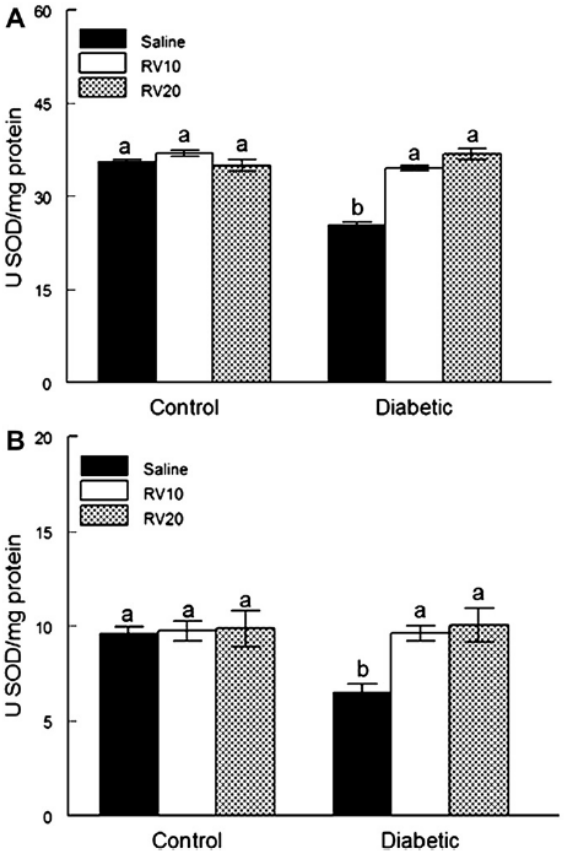

Fig. 3. SOD activity in liver (A) and kidney (B) from STZ-induced diabetic rats and those treated with resveratrol. Bars represent means ± S.D. Groups with different letters are statistically different ($P < 0.05$; $n = 8$). ANOVA-Duncan's Test.

addition, treatment with resveratrol per se did not alter significantly the CAT activity in both types of tissues, in the control/RV10 and control/RV20 groups when compared to the control/saline group.

Results found for SOD activity were similar to those obtained for CAT activity. A significant decrease in liver (Fig. 3A) and kidney (Fig. 3B) SOD was observed activity in the diabetic/saline group when compared to the control/saline group ($P < 0.05$). Treatment with resveratrol prevented this decrease in SOD activity in both tissues, in the diabetic/RV10 and diabetic/RV20 groups reaching similar values to the control/saline group ($P < 0.05$). The administration of resveratrol per se caused no significant effect on SOD activity in the control/RV10 or control/RV20 groups when compared to the control/saline group.

3.4. Vitamin C and NPSH levels

Table 2 shows the hepatic and renal vitamin C and NPSH content. As can be seen, in both liver and kidney vitamin C and NPSH content was significantly reduced in the diabetic/saline group when compared to the control/saline group ($P < 0.05$). However, the treatment with resveratrol was able to prevent the reduction in the hepatic and renal content of these non enzymatic antioxidants in the diabetic/RV10 and diabetic/RV20 group when compared to diabetic/saline group ($P < 0.05$). When resveratrol was given per se, no significant difference in liver and kidney was observed in the in the control/RV10 or control/RV20 groups when compared to control/saline group.

Table 2
Non-protein thiols (NPSH) and ascorbic acid levels in liver and Kidney of STZ-induced diabetic rats and treated with resveratrol (RV). Data are expressed as means ± S.D. Different letters in the same line indicate differences among the groups. ($P < 0.05$; $n = 8$ animals per group). ANOVA-Duncan's test.

Groups	NPSH (μmol SH/g tissue)		AsA (μg ascorbic acid/g tissue)	
	Liver	Kidney	Liver	Kidney
Control/saline	3.67 ± 1.28^{a}	3.35 ± 0.33^{a}	227.61 ± 14.81^{a}	161.12 ± 11.21^{a}
Control/RV10	3.80 ± 0.86^{a}	2.87 ± 0.24^{a}	235.31 ± 11.38^{a}	163.42 ± 6.86^{a}
Control/RV20	3.61 ± 0.90^{a}	2.86 ± 0.27^{a}	245.43 ± 15.02^{a}	156.72 ± 9.51^{a}
Diabetic/saline	2.26 ± 0.69^{b}	1.97 ± 1.26^{b}	181.36 ± 12.08^{b}	129.77 ± 7.26^{b}
Diabetic/RV10	3.82 ± 0.56^{a}	3.20 ± 1.01^{a}	199.11 ± 7.52^{c}	159.09 ± 6.17^{a}
Diabetic/RV20	3.46 ± 0.35^{a}	2.87 ± 0.94^{a}	233.46 ± 10.01^{a}	164.05 ± 6.67^{a}

3.5. δ-ALA-D activity

Liver and kidney δ-ALA-D (Fig. 4A and B, respectively) activity presented a significant decrease in the diabetic/saline group when compared to the control/saline group ($P < 0.05$). The treatment

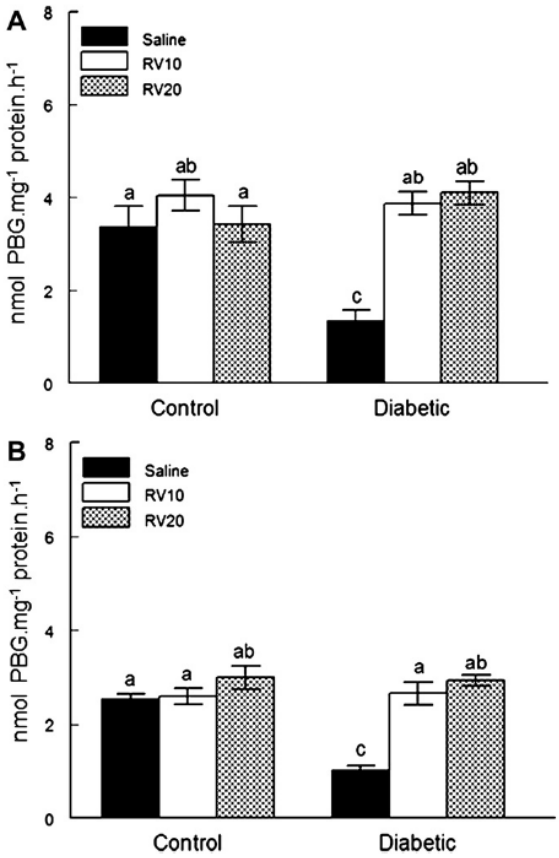

Fig. 4. δ-ALA-D activity in liver (A) and kidney (B) from STZ-induced diabetic rats and those treated with resveratrol. Bars represent means ± S.D. Groups with different letters are statistically different ($P < 0.05$; $n = 8$). ANOVA-Duncan's Test.

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Table 3
Hepatic and renal markers of STZ-induced diabetic rats and treated with resveratrol (RV). Data are expressed as means ± S.D. Different letters in the same column indicate differences among the groups. ($P < 0.05$; $n = 8$ animals per group). ANOVA-Duncan's test.

Groups	ALT[a]	AST[a]	γ-GT[a]	Urea[b]	Creatinine[b]
Control/saline	179.1 ± 39.3[a]	64.0 ± 13.5[a]	7.7 ± 0.6[a]	52.1 ± 3.8[a]	0.41 ± 0.05[a]
Control/RV10	192.1 ± 45.2[a]	55.1 ± 6.5[a]	9.1 ± 1.0[a]	44.1 ± 1.9[a]	0.38 ± 0.08[a]
Control/RV20	176.2 ± 13.3[a]	57.8 ± 8.9[a]	9.5 ± 1.3[a]	54.2 ± 4.5[a]	0.44 ± 0.05[a]
Diabetic/saline	323.4 ± 77.3[b]	94.4 ± 19.6[b]	12.3 ± 1.7[b]	86.4 ± 9.5[b]	0.60 ± 0.09[b]
Diabetic/RV10	192.1 ± 24.8[a]	68.2 ± 9.4[ab]	8.6 ± 3.8[a]	62.2 ± 7.2[a]	0.48 ± 0.06[a]
Diabetic/RV20	197.8 ± 64.5[a]	61.3 ± 11.2[a]	8.4 ± 0.92[a]	61.5 ± 6.9[a]	0.35 ± 0.08[a]

[a] Data of enzyme activities ALT, AST and γ-GT are presented as U/l.
[b] Data of renal markers (urea and creatinine) are represented as mg/dl.

with resveratrol prevented this decrease in δ-ALA-D activity in both types of tissues in the diabetic/RV10 and diabetic/RV20 groups when compared to the diabetic/saline group ($P < 0.05$). Administration of resveratrol per se did not modify significantly hepatic and renal δ-ALA-D activity in the control/RV10 or control/RV20 groups when compared to the control/saline group.

3.6. Biochemical analysis

The activities of serum enzymes AST, ALT and γ-GT were significantly higher in the diabetic/saline group when compared to the control/saline group ($P < 0.05$) (Table 3). The treatment with resveratrol prevented significantly the rise of AST, ALT and γ-GT activities in the diabetic/RV10 and diabetic/RV20 groups ($P < 0.05$). When resveratrol was given per se, no significant differences were observed in the control/RV10 or control/RV20 groups when compared to the control/saline group.

Levels of renal function markers, urea and creatinine also presented a significant increase in diabetic/saline group when compared to control/saline group ($P < 0.05$) (Table 3). However, the administration of resveratrol prevented this increase of urea and creatinine levels in the diabetic/RV10 and diabetic/RV20 groups ($P < 0.05$). The treatment with resveratrol per se caused no effect in urea or creatinine levels in the control/RV10 or control/RV20 groups when compared to the control/saline group.

Cholesterol and triglycerides levels were significantly increased in the diabetic/saline group when compared to the control/saline group ($P < 0.05$) (Table 4). Treatment with resveratrol prevented the increase of cholesterol and triglyceride levels in the diabetic/RV10 and diabetic/RV20 groups ($P < 0.05$). The administration of resveratrol per se did not alter significantly the cholesterol or triglyceride levels in the control/RV10 and control/RV20 groups when compared to the control/saline group.

4. Discussion

Extensive evidence has shown that chronic hyperglycemia leads to a series of biochemical events resulting in the production of high levels of ROS and eventual oxidative stress [2–5]. STZ-induced diabetes is a well-characterized experimental model for type 1 diabetes due to its ability to selectively destroy pancreatic islet β-cells leading insulin deficiency and hyperglycemia [40]. Diabetogenic effect of STZ is mainly attributed to the excess production of ROS leading to toxicity in pancreatic cells which reduces the synthesis and the release of insulin, while affecting organs such as liver, kidney, and hematopoietic system [40]. In view of this, several experimental and clinical studies have indicated the potential usefulness of polyphenolic compounds as antioxidants therapeutic agents for the prevention and/or reduction of oxidative damage in diabetes [41–43]. Thus, the effects of resveratrol on oxidative stress in liver and kidney, as well as on blood biochemical parameters of STZ-induced diabetic rats were investigated in this study.

Table 4
Cholesterol and triglycerides levels of STZ-induced diabetic rats and treated with resveratrol (RV). Data are expressed as means ± S.D. Different letters in the same column indicate differences among the groups. ($P < 0.05$; $n = 8$ animals per group). ANOVA-Duncan's test.

Groups	Cholesterol (mmol/L)	Triglycerides (mmol/L)
Control/saline	1.56 ± 0.12[a]	0.77 ± 0.05[a]
Control/RV10	1.27 ± 0.05[a]	0.68 ± 0.06[a]
Control/RV20	1.64 ± 0.17[a]	0.65 ± 0.05[a]
Diabetic/saline	2.17 ± 0.06[b]	1.00 ± 0.04[b]
Diabetic/RV10	1.57 ± 0.20[a]	0.77 ± 0.07[a]
Diabetic/RV20	1.75 ± 0.21[ab]	0.74 ± 0.10[a]

Lipid peroxidation is considered a hallmark of oxidative stress, in which ROS interact with polyunsaturated fatty acids, and leads to the formation of lipid products such as MDA and 4-HNE (4-hydroxynonenal), which then causing damages to the membrane components of the cell, cell necrosis and inflammation [44]. Extensive evidence has demonstrated that the increase of lipid peroxidation plays an important role in the progression of diabetes by altering the transbilayer fluidity gradient, which could hamper the activities of membrane-bound enzymes and receptors [3,45]. In our study, there were significant increases in lipid peroxidation in liver and kidney of diabetic rats, as measured by TBARS formation (Fig. 1A and B). These results are in agreement with several studies that have reported an increase in TBARS levels in kidney, liver, serum and erythrocytes of animal with experimental diabetes [11,43,46,47]. Taken together these findings suggest that the exposure to high glucose levels may elevate the generation of ROS through the non-enzymatic glycation of protein and glucose autoxidation. As a consequence, it provokes damage in structural and functional integrity of hepatic and renal tissues, as evidenced by an increase in the oxidative deterioration of the lipids of cellular membrane in diabetic state.

In addition, the increased lipid peroxidation under diabetic conditions could be due to increased oxidative stress in the cell as a result of the depletion of antioxidant defense systems [48]. Antioxidant enzymes form the first line of defense against ROS in the organism include the enzymes SOD, CAT and GSH-Px, which play an important role in scavenging the toxic intermediate of incomplete oxidation. CAT and SOD are the two major enzymes that remove ROS *in vivo*. SOD catalyzes the dismutation of superoxide anion ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2), which is then degraded to H_2O by CAT or by glutathione peroxidase. A decrease in the activity of these antioxidants may lead to an excess of availability of $O_2^{\bullet -}$ and H_2O_2, which in turn generates hydroxyl radicals, resulting in initiation and propagation of lipid peroxidation [2,9,48,49]. In line with this, in our study, it was observed a decrease in the activities of antioxidants enzymes SOD and CAT in liver and kidney of diabetic rats associated with a concomitant increase in lipid peroxidation in these tissues (Figs. 2A, B and 3A, B). Corroborating with these results, other studies have demonstrated

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3.2. ARTIGO II

Effects of resveratrol on nucleotide degrading enzymes in streptozotocin-induced diabetic rats

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Life science

that the activity of the antioxidant enzymes, such as SOD and CAT are reduced in tissues of diabetic rats. This may result in a number of deleterious effects due to the accumulation of ROS [4]. One possible mechanism for this reduction in SOD and CAT activities may be due to the inactivation caused by the excess of free radicals and/or by non-enzymatic glycation due to the persistent hyperglycemia, which has been extensively reported to occur in diabetes [2–5,50].

On the other hand, our study demonstrated that the treatment with resveratrol (10 and 20 mg/kg) prevented the increase in TBARS levels and the reduction in SOD and CAT activity in liver and kidney of STZ-induced diabetic rats, indicating a possible role of this polyphenol in free radical inactivation and in the antioxidant defense (Figs. 1A, B–3A, B). These results are consistent with reductions in oxidative stress found in other studies, where the resveratrol treatment greatly ameliorated antioxidants enzyme activities and prevented the rise in lipid peroxides in tissue and blood cells of diabetic animals [24,51–54]. In addition, Cao and Li [55] and Leonard et al. [56] reported that resveratrol is able to up-regulate mRNA expression for antioxidant enzymes and decrease the activation of nuclear factor NF-kB (ROS-sensitive transcription factor). Based on these findings, we can suggest that resveratrol is able to modulate SOD and CAT activities, which may be very important since it increases the scavenging capacity of ROS of liver and kidney providing higher protection against oxidative damage induced by diabetes in these tissues. In fact, the reactivation in SOD activity promoted by resveratrol may accelerate the dismutation of $O_2^{\bullet -}$ to H_2O_2, which is quickly removed by CAT protecting the hepatic and renal tissues of diabetic rats against highly reactive and toxic hydroxyl radicals and consequently preventing the lipid peroxidation [20,56].

Numerous investigations have reported that resveratrol inhibits effectively the lipid peroxidation of cellular membranes, the protein oxidation as well as the DNA damage due its ability to directly scavenge various free radicals, including superoxide radicals and peroxyl and hydroxyl radicals [20,56,57]. Moreover, it showed to reduce α-tocopheroxyl radical to regenerate the endogenous tocopherol, which further strengthens the antioxidant defense mechanism [58]. The capacity of resveratrol to scavenge ROS may be attributed to a hydrogen-electron donation from its hydroxyl groups [59]. It has been reported that due to hydroxylated structure of resveratrol, it can form a radical derivative stabilized by the delocalization of two electrons between the two aromatic cycles and the methylene bridge joining these two cycles [60]. It is important to observe that due to its highly lipophilic character resveratrol is able to bind the lipoprotein particles suggesting that this event improved its antioxidant activity [61].

Simultaneously with the decrease in the activity of the antioxidants enzymes, ruined antioxidant competence of non-enzymatic antioxidants such as non-protein thiols and vitamin C have been reported in diabetes [62–64]. In our study, a marked decrease in NPSH and vitamin C content was observed in liver and kidney of diabetic rats, as reported by other studies [52,63,64] (Table 2). The decrease in hepatic and renal NPSH levels in diabetic rats may represent a depletion of this antioxidant due to its increased utilization in removing H_2O_2 and other peroxides produced in excess due to oxidative stress [65]. On the other hand, the reduction in vitamin C content in the diabetes may be attributed to elevated utilization of this antioxidant in trapping the oxyradicals and/or to a decrease in the GSH level, since GSH is required for the recycling of ascorbic acid [66]. This deficiency in NPSH levels, mainly GSH, and in the vitamin C content found in our study can increase the susceptibility to oxidative damage in hepatic and renal cells and may contribute to the pathogenesis of complications associated with the chronic diabetic state [67].

In our study, the treatment with resveratrol restored NPSH and vitamin C content in liver and kidney of diabetic rats close proximity to normal levels, which in turn reveals the antioxidant potential of resveratrol (Table 2). Similarly, Palsamy et al. [43,51] also observed that resveratrol was able to prevent the decrease in GSH levels in liver and pancreas of diabetic rats. In fact, polyphenols are reported to enhance the activity of γ-glutamylcysteine synthetase and demonstrated simultaneous escalation in the intracellular GSH level [41]. In addition, data of literature demonstrated that elevated levels of GSH protects cellular proteins against oxidation through glutathione redox cycle and also directly detoxifies ROS [67]. In this line, we can suggest that a prevention of a decrease in NPSH content in liver and kidney of diabetic rats found in our study could be in part responsible for the decrease in ROS formation and in the lipid peroxidation levels and the resultant of low oxidative stress obtained *in vivo* in the animals treated with resveratrol.

Persuasive evidence has indicated that δ-ALA-D enzyme is highly sensitive to the presence of a variety of pro-oxidant elements, which oxidize -SH groups of this enzyme impairing its activity [15,68]. The inhibition of δ-ALA-D activity may prejudice heme biosynthesis and can result in the accumulation of aminolevulinic acid which, under physiologically conditions, can have pro-oxidant effects contributing to the oxidative stress [69]. In line with this, in the present study, the activity of δ-ALA-D was significantly decreased in hepatic and renal tissues of diabetic rats (Fig. 4A and B). Our results are in accordance with data found in human and experimental diabetes, where a significant inhibition of δ-ALA-D is described and has been related mainly to high glucose levels and overproduction of ROS [16,64,70]. Thus, we can suggest that the inhibition of δ-ALA-D activity in liver and kidney of diabetic rats may be a consequence of either the glycation in the lysine residue from the active site of δ-ALA-D, which is involved in the formation of the Schiff basis with the first molecule of ALA [71], or by oxidation of essential reduce cysteinyl residues of the enzyme by ROS [64,68,72]. Furthermore, the decrease in δ-ALA-D activity found in our study may be linked to the significant reduction in the antioxidant defenses in hepatic and renal tissues of diabetic rats, especially in NPSH content, which is responsible for preventing the oxidation of the sulphydryl groups necessary for the activity of this enzyme [15].

The treatment with resveratrol at doses of 10 and 20 mg/kg was able to prevent the inhibition in δ-ALA-D activity in liver and kidney of diabetic rats suggesting that this compound can prevent the oxidation of essential -SH groups located at its active site of δ-ALA-D and consequently its inhibition (Fig. 4A and B). Indeed, in our study resveratrol prevented the reduction of NPSH levels in hepatic and renal tissues in STZ- induced diabetic rats; hence, it could be expected to protect other endogenous thiols such as those found in δ-ALA-D enzyme. Consequently, we can suggest that the prevention of a decrease in NPSH content as well as a decrease of oxidative stress in diabetic rats by resveratrol could be associated with a prevention of a decrease of δ-ALA-D activity. Furthermore, this study clarifies that the inhibition of δ -ALA-D enzyme is not closely related to the development of hyperglycemia in rats, pointing out the importance of antioxidants as resveratrol to minimize deleterious effects of diabetes in activity of this biologically important enzyme.

Another important aspect to be discussed in this study is serum aminotransferases activities, which have long been considered as sensitive indicators of hepatic damage [73]. Injury to the hepatocytes alters their transport function and membrane permeability, leading to leakage of enzymes from the cells [73,74]. Therefore, the marked release of AST and ALT from liver cytosol into circulation and an increase of γ-GT, a membrane enzyme, indicate severe damage to

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hepatic tissue membranes during the diabetes [74]. Thus, increased activities of AST, ALT, and γ-GT found in this study may be interpreted as a result of the liver cell destruction or changes in the membrane permeability indicating severe hepatocellular damage is induced by diabetes, which is in accordance with other reports [64,74,75] (Table 3). The administration of resveratrol in doses of 10 and 20 mg/Kg was able to protect against the increase in the activity of these enzymes in diabetic rats, demonstrating the protective effect of this polyphenol against hepatic damage induced by diabetic state (Table 3). These results are in agreement with those found in studies using resveratrol [51,76] and other antioxidants as rutin [76], caffeic acid and quercetin [77] and can be attributed to the capability of resveratrol to conserve the membrane integrity of cellular organelles in the diabetic state. Indeed, in our study resveratrol was able to prevent the lipid peroxidation in hepatic tissue.

In addition to the hepatic damage, in the current study, diabetic rats also presented renal damages that were evidenced by the elevation in serum urea and creatinine levels, which are considered as significant markers of renal dysfunction [78] (Table 3). Of great importance, resveratrol prevented the increase in the levels of urea and creatinine in diabetic rats. These findings suggest that resveratrol possesses the potential to attenuate renal injury caused by hyperglycemic state and this can be associated directly with the antioxidant capacity of this polyphenol, which protects the kidneys against oxidative damage, as evidenced in this and other studies [79,80].

Another important aspect to be discussed is that, in contrast with our results, Hassan-Khabbar et al. [80] related that the dose of 20 mg/kg of trans-resveratrol became pro-oxidant with an aggravation of liver injury associated with a depletion of total and reduced glutathione levels and a decrease of antioxidant enzyme activities. In addition, Gadacha et al. [81] related a possible pro-oxidant effect of resveratrol at 0.8, 2, and 5 mg/kg, depending on day/night rhythm. In fact, studies has related that depending of the concentration of the phytoalexin and on the cell type, the resveratrol can exhibit pro-oxidant properties, leading to oxidative breakage of cellular DNA in the presence of transition metal ions such as copper. Recently, it has been proposed that such a pro-oxidant action could be a common mechanism for anticancer and chemopreventive properties of plant polyphenols [82]. Furthermore, it has been suggested that resveratrol can work as a pro-oxidant under low oxidative conditions, while it becomes antioxidant under strong oxidative conditions [81]. Therefore, studies have shown that resveratrol possess biphasic function [83]. However, its pro-oxidant or antioxidant action appears to be depending on the dose, environmental factors, interactions with other compounds, medications, cell types and different pathological conditions [81,84]. For example, in our study the dose of 20 mg/kg had an important antioxidant effect in diabetic rats, while in other studies, in different pathologic conditions, the same dose showed pro-antioxidant effects [80]. Thus, more research is needed to understand the action of resveratrol on all cell types and conditions, and the optimum therapeutic concentration that applies to each condition in order to gain a complete understanding of the biphasic response of resveratrol.

Literature data have showed that the diabetes is usually associated with abnormal high levels of serum lipids, which may contribute to the development and progression of micro- and macrovascular complications [85]. In our study, there is a significant rise in serum cholesterol and triglycerides levels in diabetic rats (Table 4). However, when diabetic rats received resveratrol the levels of serum triglycerides and cholesterol presented a significant decrease. These results are supported by other works that have reported that administration of resveratrol improved lipid profile including reduction of serum cholesterol levels [86]. In addition, resveratrol appears to be beneficially modulating the lipid and lipoprotein levels, inhibiting hepatic triglycerides synthesis and reducing cholesterol and triglycerides accumulation in liver [87]. Considering the cholesterol lowering property of resveratrol, it has been suggested that it may act as inhibitor for some enzymes such as hydroxy methyl glutaryl CoA reductase, which participates in the cholesterol synthesis [86,87]. Based on these results, we can suggest that resveratrol improves the dyslipidemia while inhibiting the progression of hepatic and renal dysfunction in STZ-induced diabetic rats.

An important datum of this study is that despite the absence of the effect of resveratrol in the reduction of hyperglycemia of the diabetic rats treated with this polyphenol, there was a reduction of death of these animals when compared with the animals of diabetic/saline group. Indeed, only about 50% of the animals of diabetic/saline group survived until the end of experiment, while in the groups of diabetic rats treated with resveratrol there was a survival of about of 80% of the animals (data not shown). Literature data have demonstrated that resveratrol extends the lifespan of mice through the overexpression of sirtuin 1 in a yeast diet and is linked to longer life in humans [88,89]. In addition, Baur et al. [90] shows that resveratrol shifts the physiology of middle-aged mice on a high-calorie diet towards that of mice on a standard diet and significantly increases their survival. Although our study indicates an increase in survival of diabetic rats treated with resveratrol, our protocol lasted 30 days, therefore, no full response can be obtained and a longer-lasting protocol could determine the effects of resveratrol on lifespan of STZ-induced diabetic rats. Nevertheless, the treatment with resveratrol produced changes associated with longer lifespan, including an increase in antioxidant defense system and consequently a decrease of the oxidative stress in liver and kidney of diabetic rats.

In conclusion, the findings of the present study demonstrated that resveratrol treatment may provide effective protection against oxidative damage in liver and kidney STZ- induced type 1 diabetic rats, since this compound was able to ameliorate enzymatic and non-enzymatic antioxidant defense system and to prevent the lipid peroxidation in these tissues. Moreover, it is important to point out that this is the first study to investigate the effects of resveratrol on δ-ALA-D activity in diabetic rats demonstrating the important role of this enzyme as a marker of oxidative stress. Taken together, these results may contribute to a better understanding of the hepatoprotective and renoprotective role of resveratrol, emphasizing the influence of this polyphenol and other antioxidants in the diet for human health, possibly preventing hepatic and renal complications associated with diabetes mellitus.

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References

[1] American Diabetes Association, Diagnosis and classification of diabetes mellitus, Diabetes Care 33 (2010) 62–69.
[2] A.C. Maritim, R.A. Sandres, J.B. Watkins, Diabetes, oxidative stress, and antioxidants: a review, J. Biochem. Mol. Toxicol. 17 (2003) 24–39.
[3] F. Giacco, M. Brownlee, Oxidative stress and diabetic complications, Circ. Res. 107 (9) (2010) 1058–1070.
[4] M.J. Stevens, Redox-based mechanisms in diabetes, Antioxid. Redox Signal. 7 (2005) 1483–1485.
[5] J.L. Rains, S.K. Jain, Oxidative stress, insulin signaling, and diabetes, Free Radic. Biol. Med. 50 (2011) 567–575.

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[6] M. Brownlee, A radical explanation for glucose-induced beta cell dysfunction, J. Clin. Invest. 112 (2003) 1788–1790.
[7] P.S. Gill, C.S. Wilcox, NADPH oxidases in the kidney, Antioxid. Redox Signal. 8 (2006) 1597–1607.
[8] J. Shao, L. Qiao, R.C. Janssen, M. Pagliassotti, J.E. Friedman, Chronic hyperglycemia enhances PEPCK gene expression and hepatocellular glucose production via elevated liver activating protein/liver inhibitory protein ratio, Diabetes 54 (2005) 976–984.
[9] B. Halliwell, J.M.C. Gutterdige, Free Radicals in Biology and Medicine. Oxford University Press, Oxford, 1999.
[10] A.F. Fidan, Y. Dündar, The effects of Yucca schidigera and Quillaja saponaria on DNA damage, protein oxidation, lipid peroxidation, and some biochemical parameters in streptozotocin-induced diabetic rats, J. Diabet. Complications 22 (2008) 348–356.
[11] A. Likidlilid, N. Patchanans, T. Peerapatdit, C. Sriratanasathavorn, Lipid peroxidation and antioxidant enzyme activities in erythrocytes of type 2 diabetic patients, J. Med. Assoc. Thai. 93 (2010) 682–693.
[12] N.F. Wiernsperger, Oxidative stress as a therapeutic target in diabetes: revisiting the controversy, Diabet. Med. 29 (2003) 579–585.
[13] S. Sassa, ALAD porphyria, Semin. Liver Dis. 18 (1998) 95–101.
[14] D. Shemim, 5-Aminolevulinic acid dehydratase: structure, function, and mechanism, Phil. Trans. R. Soc. Lond. B 273 (1976) 109–115.
[15] C.W. Nogueira, F.A. Soares, P.C. Nascimento, D. Muller, J.B.T. Rocha, 2,3-Dimercaptopropane-1-sulfonic acid and meso-2,3-dimercaptosuccinic acid increase mercury and cadmium-induced inhibition of delta-aminolevulinate dehydratase, Toxicology 184 (2003) 85–95.
[16] B. Fernández-Cuartero, J.L. Rebollar, A. Batlle, E.R. de Salamanca, Delta aminolevulinate dehydratase (ALA-D) activity in human and experimental diabetes mellitus, Int. J. Biochem. Cell Biol. 31 (1999) 479–488.
[17] K. Bhooshan, P. Syed, I. Rizvi, Plant polyphenols as dietary antioxidants in human health and disease, Oxid. Med. Cell. Longev. 2 (2009) 270–278.
[18] Z. Francine, M. Marques, A. Markus, B.J. Morris, Resveratrol: cellular actions of a potent natural chemical that confers a diversity of health benefits, Int. J. Biochem. Cell Biol. 41 (2009) 2125–2128.
[19] K.B. Pandey, S.I. Rizvi, Anti-oxidative action of resveratrol: implications for human health, Arabian J. Chem. 4 (2011) 293–298.
[20] S.M. Lee, H. Yang, D.M. Tartar, B. Gao, X. Luo, S.Q. Ye, H. Zaghouani, D. Fang, Prevention and treatment of diabetes with resveratrol in a non-obese mouse model of type 1 diabetes, Diabetologia 54 (2011) 1136–1146.
[21] J.A. Baur, D.A. Sinclair, Therapeutic potential of resveratrol: the in vivo evidence, Nat. Rev. Drug Discov. 5 (6) (2006) 493–506.
[22] P. Saiko, A. Szakmary, W. Jaeger, T. Szekeres, Resveratrol and its analogs: defense against cancer, coronary disease and neurodegenerative maladies or just a fad? Mutat. Res. Rev. 658 (2008) 68–94.
[23] K. Szkudelska, T. Szkudelski, Resveratrol, obesity and diabetes, Eur. J. Pharmacol. 635 (2010) 1–8.
[24] K.B. Pandey, S.I. Rizvi, Protective effect of resveratrol on formation of membrane protein carbonyls and lipid peroxidation in erythrocytes subjected to oxidative stress, Appl. Physiol. Nutr. Metab. 34 (2009) 1093–1097.
[25] R. Schmatz, C.M. Mazzanti, R. Spanevello, N. Stefanello, J. Gutierres, M. Corrêa, M.M. da Rosa, M.A. Rubin, M.R.C. Schetinger, V.M. Morsch, Resveratrol prevents memory deficits and the increase in acetylcholinesterase activity in streptozotocin-induced diabetic rats, Eur. J. Pharmacol. 610 (2009) 42–48.
[26] R. Schmatz, M.R.C. Schetinger, R.M. Spanevello, C.M. Mazzanti, N. Stefanello, P.A. Maldonado, J. Gutierres, V.M. Morsch, Effects of resveratrol on nucleotide degrading enzymes in streptozotocin-induced diabetic rats, Life Sci. 84 (2009) 345–350.
[27] R. Schmatz, C.M. Mazzanti, R. Spanevello, N. Stefanello, J. Gutierres, P.A. Maldonado, M. Correa, V.M. Morsch, Ectonucleotidase and acetylcholinesterase activities in synaptosomes from the cerebral cortex of streptozotocin-induced diabetic rats and treated with resveratrol, Brain Res. Bull. 80 (2009) 371–376.
[28] M.E. Juan, M.P. Vinardell, J.M. Planas, The daily oral administration of high doses of trans-resveratrol to rats for 28 days is not harmful, J. Nutr. 132 (2002) 257–260.
[29] V. Tiwari, K.I Chopra, Resveratrol prevents alcohol-induced cognitive deficits and brain damage by blocking inflammatory signaling and cell death cascade in neonatal rat brain, J. Neurochem. 117 (2011) 678–690.
[30] E.S. Lee, M.O. Shin, S. Yoon, Resveratrol inhibits dimethylnitrosamine-induced hepatic fibrosis in rats, Arch. Pharm. Res. 33 (2010) 925–932.
[31] E.H. Siemann, L.L. Creasy, Concentration of the phytoalexin resveratrol in wine, Am. J. Enol. Viticult. 43 (1992) 49–52.
[32] D.M. Goldberg, E. Ng, J. Yan, A. Karumanchiri, G.J. Soleas, E.P. Diamandis, Regional differences in resveratrol isomer concentrations of wines from various cultivars, J. Wine Res. 7 (1996) 13–24.
[33] H. Ohkawa, N. Ohishi, K. Yagi, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal. Biochem. 95 (1979) 351–358.
[34] D.P. Nelson, L.A. Kiesow, Enthalpy of decomposition of hydrogen peroxide by catalase at 25 °C (with molar extinction coefficients of H_2O_2 solutions in the UV), Anal. Biochem. 49 (1972) 474–478.
[35] H.P. Misra, I. Fridovich, The role of superoxide anion in the autoxidation of epinephrine and simple assay for superoxide dismutase, J. Biol. Chem. 247 (1972) 3170–3175.
[36] M.C. Jacques-Silva, C.W. Nogueira, L.C. Broch, E.M.M. Flores, J.B.T. Rocha, Diphenyl diselenide and ascorbic changes deposition of selenium and ascorbic acid in liver and brain of mice, Pharmacolol. Toxicol. 88 (2001) 119–125.
[37] G.L. Ellman, Tissue sulphydryl groups, Arch. Biochem. Biophys. 82 (1959) 70–77.
[38] S. Sassa, δ-Aminolevulinic acid dehydratase assay, Enzyme 28 (1982) 133–145.
[39] M.M. Bradford, A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[40] K.K. Wu, Y. Huan, Streptozotocin-Induced diabetic models in mice and rats, Curr. Protoc. Pharmacol. 40 (2008) 1–5.
[41] R. Rodrigo, A. Miranda, L. Vergara, Modulation of endogenous antioxidant system by wine polyphenols in human disease, Clin. Chim. Acta 412 (2011) 410–424.
[42] J.S. Lee, Effects of soy protein and genistein on blood glucose, antioxidant enzyme activities, and lipid profile in streptozotocin-induced diabetic rats, Life Sci. 79 (2006) 1578–1584.
[43] P. Palsamy, S. Subramanian, Ameliorative potential of resveratrol on proinflammatory cytokines, hyperglycemia mediated oxidative stress, and pancreatic beta-cell dysfunction in streptozotocin-nicotinamide-induced diabetic rats, J. Cell Physiol. 224 (2010) 423–432.
[44] G. Stark, Functional consequences of membrane damage, J. Membr. Biol. 205 (2005) 1–16.
[45] L.F. Dmitriev, V.N. Titov, Lipid peroxidation in relation to ageing and the role of endogenous aldehydes in diabetes and other age-related diseases, Ageing Res. Rev. 9 (2010) 200–210.
[46] H.R. Yilmaz, E. Uz, N. Yucel, I. Altuntas, N. Ozcelik, Protective effect of caffeic acid phenethyl ester (CAPE) on lipid peroxidation and antioxidant enzymes in diabetic rat liver, J. Biochem. Mol. Toxicol. 18 (2004) 234–238.
[47] L. Jin, H.Y. Xue, L.J. Jin, S.Y. Li, Y.P. Xu, Antioxidant and pancreas-protective effect of aucubin on rats with streptozotocin-induced diabetes, Eur. J. Pharmacol. 582 (2008) 162–167.
[48] S.A. Santini, G. Marra, B. Giardina, Defective antioxidant defenses and enhanced susceptibility to lipid peroxidation in uncomplicated IDDM, Diabetes 46 (1997) 1853–1858.
[49] M. Kinalski, A. Sledziewski, B. Telejko, W. Zarzycki, I. Kinalska, Lipid peroxidation and scavenging enzyme activity in streptozotocin-induced diabetes, Acta Diabetol. 37 (2000) 179–183.
[50] A.K. Saxena, P. Srivastava, R.K. Kale, N.Z. Baquer, Impaired antioxidant status in diabetic rat liver. Effect of vanadate, Biochem. Pharmacol. 45 (3) (1993) 539–554.
[51] P. Palsamy, S. Sivakumar, S. Subramanian, Resveratrol attenuates hyperglycemia mediated oxidative stress, proinflammatory cytokines and protects hepatocytes ultrastructure in streptozotocin-nicotinamide-induced experimental diabetic rats, Chem. Biol. Interact. 186 (2010) 200–210.
[52] P. Palsamy, S. Subramanian, Resveratrol protects diabetic kidney by attenuating hyperglycemia-mediated oxidative stress and renal inflammatory cytokines via Nrf2-Keap1 signaling, Biochim. Biophys. Acta 1812 (2011) 719–731.
[53] A. Kumar, R.K. Kaundal, S. Iyer, S.S. Sharma, Effects of resveratrol on nerve functions, oxidative stress and DNA fragmentation in experimental diabetic neuropathy, Life Sci. 80 (2007) 1236–1244.
[54] O. Ates, S.R. Cayli, N. Yucel, E. Altinoz, A. Kocak, M.A. Durak, Y. Turkoz, S. Yologlu, Central nervous system protection by resveratrol in streptozotocin-induced diabetic rats, J. Clin. Neurosci. 14 (2007) 256–260.
[55] Z. Cao, Y. Li, Potent induction of cellular antioxidants and phase 2 enzymes by resveratrol in cardiomyocytes: protection against oxidative and electrophilic injury, Eur. J. Pharmacol. 489 (2004) 39–48.
[56] S.S. Leonard, C. Xia, B.H. Jiang, B. Stinefelt, H. Klandorf, G.K. Harris, X. Shi, Resveratrol scavenges reactive oxygen species and effects radical-induced cellular responses, Biochem. Biophys. Res. Commun. 309 (2003) 1017–1026.
[57] S. Bradamante, L. Barenghi, A. Villa, Cardiovascular protective effects of resveratrol, Cardiovasc. Drug Rev. 22 (2004) 169–188.
[58] Y.J. Cai, J.G. Fang, L.P. Ma, L. Yang, Z.L. Liu, Inhibition of free radical-induced peroxidation of rat liver microsomes by resveratrol and its analogues, Biochim. Biophys. Acta 1637 (2003) 31–38.
[59] M. Lüpez-Velez, F. Martínez-Martínez, C. Del Valle-Ribes, The study of phenolic compounds as natural antioxidants in wine, Crit. Rev. Food Sci. Nutr. 43 (2003) 233–244.
[60] D. Delmas, B. Jannin, N. Latruffe, Resveratrol: preventing properties against vascular alterations and ageing, Mol. Nutr. Food Res. 49 (2005) 377–395.
[61] L. Belguendouz, L. Fremont, M.T. Gozzelino, Interaction of trans resveratrol with plasma lipoproteins, Biochem. Pharmacol. 55 (1998) 811–816.
[62] W. Stahl, H. Sies, Antioxidant defense: vitamins E and C and carotenoids, Diabetes 46 (1997) 14–18.
[63] P. Arulselvan, S.P. Subramanian, Beneficial effects of Murraya koenigii leaves on antioxidant defense system and ultra structural changes of pancreatic β-cells in experimental diabetes in rats, Chem. Biol. Interact. 165 (2007) 155–164.
[64] I.J. Kade, V.C. Borges, L. Savegnago, E.O. Ibukun, G. Zeni, C.W. Nogueira, J.B. Rocha, Effect of oral administration of diphenyl diselenide on antioxidant status, and activity of delta aminolevulinic acid dehydratase and isoforms of lactate dehydrogenase, in streptozotocin-induced diabetic rats, Cell Biol. Toxicol. 25 (2009) 415–424.
[65] A. Sharma, S. Kharb, S.N. Chugh, R. Kakkar, G.P. Singh, Effect of glycemic control and vitamin E supplementation on total glutathione content in non-insulin-dependent diabetes mellitus, Ann. Nutr. Metab. 44 (2000) 11–13.
[66] X. Li, Z.C. Qu, J.M. May, GSH is required to recycle ascorbic acid in cultured liver cell lines, Antioxid. Redox Signal. 3 (2001) 1089–1097.

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[67] D.M. Townsend, K.D. Tew, H. Tapiero, The importance of glutathione in human disease, Biomed. Pharmacother. 57 (2003) 145–155.
[68] V.B. Brito, V. Folmer, J.C. Soares, I.D. Silveira, J.B. Rocha, Long-term sucrose and glucose consumption decreases the delta-aminolevulinate dehydratase activity in mice, Nutrition 23 (2007) 818–826.
[69] E.J. Bechara, Oxidative stress in acute intermittent porphyria and lead poisoning may be triggered by 5-aminolevulinic acid, Braz. J. Med. Biol. Res. 29 (1996) 841–851.
[70] J.B. Souza, J.B. Rocha, C.W. Nogueira, V.C. Borges, R.R. Kaizer, V.M. Morsch, V.L. Dressler, A.F. Martins, E.M. Flores, M.R. Schetinger, Delta-aminolevulinate dehydratase (delta-ALA-D) activity in diabetes and hypothyroidism, Clin. Biochem. 40 (2007) 321–325.
[71] F.A. Caballero, E.N. Gerez, C.F. Polo, E.S. Vazquez, A.M.C. Batlle, Reducing sugars trigger d aminolevulinate dehydratase inactivation:evidence of in vitro aspirin prevention, Gen. Pharmacol. 31 (1998) 441–445.
[72] V. Folmer, J.C. Soares, D. Gabriel, J.B.T. Rocha, A high fat diet inhibits deltaaminolevulinate dehydratase and increases lipid peroxidation in mice (Musmusculus), J. Nutr. 133 (2003) 2165–2170.
[73] D.W. Molander, F. Wroblewski, J.S. Ladue, Serum glutamic oxalacetic transaminase as an index of hepatocellular integrity, J. Lab. Clin. Med. 46 (1955) 831–839.
[74] H. Elizabeth, M.D. Harris, Elevated liver function tests in type 2 diabetes, Clin. Diabetes 23 (2005) 115–119.
[75] F.M. El-Demerdash, M.I. Yousef, N.I. El-Naga, Biochemical study on the hypoglycemic effects of onion and garlic in alloxan-induced diabetic rats, Food Chem. Toxicol. 43 (2005) 57–63.
[76] A.A. Fernandes, E.L. Novelli, K. Okoshi, M.P. Okoshi, B.P. Di Muzio, J.F. Guimarães, A.J. Fernandes, Influence of rutin treatment on biochemical alterations in experimental diabetes, Biomed. Pharmacother. 64 (2010) 214–219.
[77] K.H. Janbaz, S.A. Saeed, A.H. Gilani, Studies on the protective effects of caffeic acid and quercetin on chemical-induced hepatotoxicity in rodents, Phytomedicine 11 (2004) 424–430.
[78] T. de Jesus Soares, R.A. Volpini, H.D. Francescato, R.S. Costa, C.G. da Silva, T.M. Coimbra, Effects of resveratrol on glycerol-induced renal injury, Life Sci. 81 (2007) 647–656.
[79] S. Sharma, M. Anjaneyulu, S.K. Kulkarni, K. Chopra, Resveratrol, a polyphenolic phytoalexin, attenuates diabetic nephropathy in rats, Pharmacology 76 (2006) 69–75.
[80] S. Hassan-Khabbar, C.H. Cottart, D. Wendum, F. Vibert, J.P. Clot, J.F. Savouret, M. Conti, V. Nivet-Antoine, Postischemic treatment by trans-resveratrol in rat liver ischemia-reperfusion: a possible strategy in liver surgery, Liver Transpl. 14 (2008) 451–459.
[81] W. Gadacha, M. Ben-Attia, D. Bonnefont-Rousselot, E. Aouani, N. Ghanem-Boughanmi, Y. Touitou, Resveratrol opposite effects on rat tissue lipoperoxidation: pro-oxidant during day-time and antioxidant at night, Redox Rep. 14 (2009) 154–158.
[82] C.A. de la Lastra, I. Villegas, Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications, Biochem. Soc. Trans. 35 (2007) 1156–1160.
[83] S. Mukherjee, J.I. Dudley, D.K. Das, Dose-Dependency of resveratrol in providing health benefits, Dose Response 8 (2010) 478–500.
[84] F.Z. Marques, B.J. Morris, Commentary on resveratrol and hormesis: resveratrol-a hormetic marvel in waiting? Hum. Exp. Toxicol. 29 (2010) 1026–1028.
[85] P. Pushparaj, C.H. Tan, B.K.H. Tan, Effects of Averrhoa bilimbi leaf extract on blood glucose and lipids in streptozotocin-diabetic rats, J. Ethnopharmacol. 72 (2000) 69–76.
[86] S.V. Penumathsa, M. Thirunavukkarasu, S. Koneru, B. Juhasz, L. Zhan, R. Pant, Statin and resveratrol in combination induces cardioprotection against myocardial infarction in hypercholesterolemic rat, J. Mol. Cell. Cardiol. 42 (2007) 508–516.
[87] I.J. Cho, J.Y. Ahn, S. Kim, M.S. Choi, T.Y. Ha, Resveratrol attenuates the expression of HMG-CoA reductase mRNA in hamsters, Biochem. Biophys. Res. Commun. 367 (2008) 190–194.
[88] J.N. Feige, M. Lagouge, C. Canto, Specific SIRT1 activation mimics low energy levels and protects against diet-induced metabolic disorders by enhancing fat oxidation, Cell Metab. 8 (2008) 347–358.
[89] M. Stefani, M.A. Markus, R.C. Lin, M. Pinese, I.W. Dawes, B.J. Morris, The effect of resveratrol on a cell model of human aging, Ann. N.Y. Acad. Sci. 1114 (2007) 407–418.
[90] J.A. Baur, K.J. Pearson, N.L. Price, H.A. Jamieson, C. Lerin, A. Kalra, V.V. Prabhu, J.S. Allard, G. Lopez-Lluch, K. Lewis, P.J. Pistell, S. Poosala, K.G. Becker, O. Boss, D. Gwinn, M. Wang, S. Ramaswamy, K.W. Fishbein, R.G. Spencer, E.G. Lakatta, D. Le Couteur, R.J. Shaw, P. Navas, P. Puigserver, D.K. Ingram, R. de Cabo, D.A.Sinclair, resveratrol improves health and survival of mice on a high-calorie diet, Nature 16 (2006) 337–342.

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3.2. ARTIGO II

Effects of resveratrol on nucleotide degrading enzymes in streptozotocin-induced diabetic rats

Roberta Schmatz, Maria Rosa Chitolina Schetinger, Roselia Maria Spanevello, Cinthia Melazzo Mazzanti, Naiara Stefanello, Paula Acosta Maldonado, Jessié Gutierres, Maísa de Carvalho Corrêa, Eduardo Girotto, Maria Beatriz Moretto, Vera Maria Morsch

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Rats

ABSTRACT

Aims: Diabetes mellitus is associated with platelet alterations that may contribute to the development of cardiovascular complications. The present study investigates the effects of resveratrol (RSV), an important compound with cardioprotective activities, on NTPDase, ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP), 5′-nucleotidase and adenosine deaminase (ADA) activities in platelets from streptozotocin (STZ)-induced diabetic rats.
Main methods: The animals were divided into six groups (n=8): control/saline; control/RSV 10 mg/kg; control/RSV 20 mg/kg; diabetic/saline; diabetic/RSV 10 mg/kg; diabetic/RSV 20 mg/kg. RSV was administered during 30 days and after this period the blood was collected for enzymatic assay.
Key findings: The results demonstrated that NTPDase, E-NPP and 5′-nucleotidase activities were significantly higher in the diabetic/saline group (P<0.05) compared to control/saline group. Treatment with RSV significantly increased NTPDase, 5′-nucleotidase and E-NPP activities in the diabetic/RSV10 and diabetic/RSV20 groups (P<0.05) compared to diabetic/saline group. When RSV was administered per se there was also an increase in the activities of these enzymes in the control/RSV10 and control/RSV20 groups (P<0.05) compared to control/saline group. ADA activity was significantly increased in the diabetic/saline group (P<0.05) compared to control/saline group. The treatment with RSV prevented this increase in the diabetic/RSV10 and diabetic/RSV20 groups. No significant differences in ADA activity were observed in the control/RSV10 and control/RSV20 compared to control/saline group.
Significance: The present findings demonstrate alterations in nucleotide hydrolysis in platelets of STZ-induced diabetic rats and treatment with RSV was able to modulate adenine nucleotide hydrolysis, which may be important in the control of the platelet coagulant status in diabetes.

Introduction

Platelets are one of the most important blood components that participate in maintaining vascular integrity promoting the primary and secondary hemostasis that occur after vessel damage (Marcus et al., 2003). Altered platelet morphology and function have been reported in patients with diabetes mellitus and may be associated with an increased risk of developing vascular disease (Stratmann and Tschoepe, 2005). Furthermore, it has been demonstrated that platelets of these patients exhibit a greater tendency toward spontaneous aggregation and are highly hypersensitive to agonists such as ADP (Sobol and Watala, 2000; Haouari and Rosado, 2008).

Extracellular adenine nucleotides, such as adenosine triphosphate (ATP) and adenosine diphosphate (ADP), and their metabolite adenosine can modulate multiple effects on the vascular system by interacting with specific receptors in platelets and endothelial cells (Di Virgilio et al., 2001). The nucleotide ADP is the main promoter of platelet aggregation (Birk et al., 2002a) whereas adenosine is a potent inhibitor of this aggregation and an important modulator of vascular tone (Anfossi et al., 2002; Borowiec et al., 2006). Moreover, studies have demonstrated that ATP has a complex role in the regulation of platelet aggregation (Birk et al., 2002b; Rozalski et al., 2005).

Platelets express a multienzymatic complex on their surface, which is responsible for extracellular nucleotide hydrolysis. This complex

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includes the enzymes NTPDase (ecto-nucleoside triphosphate phosphohydrolase), E-NPP (ecto-nucleotide pyrophosphatase/phosphodiesterase), ecto-5′-nucleotidase and ecto-adenosine deaminase (ADA) (Fürstenau et al., 2006). NTPDases hydrolyzes ATP and ADP to AMP (Robson et al., 2006) while E-NPPs hydrolyzes 5′-phosphodiester bonds in nucleotides and their derivatives producing nucleotide monophosphate (Stefan et al., 2005). AMP resulting from the action of NTPDase and E-NPP is subsequently hydrolyzed to adenosine by ecto-5′-nucleotidase (Colgan et al., 2006; Strater, 2006). The adenosine can be directly inactivated on the cell surface through sequential action of ADA that catalyses the irreversible deamination of adenosine leading to inosine production (Blackburn and Kellems, 2005). Together, these ecto-enzymes constitute a highly organized enzymatic cascade that is able to regulate the extracellular concentrations of adenine nucleotides and nucleosides and play an important role in the maintenance of normal hemostasis and thrombogenesis, mainly by regulating the platelet aggregation status (Yegutkin, 2008).

Resveratrol (3, 5, 4′-trihydroxy-*trans*-stilbene — RSV) is a natural polyphenol found mainly in grapes and red wines (Frémont, 2000). The RSV has a variety of biological effects including anti-inflammatory (Zhu et al., 2008), antioxidant (Fabris et al., 2008) and cardioprotective activities (Bradamante and Villa, 2004; Tosaki et al., 2007). Recently, a number of studies have focused on the cardioprotective effects of RSV, demonstrating that this compound protects against vascular disease via mechanisms that may include vasodilation and prevention of excessive platelet aggregation (Olas et al., 2002; Shen et al., 2007; Fragopoulou et al., 2007).

In this context, due to the benefic properties of RSV in the vascular system and the importance of enzymes that hydrolyze adenine nucleotides in the mechanism of thromboregulation, the aim of present study was to evaluate the activity of NTPDase, E-NPP, 5′-nucleotidase and ADA in platelets from streptozotocin (STZ) -induced diabetic rats and treated with RSV, in order to investigate the potentially therapeutic use of this compound in hemostatic disorders occurring in the diabetic state.

Materials and methods

Chemicals

The substrates ATP, ADP, AMP, p-nitrophenyl thymidine 5′-monophosphate (p-Nph-5′-TMP), adenosine, as well as trizma base, sodium azide, HEPES, streptozotocin, resveratrol (3,5,4′-trihydroxy-*trans*-stilbene, approximately 99% purity) and Coomassie brilliant blue G were obtained from Sigma Chemical Co (St. Louis, MO, USA) and bovine serum albumin, K_2HPO_4, from Reagen. All the other chemicals used in this experiment were of the highest purity.

Animals

Adult male Wistar rats (70–90 days; 220–300 g) from the Central Animal House of the Federal University of Santa Maria were used in this experiment. The animals were maintained at a constant temperature (23±1 °C) on a 12 h light/dark cycle with free access to food and water. All animal procedures were approved by the Animal Ethics Committee from the Federal University of Santa Maria (protocol under number: 21/2007).

Experimental induction of diabetes

Diabetes was induced by a single intraperitoneal injection of 55 mg/kg streptozotocin (STZ) (Kumar et al., 2007), diluted in 0.1 M sodium-citrate buffer (pH 4.5). The age-matched control rats received an equivalent amount of the sodium-citrate buffer. STZ-treated rats received 5% of glucose instead of water for 24 h after diabetes induction in order to reduce death due to hypoglycemic shock. Blood samples were taken from the tail vein 48 h after STZ or vehicle injection. Glucose levels were measured with a portable glucometer (ADVANTAGE, Boehringer Mannheim, MO, USA). Only animals with fasting glycemia over 300 mg/dL were considered diabetic and used for the present study. During the experiment the levels of blood glucose were verified four times (2, 10, 20, and 30 days after the beginning of treatment). The animals that maintained fasting glycemia higher than 300 mg/dL were considered diabetic and selected for enzymatic assays.

Treatment with resveratrol (RSV)

The animals were randomly divided into six groups (eight rats per group): control/saline; control/RSV 10 mg/kg; control/RSV 20 mg/kg; diabetic/saline; diabetic/RSV 10 mg/kg; diabetic/RSV 20 mg/kg. One week after diabetes induction, the animals belonging to the control/RSV10 group and diabetic/RSV10 group received 10 mg/kg of RSV intraperitoneally and the animals from the control/RSV20 and diabetic/RSV20 groups received 20 mg/kg of RSV, while the animals from the control/saline and diabetic/saline groups received saline solution intraperitoneally. RSV was freshly prepared in 25% ethanol and was administered between 10 and 11 a.m. once a day during 30 days, at a volume not exceeding 0.1 mL/100 g rat weight. After the treatment period, the animals were submitted to euthanasia, and the blood was collected by cardiac puncture for platelet preparation.

Platelet preparation

Platelet-Rich Plasma (PRP) was prepared by the method of Lunkes et al. (2004) with the following minor modifications. Total blood was collected by cardiac puncture with 0.120 M sodium citrate as anticoagulant. The total blood–citrate system was centrifuged at 160 ×g during 15 min. After this, the PRP was centrifuged at 1400 ×g for 30 min and washed twice with 3.5 mM HEPES buffer, pH 7.0, containing 142 mM NaCl, 2.5 mM KCl and 5.5 mM glucose. The platelet pellets were resuspended in HEPES buffer and used to determine enzymatic activities.

NTPDase and 5′-nucleotidase activity determination

The NTPDase enzymatic assay was carried out in a reaction medium containing 5 mM $CaCl_2$, 100 mM NaCl, 4 mM KCl, 5 mM glucose and 50 mM Tris–HCl buffer, pH 7.4, at a final volume of 200 µL as described by Lunkes et al. (2004). For AMP hydrolysis, the 5′-nucleotidase activity was carried out as previously described by Lunkes et al. (2004), except that the 5 mM $CaCl_2$ was replaced by 10 mM $MgCl_2$. Twenty microliters of the enzyme preparation (8–12 µg of protein) was added to the reaction mixture and the pre-incubation proceeded for 10 min at 37 °C. The reaction was initiated by the addition of ATP or ADP at a final concentration of 1.0 mM, and AMP at a concentration final of 2 mM. The time of incubation was 60 min. Both enzyme assays were stopped by the addition of 200 µL of 10% tricloroacetic acid (TCA) to provide a final concentration of 5%. Subsequently, the tubes were chilled on ice for 10 min. Released inorganic phosphate (Pi) was assayed by the method of Chan et al. (1986) using malachite green as the colorimetric reagent and KH_2PO_4 as standard. Controls were carried out to correct for non-enzymatic hydrolyses of nucleotides by adding enzyme preparation after TCA addition. All samples were run in triplicate. Enzyme specific activities are reported as nmol Pi released/min/mg of protein.

E-NPP activity determination — measurement of p-Nph-5′-TMP hydrolysis in platelets

Ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) activity from platelets was assessed using p-nitrophenyl 5′-thymidine

monophosphate (p-Nph-5′-TMP) as substrate as described by Fürstenau et al. (2006). The reaction medium containing 50 mM Tris–HCl buffer, 120 mM NaCl, 5.0 mM KCl, 60 mM glucose, and 5.0 mM $CaCl_2$, pH 8.9, was preincubated with approximately 20 mg per tube of platelet protein for 10 min at 37 °C in a final volume of 200 mL. The enzyme reaction was started by the addition of p-Nph-5′-TMP at a final concentration of 0.5 mM. After 80 min of incubation, 200 mL NaOH 0.2 N was added to the medium to stop the reaction. The amount of p-nitrophenol released from the substrate was measured at 400 nm using a molar extinction coefficient of 18.8×10^{-3}/M/cm. All samples were performed in triplicate. Enzyme activities were expressed as nmol of p-nitrophenol released per minute per milligram of protein (nmol p-nitrophenol released/min/mg protein).

Adenosine deaminase activity determination (ADA)

ADA from platelets was determined according to Guisti and Galanti (1984) based on the Bertholet reaction, that is, the formation of colored indophenol complex from ammonia liberated from adenosine and quantified spectrophotometrically. Briefly, 50 µL of platelets reacted with 21 mmol/L of adenosine pH 6.5 and was incubated at 37 °C for 60 min This method is based on the direct production of ammonia when ADA acts in excess of adenosine. The protein content used for the platelet experiment was adjusted to between 0.7 and 0.9 mg/mL. Results were expressed in units per liter (U/L). One unit (1 U) of ADA is defined as the amount of enzyme required to release 1 mmol of ammonia per minute from adenosine at standard assay conditions.

Protein determination

Protein content was determined according to Bradford (1976), using bovine serum albumin as standard.

Statistical analysis

The statistical analysis used was two-way ANOVA, followed by Scheffe's post hoc test, using the statistical program, SPSS 10.0 for Windows. Effects were considered significant at values of $P<0.05$. All data were expressed as mean±SEM.

Results

The body weight and blood glucose levels determined at the end of the experiment are presented in Table 1. Statistical analysis revealed a significant effect for diabetes [$F(2,30)=185.4$; $P<0.001$] in blood glucose, but no significant diabetes vs resveratrol interaction. Post hoc analysis revealed that the blood glucose levels for the diabetic/saline group were significantly increased when compared to the control/saline group. However, the treatment with RSV had no effects on glucose levels in the diabetic/RSV10 and diabetic/RSV20 groups, which remained increased, when compared to the control/saline group. Similarly, no significant differences in glucose levels were observed when RSV was administered per se in the control/RSV10 and control/RSV20 groups when compared to the control/saline group. In relation to body weight, statistical analysis showed a significant control or diabetic vs saline or RSV (10 and 20 mg/kg) interaction [$F(2,30)=4.08$; $P<0.05$]. Post hoc analysis revealed a significant decrease in the body weight in the diabetic/saline group when compared to the control/saline group. Treatment with RSV had no effect on body weight in the diabetic/RSV10 and diabetic/RSV20 groups which remained reduced in relation to the control/saline group. Similarly, when administered per se RSV also had no effect on body weight in the control/RSV10 and control/RSV20 groups when compared to the control/saline group.

Table 1
Body weight and blood glucose levels of STZ-induced diabetic rats and those treated with resveratrol at the end of the experiment

Groups	Body weight (g)	Glucose (mg/dL)
Control/saline	284±8.15[a]	111±11.19[a]
Control/RSV 10 mg/kg	269±9.40[a]	110±5.87[a]
Control/RSV 20 mg/kg	261±4.68[a]	104±14.27[a]
Diabetic/saline	189±15.44[b]	452±51.26[b]
Diabetic/RSV 10 mg/kg	202±17.47[b]	460±40.24[b]
Diabetic/RSV 20 mg/kg	202±11.41[b]	418±49.52[b]

Groups with different letters are statistically different ([a,b]$P<0.05$, $n=6$–8).

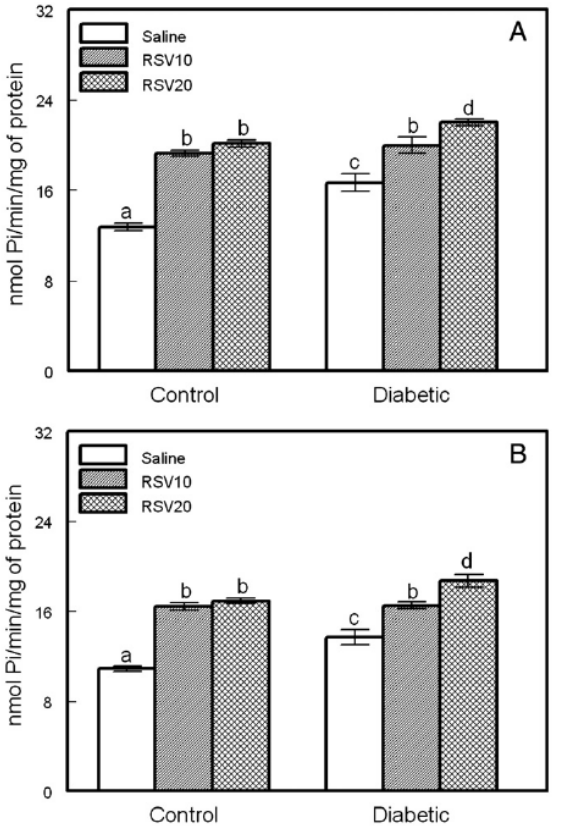

Fig. 1. NTPDase activity in platelets of rats using ATP (A) and ADP (B) as substrate (nmol Pi/min/mg of protein). Bars represent means±SEM. Groups with different letters are statistically different ($P<0.05$; $n=8$).

The results of the present study demonstrate that the cascade of ecto-enzymes was altered both in the diabetic state and after treatment with RSV. The results obtained for NTPDase activity are shown in Fig. 1. Statistical analysis showed a significant control or diabetic vs saline or RSV (10 and 20 mg/kg) interaction [$F(2,30)=14.2$; $P<0.001$] for ATP hydrolysis (Fig. 1A). Post hoc analysis revealed that NTPDase activity with ATP as substrate was significantly increased in the diabetic/saline group when compared to the control/saline group. The treatment with RSV significantly increased ATP hydrolysis in the diabetic/RSV10 and diabetic/RSV20 groups when compared to the diabetic/saline group. On the other hand, treatment with RSV per se significantly increased ATP hydrolysis in the control/RSV10 and control/RSV20 groups when compared to control/saline group. Statistical analysis of ADP hydrolysis data showed a significant control or diabetic vs saline or RSV (10 and 20 mg/kg) interaction [$F(2,30)=$

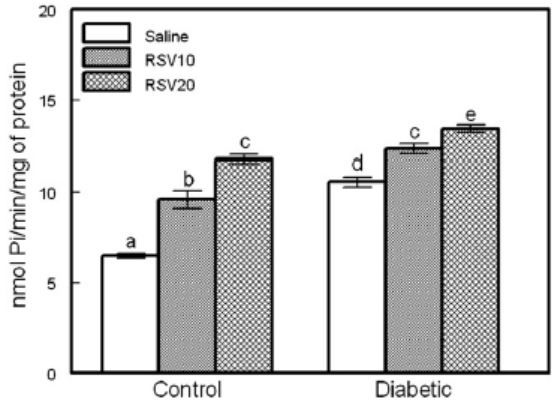

Fig. 2. 5′-Nucleotidase activity in platelets of rats using AMP as substrate (nmol Pi/min/mg of protein). Bars represent means ± SEM. Groups with different letters are statistically different ($P<0.05$, $n=8$).

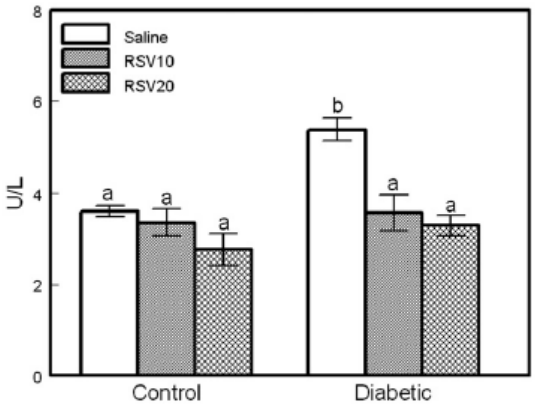

Fig. 4. ADA activity in platelets of rats (U/L). Bars represent means ± SEM. Groups with different letters are statistically different ($P<0.05$, $n=8$).

4.55; $P<0.05$] (Fig. 1B). Post hoc comparisons revealed a significant increase in ADP hydrolysis in the diabetic/saline group when compared to the control/saline group. Treatment with RSV significantly increased ADP hydrolysis in the diabetic/RSV10 and diabetic/RSV20 when compared to the diabetic/saline group. RSV produced a significant increase in ADP hydrolysis per se in the control/RSV10 and control/RSV20 groups compared to the control/saline group.

In addition, the results obtained for 5′-nucleotidase activity were similar to those found for NTPDase activity (Fig. 2). Statistical analysis showed a significant control or diabetic vs saline or RSV (10 and 20 mg/kg) interaction [$F(2,30)=3.79$; $P<0.05$] for AMP hydrolysis. Post hoc analysis revealed that AMP hydrolysis was also significantly increased in the diabetic/saline group when compared with the control/saline group. Treatment with RSV significantly increased AMP hydrolysis in the diabetic/RSV10 and diabetic/RSV20 groups when compared to the diabetic/saline group. Moreover, treatment with RSV per se provoked a significant increase in AMP hydrolysis in the control/RSV10 and control/RSV20 groups when compared to the control/saline group.

In relation to the E-NPP enzyme, statistical analysis showed a significant control or diabetic vs saline or RSV (10 and 20 mg/kg) interaction [$F(2,30)=3.72$; $P<0.05$]. In fact, post hoc analysis revealed a significant increase in E-NPP activity in the diabetic/saline group when compared to the control/saline group (Fig. 3). Treatment with RSV significantly increased E-NPP activity in the diabetic/RSV10 and diabetic/RSV20 when compared to the diabetic/saline group. Similarly, treatment with RSV per se also significantly increased E-NPP activity in the control/RSV10 and control/RSV20 groups when compared to the control/saline group.

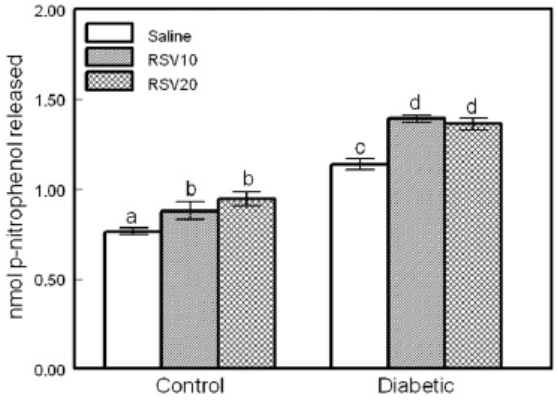

Fig. 3. E-NPP activity in platelets of rats using p-Nph-5′TMP as substrate (nmol p-nitrophenol released). Bars represent means ± SEM. Groups with different letters are statistically different ($P<0.05$, $n=8$).

The results obtained for ADA activity are presented in Fig. 4. Statistical analysis showed a significant control or diabetic vs saline or RSV (10 and 20 mg/kg) interaction [$F(2,30)=5.97$; $P<0.05$] for ADA activity. In addition, post hoc analysis revealed that ADA activity in the platelets was significantly increased in the diabetic/saline group when compared to the control/saline group. However, treatment with RSV significantly prevented the increase in ADA activity in the diabetic/RSV10 and diabetic/RSV20 groups. When RSV was given per se there were no significant differences in ADA activity in the control/RSV10 and control/RSV20 groups when compared to the control/saline group.

It is important to note that controls were performed to correct for vehicle (ethanol) interference, and no differences between vehicle and control enzyme activities were observed (data not shown).

Discussion

Around 80% of diabetic patients die from major thrombotic complications such as atherosclerosis, stroke and myocardial infarction (Zimmet et al., 2001). Plasma and cellular components of the homeostatic system are often abnormal in diabetic patients, and some of these abnormalities may play a role in the high risk of thrombosis in these patients (Carr, 2001; Sobol and Watala, 2000). In fact, multiple studies have reported the activation of platelets during diabetes mellitus, as well as the involvement of platelet aggregation in the development of vascular complications associated with this pathology (Vinik et al., 2001; Colwell and Nesto, 2003; Haouari and Rosado, 2008). Although recent studies have investigated the biologic activities of resveratrol and their possible protective effect on complications associated with diabetes mellitus (Ates et al., 2000; Kumar et al., 2007; Chi et al., 2007), the effects of RSV in hydrolyzing of the nucleotides and nucleosides from platelets, in animal models of diabetes still remain unknown. In this study, we evaluated the effect of RSV, on the ecto-enzyme cascade, constituted by NTPDase, E-NPP, 5′-nucleotidase, and ADA from platelets of diabetic rats experimentally induced with STZ.

The results of the present study demonstrated that NTPDase, E-NPP, 5′-nucleotidase and ADA activities were increased in diabetic rats (Figs. 1–4). Previous studies carried out in our laboratory also demonstrated an increase in NTPDase and 5′-nucleotidase activities in platelets of diabetic rats experimentally induced with alloxan, as

well as in patients with diabetes type 2 (Lunkes et al., 2003, 2004). Taken together, these findings indicate the up-regulation of ecto-enzymes, implying that these enzymes play an important role in the control of cellular responses induced by the diabetic state.

Extracellular nucleotides such as ATP and ADP and their nucleoside adenosine are known to regulate the vascular response to endothelial damage by exerting a variety of effects on platelets (Birk et al., 2002a). The metabolism of these extracellular nucleotides of adenine in platelets occurs by the action of the surface-located enzyme cascade constituted by NTPDase, E-NPP, 5′-nucleotidase and ADA (Frassetto et al., 1993; Fürstenau et al., 2006). These enzymes have an important role in thromboregulation process and alterations in their activities have been observed in various diseases suggesting that this could be an important physiological and pathological parameter (Lunkes et al., 2003; Silva et al., 2005; Araújo et al., 2005; Spanevello et al., 2006; Maldonado et al., 2008).

The increase in NTPDase, E-NPP and 5′-nucleotidase activities found in this study could be related to a compensatory organic response. One explanation may be that the rapid ATP and ADP hydrolysis favors adenosine production. It is known that ATP promotes vasoconstriction in the vascular endothelium and ADP activates platelet aggregation, whereas adenosine induces vasodilatation and the inhibition of platelet aggregation (Soslau and Youngprapakorn, 1997; Birk et al., 2002a,b). Consequently, the organism could be avoiding thrombotic processes by depleting ADP and enhancing adenosine production.

In addition to the enhancement of ectonucleotidase activities, our results demonstrated that ADA activity also was increased in platelets of diabetic rats (Fig. 4). Corroborating with these results, Rutkiewicz and Górski (1990) also found a significant elevation in ADA activity in tissues of diabetic rats induced with STZ. Additionally, studies have suggested that insulin is involved in the regulation of ADA activity in diabetes, and insulin administration is capable of decreasing the elevated activity of this enzyme in these tissues, however the mechanism involved in this phenomenon is unclear at the present time (Rutkiewicz and Górski, 1990; Kurtul et al., 2004; Prakash et al., 2006). Thus, we can suggest that the increase in ADA activity in diabetes could be explained, in part, by insulin action. In relation to our results, we also demonstrated that ADA activity is increased in platelets of the diabetic rats, which may be related with insulin deficits, since high concentrations of glucose in the curve in vitro did not alter ADA activity (data not shown). Based on these findings, we may suggest that a rapid deamination of adenosine by ADA causes a decrease in the level of adenosine in circulation and may be associated with the development of vascular complications observed in the diabetic state, since adenosine has an important role in preventing the thrombotic process.

When treatment with RSV (10 and 20 mg/kg) was associated with diabetes an increase in NTPDase, E-NPP and 5′-nucleotidase platelets activities was observed (Figs. 1–3). These results demonstrated that the modulation of these enzymes caused by RSV has a beneficial effect on the diabetic state, since the increase in ADP hydrolysis protects platelets from excessive aggregation, contributes to the control of hemostasis and prevents pro-thrombotic conditions observed in diabetes. Another aspect to be discussed is that ADA activity in diabetic rats treated with RSV was equal to that found in the control group. Based on our findings, we can suggest that treatment with RSV is able to maintain a high level of adenosine in the extracellular environment, which promotes vasodilation and has an important protective role under pathophysiological conditions caused by the hyperglycemic state. In fact, various studies have demonstrated the benefic effect of RSV against a number of vascular disorders such as atherosclerosis and myocardial infarction (Bradamante et al., 2003; Hung et al., 2004; Penumathsa et al., 2007). In the same vein, our results sustain and corroborate the benefits of RSV on vascular system during the diabetic state.

An important data observed in our study is that RSV per se promoted an increase in NTPDase, E-NPP and 5′-nucleotidase activities, at both concentrations tested while this compound reduces the ADA activity to equal levels that found in the control animals. Similar results were found by Spier et al. (2007), who observed that RSV also increased the hydrolysis of ATP and ADP in rat serum, in vitro. These findings demonstrate that the activation of ectonucleotidases promoted by this polyphenol tends to rapidly inactivate the released ATP/ADP with respective generation of adenosine and this way can partly explain the properties of inhibitors of platelet aggregation and vasodilators attributed to the RSV.

Thus, based on the results found in this study, we can suggest that one of the mechanisms by which RSV exerts its cardioprotective effects is through the modulation of the cascade of ecto-enzymes responsible for the hydrolysis of nucleotides of adenine and adenosine. Furthermore, these results may contribute to the understanding of the cardioprotective role of RSV, supporting theories that the regular consumption of the products containing this polyphenol is associated with a reduction and prevention of the risk of cardiovascular disease (Frémont, 2000; Kris-Etherton et al., 2002; Olas et al., 2002; Walle, 2004; Spier et al., 2007; Penumathsa et al., 2007; Matsuoka et al., 2008).

Conclusion

In summary, the results found in the present study demonstrate alterations in nucleotide hydrolysis in platelets of STZ-induced diabetic rats, which might reinforce the abnormal hemostasis caused by the diabetic state. In addition, this is the first study to investigate the in vivo effect of RSV on the activity of NTPDase, E-NPP, 5′-nucleotidase and ADA associated with experimental diabetes. This compound modulates adenine nucleotide hydrolysis and may be important in the control of the platelet coagulant status. These results confirmed our expectation in relation to the benefic effects of RSV in the diabetes. Thus, we can suggest that the RSV is a natural and promising compound that should be investigated in future studies with the intention of finding a better therapy to benefit patients with hemostatic disorders caused by the hyperglycemic state.

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References

Anfossi, G., Russo, I., Massuco, P., Mattielo, L., Cavalot, F., Balbo, A., Trovati, M., 2002. Adenosine increases human platelet levels of 3′,5′-cGMP through role in this antiagreggating effect. Thrombosis Research 105, 71–78.

Araújo, M., Rocha, J., Morsch, A., Zanin, R., Bauchspiess, R., Morsch, V., Schetinger, M.R.C., 2005. Enzymes that hydrolyze adenine nucleotides in platelets from breast cancer patients. Biochimica et Biophysica Acta 1740 (3), 421–426.

Ates, O., Cayli, S.R., Yucel, N., Altinoz, E., Kocak, A., Durak, M.A., Turkoz, Y., Yologlu, S., 2000. Central nervous system protection by resveratrol in streptozotocin-induced diabetic rats. Journal of Clinical Neuroscience 14 (3), 256–260.

Birk, A.V., Broekman, J., Gladek, E.M., Robertson, H.D., Drosopoulos, J.H.F., Marcus, A.J., Szeto, H., 2002a. Role of a novel soluble nucleotide phosphohydrolase from sheep plasma in inhibition of platelet reactivity: hemostasis, thrombosis, and vascular biology. Journal of Laboratory and Clinical Medicine 139 (2), 116–124.

Birk, A.V., Broekman, J., Gladek, E.M., Robertson, H.D., Drosopoulos, J.H.F., Marcus, A.J., Szeto, H., 2002b. Role of extracellular ATP metabolism in regulation of platelet reactivity. Journal of Laboratory and Clinical Medicine 140 (3), 166–175.

Blackburn, M.R., Kellems, R.E., 2005. Adenosine deaminase deficiency: metabolic basis of immune deficiency and pulmonary inflammation. Advances in Immunology 86, 1–41.

Borowiec, A., Lechward, K., Tkacz-Stachowska, K., Skladanowski, A.C., 2006. Adenosine as a metabolic regulator of tissue function: production of adenosine by cytoplasmic 5′-nucleotidases. Acta Biochimica Polonica 53, 269–278.

Bradamante, S., Barenghi, L., Piccinini, F., Bertelli, A.A.E., Jonge, R.D., Beemster, P., Jong, J.W.D., 2003. Resveratrol provides late-phase cardioprotection by means of a nitric oxide- and adenosine-mediated mechanism. European Journal of Pharmacology 465 (1–20), 115–123.

Bradamante, S., Villa, A., 2004. Cardiovascular protective effects of resveratrol. Cardiovascular Drug Reviews 22 (3), 169–188.

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Carr, M.E., 2001. Diabetes mellitus: a hypercoagulable state. Journal of Diabetes and its Complications 15 (1), 44–54.

Chan, K., Delfert, D., Junger, K.D., 1986. A direct colorimetric assay for Ca^{2+}-ATPase activity. Analytical Biochemistry 157 (2), 375–378.

Chi, T.C., Chen, W.P., Chi, T.L., Kuo, T.F., Lee, S.S., Cheng, J.T., Su, M.J., 2007. Phosphatidylinositol-3-kinase is involved in the antihyperglycemic effect induced by resveratrol in streptozotocin-induced diabetic rats. Life Sciences 80 (18), 1713–1720.

Colgan, S.P., Eltzschig, H.K., Eckle, T., Thompson, L.F., 2006. Physiological roles for ecto-5′-nucleotidase (CD73). Purinergic Signalling 2 (2), 351–360.

Colwell, J.A., Nesto, R.W., 2003. The platelet in diabetes: focus on prevention of ischemic events. Diabetes Care 26 (7), 2181–2188.

Di Virgilio, F., Chiozzi, P., Ferrari, D., Falzoni, S., Sanz, J.M., Morelli, A., Torboli, M., Bolognesi, G., Baricordi, O.R., 2001. Nucleotide receptors: an emerging family of regulatory molecules in blood cells. Blood 97, 587–600.

Fabris, S., Momo, F., Ravagnan, G., Stevanato, R., 2008. Antioxidant properties of resveratrol and piceid on lipid peroxidation in micelles and monolamellar liposomes. Biophysical Chemistry 135 (1–3), 76–83.

Fragopoulou, E., Nomikos, T., Karantonis, H.C., Apostolakis, C., Pliakis, E., Samiotaki, M., Panayotou, G., Antonopoulou, S., 2007. Biological activity of acetylated phenolic compounds. Journal of Agricultural and Food Chemistry 55 (1), 80–89.

Frassetto, S.S., Dias, R.D., Sarkis, J.J.F., 1993. Characterization of an ATP diphosphohydrolase activity (EC 3615) in rat blood platelets. Molecular and Cellular Biochemistry 129 (1), 47–55.

Frémont, L., 2000. Biological effects of resveratrol. Life Sciences 66 (8), 663–673.

Fürstenau, C.R., Trentin, D.S., Barreto-Chaves, M.L.M., Sarkis, J.J.F., 2006. Ecto-nucleotide pyrophosphatase/phosphodiesterase as part of a multiple system for nucleotide hydrolysis by platelets from rats: kinetic characterization and biochemical properties. Platelets 17, 84–91.

Guisti, G., Galanti, B., 1984. Colorimetric method. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis. Verlag Chemie, Weinheim, pp. 315–323.

Haouari, M.E., Rosado, J.A., 2008. Platelet signalling abnormalities in patients with type 2 diabetes mellitus: a review. Blood Cells, Molecules, and Diseases 41 (1), 119–123.

Hung, L.M., Su, M.J., Chen, J.K., 2004. Resveratrol protects myocardial ischemia–reperfusion injury through both NO-dependent and NO-independent mechanisms. Free Radical Biology and Medicine 36 (6), 774–781.

Kris-Etherton, P.M., Hecker, K.D., Bonanome, A., Coval, S.M., Binkoski, A.E., Hilpert, K.F., Griel, A.E., Etherton, T.D., 2002. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. The American Journal of Medicine 113 (9), 71–88.

Kumar, A., Kaundal, R.K., Iyer, S., Sharma, S.S., 2007. Effects of resveratrol on nerve functions, oxidative stress and DNA fragmentation in experimental diabetic neuropathy. Life Sciences 80 (13), 1236–1244.

Kurtul, N., Pence, S., Akarsu, E., Kocoglu, H., Aksoy, Y., Aksoy, H., 2004. Adenosine deaminase activity in the serum of type 2 diabetic patients. Acta Medica 47 (1), 33–35.

Lunkes, G., Lunkes, D., Morsch, V., Mazzanti, C., Morsch, A., Miron, V., Schetinger, M.R., 2004. NTPDase and 5′-nucleotidase in rats alloxan-induced diabetes. Diabetes Research and Clinical Practice 65 (1), 1–6.

Lunkes, G.I., Lunkes, D., Stefanello, F., Morsch, A., Morsch, V., Mazzanti, C.M., Schetinger, M.R.C., 2003. Enzymes that hydrolyze adenine nucleotides in diabetes and associated pathologies. Thrombosis Research 109 (4), 189–194.

Maldonado, P.A., Corrêa, M.C., Becker, L.V., Flores, C., Moretto, M.B., Morsch, V., Schetinger, M.R.C., 2008. Ectonucleotide pyrophosphatase/phosphodiesterase (E-NPP) and adenosine deaminase (ADA) activities in patients with uterine cervix neoplasia. Clinical Biochemistry 41 (6), 400–406.

Marcus, A.J., Broekman, M.J., Drosopoulos, J.H.F., Islam, N., Pinsky, D.J., Sesti, C., 2003. Metabolic control of excessive extracellular nucleotide accumulation by CD39/ecto-nucleotidase-1: implications for ischemic vascular diseases. Journal of Pharmacology and Experimental Therapeutics 305, 9–16.

Matsuoka, A., Kodama, Y., Fukuhara, K., Honda, S., Hayashi, M., Sai, K., Hasebe, M., Fujiwara, Y., 2008. A pilot study of evaluation of the antioxidative activity of resveratrol and its analogue in a 6-month feeding test in young adult mice. Food and Chemical Toxicology 46 (3), 1125–1130.

Olas, B., Wachowicz, B., Saluk-Juszczak, J., Zielinski, T., 2002. Effect of resveratrol, a natural polyphenolic compound, on platelet activation induced by endotoxin or thrombin. Thrombosis Research 107, 141–145.

Penumathsa, S.V., Thirunavukkarasu, M., Koneru, S., Juhasz, B., Zhan, L., Pant, R., Menon, V.P., Otani, H., Maulik, N., 2007. Statin and resveratrol in combination induces cardioprotection against myocardial infarction in hypercholesterolemic rat. Journal of Molecular and Cellular Cardiology 42 (3), 508–516.

Prakash, M.S., Chennaiah, S., Murthy, Y.S.R., Anjaiah, E., Rao, S.A., Suresh, C., 2006. Altered adenosine deaminase activity in type 2 diabetes mellitus. Journal Indian Academy of Clinical Medicine 7 (2), 114–121.

Robson, S.C., Sevigny, J., Zimmermann, H., 2006. The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. Purinergic Signalling 2, 409–430.

Rozalski, M., Nocun, M., Watala, C., 2005. Adenosine diphosphate receptors on blood platelets — potential new targets for antiplatelet therapy. Acta Biochimica Polonica 52 (2), 411–415.

Rutkiewicz, J., Górski, J., 1990. On the role of insulin in regulation of adenosine deaminase activity in rat tissues. FEBS Letters 271 (1–2), 79–80.

Shen, M.Y., George, H., Chang, L.L., Tsorng, F.H., Kuang, L.H., Duen, C.S., Joen, S.R., 2007. Inhibitory mechanisms of resveratrol in platelet activation: pivotal roles of p38 MAPK and NO/cyclic GMP. British Journal of Haematology 139 (3), 475–485.

Silva, A.C., Morsch, A.L.B., Zanin, R.F., Côrrea, M.C., Arantes, L.C., Araújo, M.C., Morsch, V.M., Schetinger, M.R.C., 2005. Enzymes that hydrolyze adenine nucleotides in chronic renal failure: relationship between hemostatic defects and renal failure severity. Biochimica et Biophysica Acta 1741 (3), 282–288.

Sobol, A.B., Watala, C., 2000. The role of platelets in diabetes-related vascular complications. Diabetes Research and Clinical Practice 50 (1), 1–16.

Soslau, G., Youngprapakorn, D., 1997. A possible dual physiological role of extracellular ATP in modulation of platelet aggregation. Biochimica et Biophysica Acta 1355 (2), 131–140.

Spanevello, R.M., Mazzanti, C.M., Maldonado, P., Zanin, R., Morsch, A., Hannel, L., Mazzanti, A., Festugatto, R., Graça, D., Schmatz, R., Loro, V.L., Schetinger, M.R.C., Morsch, V., 2006. Activities of enzymes that hydrolyze adenine nucleotides in platelets from rats experimentally demyelinated with ethidium bromide and treated with interferon β. Life Science 80 (12), 1109–1114.

Spier, A.P., Bavaresco, C.S., Wyse, A.T.S., Carvalho, D., Sarkis, J.J.F., 2007. Effects of resveratrol and purple grape juice on nucleotide hydrolysis by adult rat serum. Food Chemistry 103 (2), 565–571.

Stefan, C., Jansen, S., Bollen, M., 2005. NPP-type ectophosphodiesterases: unity in diversity. Trends in Biochemical Sciences, 30 (10), 542–550.

Strater, N., 2006. Ecto-5-nucleotidase: structure function relationships. Purinergic Signalling 2 (2), 343–350.

Stratmann, B., Tschoepe, D., 2005. Pathobiology and cell interactions of platelets in diabetes. Diabetes and Vascular Disease Research 2, 16–23.

Tosaki, A., Juhasz, B., Bak, I., Das, S., Das, K., Varga, E., Lekli, I., 2007. Cardioprotective effects of resveratrol in isolated Zucker obese rat hearts. Journal of Molecular and Cellular Cardiology 42 (6), 211–220.

Vinik, A.I., Erbas, T., Park, T.S., Nolan, R., Pittenger, G.L., 2001. Platelet dysfunction in type 2 diabetes. Diabetes Care 24, 1476–1485.

Walle, T., 2004. Absorption and metabolism of flavonoids. Free Radicals Biology and Medicine 36, 829–837.

Yegutkin, G.G., 2008. Nucleotide- and nucleoside-converting ectoenzymes: important modulators of purinergic signalling cascade. Biochimica et Biophysica Acta (BBA) — Molecular Cell Research 1783 (5), 673–694.

Zhu, J., Yong, W., Wu, X., Yu, Y., Iv, J., Liu, C., Mao, X.M., Zhu, Y., Xu, K., Han, X., Liu, C., 2008. Anti-inflammatory effect of resveratrol on TNF-α-induced MCP-1 expression in adipocytes. Biochemical and Biophysical Research Communications 369 (2), 471–477.

Zimmet, P., Alberti, K.G., Shaw, J., 2001. Global and societal implications of the diabetes epidemic. Nature 414, 782–787.

3.3. MANUSCRITO I

Moderate red wine and grape juice consumption modulate the hydrolysis of the adenine nucleotides and decrease platelet aggregation in streptozotocin-induced diabetic rats

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Moderate red wine and grape juice consumption modulate the hydrolysis of the adenine nucleotides and decrease platelet aggregation in streptozotocin-induced diabetic rats

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Abstract

The present study investigated the effects ex vivo of the moderate red wine (RW) and grape juice (GJ) consumption and the effects in vitro of the polyphenols resveratrol, acid caffeic, gallic acid, quercetin, and rutin on NTPDase, ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP), 5'-nucleotidase and adenosine deaminase (ADA) activities in platelets and platelet aggregation from streptozotocin (STZ)-induced diabetic rats. For the experiment ex vivo, the animals were divided into six groups (n=10): control/saline, control/GJ, control/RW, diabetic/saline, diabetic/GJ, and diabetic/RW. RW and GJ were administered for 45 days and after this period the blood was collected for experimental determinations. Results showed that NTPDase, E-NPP, 5′-nucleotidase and ADA activities as well as platelet aggregation were increased in the diabetic/saline group compared to the control/saline group *(P<0.05).* Treatment with RW and GJ increased NTPDase, E-NPP and 5′-nucleotidase activities and prevented the increase in the ADA activity in the diabetic/GJ and diabetic/RW groups compared to the diabetic/saline group *(P<0.05).* Platelet aggregation was also decreased by the treatment with RW and GJ in the diabetic/GJ and diabetic/RW groups compared to the diabetic/saline group *(P<0.05).* In the in vitro tests, resveratrol, caffeic acid, and gallic acid increased ATP, ADP and AMP hydrolysis, while quercetin and rutin decreased the hydrolysis of these nucleotides in platelets of diabetic rats *(P<0.05).* The ADA activity and platelet aggregation were reduced in platelets of diabetic rats in the presence of all polyphenols tested *in vitro (P<0.05).* These findings suggest that RW and GJ as well as resveratrol, acid caffeic, gallic acid, quercetin, and rutin were able to modulate the ectonucleotidases and ADA activities. Moreover, a decrease in the platelet aggregation was observed and it could contribute to the prevention and reduction of platelet abnormality and consequently vascular complications in diabetic state.

Keywords: diabetes; platelets; red wine; grape juice; ectonucleotidases;

1. Introduction

Diabetes mellitus, one of the most prevalent endocrine disorder worldwide, is characterized by hyperglycemia resulting in short-term metabolic changes in lipid and protein metabolism and in long-term a series of vascular alterations [1]. These vascular alterations include diabetes-specific complications such as retinopathy, nephropathy, and neuropathy as well as macro vascular complications such as atherosclerosis, potentially resulting in heart disease, stroke, and peripheral vascular disease [1].

The pathogenesis of the vascular complications in diabetes is complex and has several potential contributors including alterations in platelet morphology and function [2]. In fact, growing evidence suggests that platelets of diabetic patients are larger and hyperreactive, and consequently present deregulation of several signaling pathways leading to an increase of adhesion, activation, and aggregation [2, 3]. Moreover, it has been demonstrated that the platelets of these patients are more prone to spontaneous aggregation and are highly hypersensitive to agonists such as thrombin, collagen, and ADP [4, 5].

Extracellular nucleotides such as ATP, ADP, and their nucleoside derivative, adenosine, have become clearly recognized for their important role in modulating processes linked to vascular inflammation and thrombosis [6, 7]. In the vascular system, these molecules can recruit and activate platelets, activate endothelial cells, and induce vasoconstriction [7]. The nucleotide ADP acts upon platelets, promoting their aggregation and modifying their shape [8], while ATP has been postulated to be a competitive inhibitor of ADP-induced platelet aggregation [9]. In addition, adenosine, produced by the nucleotide catabolism, is recognized as an important modulator of vascular tone and a potent inhibitor of platelet aggregation [10, 11].

The importance of adenine nucleotides in the vascular system is greatly correlated with the essential role of a multienzymatic complex present on the platelet membrane, which provides an adequate control of these signaling molecules in the extracellular medium [12]. This complex includes the enzymes NTPDase (ectonucleoside triphosphate phosphohydrolase), E-NPP (ectonucleotide pyrophosphatase/phosphodiesterase), ecto-5′-nucleotidase, and ecto-adenosine deaminase (ADA) [12]. NTPDase hydrolyzes ATP and ADP into AMP [13], while the

E-NPP are responsible for hydrolyzing 5’-phosphodiester bonds in nucleotides and their derivatives, resulting in the production of monophosphate nucleotides [14]. AMP produced from the action of NTPDase and E-NPP is subsequently hydrolyzed into adenosine by the action of 5’-nucleotidase [15]. The resulting adenosine can be directly inactivated through the action of ADA, which catalyzes the irreversible deamination of adenosine [16]. Together, these ecto-enzymes constitute a highly organized enzymatic cascade that is able to regulate the extracellular concentrations of adenine nucleotides and nucleosides and play an important role in the maintenance of normal hemostasis and thrombogenesis, mainly by regulating the platelet aggregation status [12, 17].

Epidemiological studies have associated the consumption of grapes and their derived products such as red wine (RW) and juice with a wide variety of health beneficial effects, in particular the reduced risk of cardiovascular diseases [18-20]. These beneficial effects have been observed as a result of chronic and moderate RW and grape juice (GJ) consumption and have been attributed mainly to polyphenolic compounds present in these products [21-23]. Indeed, grapes contain a high concentration and a great variety of polyphenols, such as flavonoids (catechin, epicatechin, quercetin, rutin, anthocyanins, and procyanidins), and non-flavonoids, such as resveratrol (3,5,4’-trihydroxy-stilbene), galic acid, and cafeic acid, which are mainly found in red grape products [21, 24]. These polyphenols have showed to exert a number of important biological activities in the cardiovascular system, such as antioxidant activity, inhibition of platelet aggregation and adhesion, vasodilator activity, modulation of lipid metabolism, inhibition of low-density lipoprotein oxidation, and diminishing of smooth muscle cell migration and proliferation [18-24]. In addition, recent data of our research group have demonstrated protective effects of resveratrol in the experimental diabetes [25-28]. However, the effect of RW and GJ on the ecto-enzyme activity in platelets of diabetic rats still has not been reported in literature.

Therefore, considering the benefic effects of moderate RW and GJ consumption and the importance of the enzymes that hydrolyze adenine nucleotides and nucleosides in the mechanism of thromboregulation, the aim of the present study was to evaluate the platelet aggregation and NTPDase, E-NPP, 5’-nucleotidase and ADA activities in platelets from streptozotocin (STZ)-induced diabetic rats that received RW or GJ. Finally, we also evaluated the effect of resveratrol, caffeic acid,

gallic acid, rutin, and quercetin in platelet aggregation and in the ecto-enzyme activities in platelets of diabetic rats under *in vitro* conditions in order to investigate the interaction of these of polyphenolic compounds of grape in hemostatic disorders in the diabetic state.

2. Materials and methods

2.1. Chemicals

The substrates ATP, ADP, AMP, p-nitrophenyl thymidine 5′- monophosphate (p-Nph-5′-TMP), adenosine, as well as trizma base, sodium azide, HEPES, streptozotocin and polyphenolic compounds resveratrol, rutin, quercetin, galic acid and caffeic acid (all with approximately 99.9% purity) and Coomassie brilliant blue G were obtained from Sigma Chemical Co (St. Louis, MO, USA) and bovine serum albumin, K_2HPO_4, from Reagen. All the other chemicals used in this experiment were of the highest purity.

2.2. Animals

Adult male Wistar rats (70-90 days; 220-300 g) from the Central Animal House of the Federal University of Santa Maria were used in this experiment. The animals were maintained at a constant temperature (23±1°C) on a 12 h light/dark cycle with free access to food and water. All animal procedures were approved by the Animal Ethics Committee from the Federal University of Santa Maria (protocol under number: 21/2007).

2.3. Experimental induction of diabetes

Diabetes was induced by a single intraperitoneal injection of 65 mg/kg streptozotocin (STZ) [25], diluted in 0.1 M sodium-citrate buffer (pH 4.5). The age-matched control rats received an equivalent amount of the sodium-citrate buffer. STZ-treated rats received 5% of glucose instead of water for 24 h after diabetes induction in order to reduce death due to hypoglycemic shock. Blood samples were

taken from the tail vein 72 h after STZ or vehicle injection. Glucose levels were measured with a portable glucometer (ADVANTAGE, Boehringer Mannheim, MO, USA). Only animals with fasting glycemia over 300 mg/dL were considered diabetic and used for the present study. During the experiment the levels of blood glucose were verified four times (10, 20, 30 and 45 days after the beginning of treatment). The animals that maintained fasting glycemia higher than 300 mg/dL were considered diabetic and selected for assays.

2.4. Grape juice and red wine composition

Organic red GJ of the Bordo variety was obtained from the city of Garibaldi (Rio Grande do Sul, Brazil), in the main grape-growing region of the state. Grapes were cultivated in 2007 and the juice was prepared in the same year. Tannat RW was obtained from the city of Santana do Livramento (Rio Grande do Sul, Brazil), and produced from grapes of the Tannat variety cultivated in the season 2008/2009, containing an alcoholic degree of 12.51%. The concentrations of the main phenolic compounds in the RW and GJ were determined by high-performance liquid chromatography (HPLC) analysis as described by Machado et al. [29]. The concentrations of resveratrol, quercitin, rutin, gallic acid, and caffeic acid in both grape products are shown in Table 1.

2.5. Treatment with red wine and grape juice

The animals were randomly divided into six groups (N=10): control/saline, control/GJ, control/RW, diabetic/saline, diabetic/GJ, and diabetic/RW. The treatment with RW and GJ began two weeks after the diabetes induction. The animals from the control/saline and diabetic/saline groups received saline solution via gavage. The animals belonging to the control/GJ and diabetic/GJ groups received GJ, whereas the animals from the control/RW and diabetic/RW groups received RW. RW and GJ were administered via gavage, between 10 and 11 a.m., once a day for 45 days, at a volume equal to 4.28 mL/kg body weight of rats. This dose is equivalent to a person of 70 kg consuming daily 300 ml (2 glasses) of RW or GJ. The dose of RW and GJ used in this study represents a moderate human dose, which is consistent with the

epidemiology data that light and moderate alcohol consumption reduces the incidence of cardiovascular diseases [30, 31]. After the treatment period, the animals were previously anesthetized with halothane and submitted to euthanasia, and the blood was collected by cardiac puncture for platelet-rich plasma preparation and other biochemical determinations.

In order to correct the interference of ethanol present in RW, a group of control rats and another group of diabetic rats received a solution of ethanol 12.51%, equivalent to the alcoholic degree of RW utilized in this experiment. However, no significant differences in the control/ethanol and diabetic/ ethanol groups were observed to any parameters analyzed when compared to control/saline and diabetic/saline groups, respectively (data not shown).

2.6. Platelet preparation

Platelet-Rich Plasma (PRP) was prepared by the method of Lunkes et al. [32] with the following minor modifications. Total blood was collected by cardiac puncture with 0.120 M sodium citrate as anticoagulant. The total blood–citrate system was centrifuged at 160 ×g during 15 min. After this, the PRP was centrifuged at 1400 ×g for 30 min and washed twice with 3.5 mM HEPES buffer, pH 7.0, containing 142 mM NaCl, 2.5mM KCl and 5.5mM glucose. The platelets pellets were resuspended in HEPES isosmolar buffer and protein was adjusted to 0.4-0.6 mg/mL and used to determine enzymatic activities.

2.7. NTPDase and 5′-nucleotidase activity determination

The NTPDase enzymatic assay was carried out in a reaction medium containing 5 mM $CaCl_2$, 100 mM NaCl, 4 mM KCl, 5 mM glucose and 50 mM Tris-HCl buffer, pH 7.4, at a final volume of 200 µL as described by Lunkes et al [32]. For AMP hydrolysis, the 5′- nucleotidase activity was carried out as previously described by Lunkes et al.[32], except that the 5 mM $CaCl_2$ was replaced by 10mM $MgCl_2$. Twenty microliters of the enzyme preparation (8-12 µg of protein) was added to the reaction mixture and the pre-incubation proceeded for 10 min at 37 °C. The reaction

was initiated by the addition of ATP or ADP at a final concentration of 1.0mM, and AMP at a concentration final of 2 mM. The time of incubation was 60 min. Both enzyme assays were stopped by the addition of 200 µL of 10% tricloroacetic acid (TCA) to provide a final concentration of 5%. Subsequently, the tubes were chilled on ice for 10 min. Released inorganic phosphate (Pi) was assayed by the method of Chan et al. [33] using malachite green as the colorimetric reagent and KH_2PO_4 as standard. Controls were carried out to correct for non-enzymatic hydrolyses of nucleotides by adding enzyme preparation after TCA addition. All samples were run in triplicate. Enzyme specific activities are reported as nmol Pi released/min/mg of protein.

2.8. E-NPP activity determination- measurement of p-Nph-5′-TMP hydrolysis in platelets

Ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) activity from platelets was assessed using p-nitrophenyl 5′-thymidinemonophosphate (p-Nph-5′-TMP) as substrate as described by Fürstenau et al. [34]. The reaction medium containing 50 mM Tris-HCl buffer, 120 mM NaCl, 5.0 mM KCl, 60 mM glucose, and 5.0 mM $CaCl_2$, pH 8.9, was preincubated with approximately 20 mg per tube of platelet protein for 10 min at 37 °C in a final volume of 200 mL. The enzyme reaction was started by the addition of p-Nph- 5′-TMP at a final concentration of 0.5mM. After 80min of incubation, 200 mL NaOH 0.2 N was added to the medium to stop the reaction. The amount of p-nitrophenol released from the substrate was measured at 400 nm using a molar extinction coefficient of 18.8×10- 3/M/cm. All samples were performed in triplicate. Enzyme activities were expressed as nmol of p-nitrophenol released per minute per milligram of protein (nmol p-nitrophenol released/min/mg protein).

2.9. Adenosine deaminase activity determination (ADA)

ADA activity from platelets was determined according to Guisti and Galanti [35] based on the Bertholet reaction, that is, the formation of colored indophenol complex from ammonia liberated from adenosine and quantified

spectrophotometrically. Briefly, 50 µL of platelets reacted with 21 mmol/L of adenosine pH 6.5 and was incubated at 37 °C for 60 min This method is based on the direct production of ammonia when ADA acts in excess of adenosine. The protein content used for the platelet experiment was adjusted to between 0.7 and 0.9 mg/mL. Results were expressed in units per liter (U/L). One unit (1 U) of ADA is defined as the amount of enzyme required to release 1 mmol of ammonia per minute from adenosine at standard assay conditions.

2.10. Platelet aggregation

The platelet aggregation profile was evaluated by the method of Born [36] by measuring turbidity with a Chrono-log optical aggregometer (AGGRO/lINK® Model 810-CA software for Windows ver. 5.1) using ADP at a concentration of 5 and 10 µmol/l as agonist. The results were expressed as percentage of aggregation.

2.11. Experiments performed in vitro

Five polyphenols present in RW and GJ were chosen due to their biological and pharmacological effects, and their contents were determined by HPLC (Table 1) [29]. The *in vitro* effects of resveratrol, quercetin, rutin, caffeic acid, and gallic acid on the platelet aggregation as well as NTPDase, 5′-nucleotidase and ADA activities were evaluated. Isolated platelets from diabetic rats and control rats were incubated with different concentrations of these compounds (0, 1, 25, 50, 100, and 200 µM) in the medium reaction as previously described above. In general, *in vitro* effects of resveratrol and other polyphenols are observable at concentrations ranging from 1 to 100 µM [37, 38]. The concentrations of resveratrol, quercetin, rutin, and caffeic acid were diluted in ethanol 25%, while the gallic acid was diluted in water. The final concentrations of ethanol 25%, when tested alone in the incubation medium, did not affect the enzyme activities (data not shown).

Likewise, was tested the effects *in vitro* of the combination of resveratrol, caffeic acid, quercetin and rutin in the concentrations of 20, 100 and 200 µM [38] on NTPDase, 5′-nucleotidase activities. The concentration of 20 µM was obtained by

combining, of the forth compounds in the concentration of 5 μM each. The concentration of 100 μM was resulting of combinations of the forth phenols in the concentration of 25 μM each. The concentration of 200 μM was obtained by combining of the forth compounds in the concentration of 50 μM each [38].

2.12. Glycated hemoglobin

The levels of glycated-hemoglobin were determined in plasma by using commercial Kits (Labtest, Minas Gerais, Brazil).

2.13. Protein determination

Protein content was determined according to Bradford [35], using bovine serum albumin as standard.

2.14. Statistical analysis

Statistical analysis was done by the commercial SPSS package for Windows©. All data were expressed as mean ± S.D. Data were analyzed statistically by one-way ANOVA followed by the Duncan's multiple tests. Differences were considered significant when the probability was $P<0.05$.

3. Results

3.1. Blood glucose and body weight

The body weight and blood glucose levels determined at the end of the experiment are presented in Table 2. Post hoc analysis revealed that the blood glucose levels for the diabetic/saline group were significantly increased when compared to the control/saline group ($P<0.05$). However, the treatment with RW significantly decreased the glucose levels in the diabetic/RW group ($P<0.05$), while the treatment with GJ had no effect on the glucose levels when compared to the

diabetic/saline group. No significant differences in the glucose levels were observed when RW and GJ were administered per se in the control/RW and control/GJ groups when compared to the control/saline group.

Post hoc analysis revealed a significant decrease in the body weight in the diabetic/saline group when compared to the control/saline group (P<0.05). Treatment with RW and GJ had no effect on the body weight in the diabetic/RW and diabetic/GJ groups which remained reduced in relation to the control/saline group. Similarly, when administered per se RW and GJ also had no effect on the body weight in the control/RW and control/GJ groups when compared to the control/saline group.

3.2. Glycated hemoglobin levels

Glycated hemoglobin levels were significantly increased in the diabetic/saline group when compared to the control/saline group (P<0.05) (Table 2). Treatment with RW and GJ prevented the increase of the levels of glycated hemoglobin in the diabetic/GJ and diabetic/RW groups (P<0.05). The administration of RW and GJ per se did not alter significantly the glycated hemoglobin levels in the control/GJ and control/RW groups when compared to the control/saline group.

3.3. Ex vivo effects of RW and GJ on NTPDase, 5'-nucelotidase, E-NPP and ADA activities

The results of the present study demonstrate that the cascade of ecto-enzymes was altered in the diabetic state and after treatment with RW and GJ (P< 0.05). Fig. 1 shows the results obtained for the NTPDase activity. As can be observed, the NTPDase activity with ATP as substrate was increased 47% in the diabetic/saline group when compared to the control/saline group (Fig. 1A). Treatment with both RW and GJ increased ATP hydrolysis about 48% in the diabetic/RW group and 29% in the diabetic/GJ group when compared to the diabetic/saline group (P < 0.05). Moreover, when RW and GJ were administered *per se*, ATP hydrolysis was increased by 78% and 61% in the control/RW and control/GJ groups, respectively, in comparison to the control/saline group (P< 0.05).

In relation to ADP hydrolysis, an increase of 40 % in the diabetic/saline group was observed when compared to the control/saline group ($P< 0.05$) (Fig. 1B). In addition, treatment with RW and GJ increased ADP hydrolysis by 48% and 27% in the diabetic/RW and diabetic/GJ groups, respectively, when compared to the diabetic/saline group ($P< 0.05$). The administration of RW and GJ *per se* increased ADP hydrolysis by 71% in the control/RW group and by 45% in control/GJ group when compared to the control/saline group.

The results obtained for the 5'-nucleotidase activity were similar to those found for the NTPDase activity (Fig. 1C). Post hoc analysis revealed that AMP hydrolysis was increased 97% in the diabetic/saline group when compared to the control/saline group ($P< 0.05$). Treatment with RW and GJ increased AMP hydrolysis about 68% in the diabetic/RW and 37% in the diabetic/GJ group, when compared to the diabetic/saline group ($P<0.05$). Moreover, when RW and GJ were given *per se*, the hydrolysis of AMP was increased by 72% and 45% in the control/RW and control/GJ groups, respectively, in comparison to the control/saline group ($P< 0.05$).

The results obtained for the E-NPP activity are presented in Fig. 2 A. As can be observed, the activity of this enzyme in platelets was increased 146% in the diabetic/saline group when compared to the control/saline group. In addition, the administration of RW produced an increase of 112% in the E-NPP activity in the diabetic/RW group when compared to the diabetic/saline group. No significant differences were observed in the E-NPP activity between the diabetic/GJ and diabetic/saline groups. The treatment with RW *per se* increased the E-NPP activity about 138% in the control/RW group compared to the control/saline group. However, no significant differences in the E-NPP activity in the control/GJ group were observed when compared to the control/saline group.

Fig. 2B shows the results obtained for the ADA activity. Post hoc analysis revealed that the activity of this enzyme in platelets was increased 114% in the diabetic/saline group when compared to the control/saline group ($P< 0.05$). However, the treatment with RW and GJ significantly prevented the increase in the ADA activity in the diabetic/RW and diabetic/GJ groups when compared to the diabetic/saline group ($P< 0.05$). No significant differences in the ADA activity were

observed in the control/RW and control/GJ in comparison with the control/saline group.

3.4. Platelet aggregation

Table 3 presents the results obtained for platelet aggregation. As can be observed, platelet aggregation was significantly increased in the diabetic/saline group when compared with the control/saline group (*P<0.05*). The treatment with RW and GJ significantly reduced the platelet aggregation at both tested concentrations of ADP in the diabetic/RW and diabetic/GJ groups when compared to the diabetic/saline group, and presented similar values to the control/saline group (*P*<0.05). When RW was given *per se*, platelet aggregation was significantly reduced in control/RW group in comparison to the control/saline group (*P*<0.05). A significant decrease in platelet aggregation at 5 umol of ADP was observed in the control/GJ group, while no significant alteration in platelet aggregation at 10 umol of ADP was observed in the same group when compared with the control/saline group.

3.5. In vitro effects of polyphenols on ATP, ADP and AMP hydrolysis

The effect of resveratrol, caffeic acid, gallic acid, quercetin, and rutin on the NTPDase and 5'-nucleotidase activities are presented in the Fig. 3 and 4. As can be observed, ATP, ADP and AMP hydrolysis in platelets of diabetic group was significantly increased when compared to the control group (*P< 0.05*). However, when platelets of diabetic rats were incubated with 50, 100 and 200 μM of resveratrol a significant increase in the ATP hydrolysis, proportional to the increase of the concentrations was observed when compared to the diabetic group (*P< 0.05*) (Fig. 3). ADP and AMP hydrolysis in platelets of diabetic rats presented a significant increase in all tested concentrations of resveratrol (in the range of 1-200 μM) in comparison to the diabetic group (*P< 0.05*). When platelets of control rats were incubated with resveratrol the hydrolysis of ATP, ADP, and AMP was significantly increased in all tested concentrations when compared to the control enzyme activity (*P< 0.05*).

Caffeic acid, another polyphenol tested, significantly increased hydrolysis of ATP and ADP in platelets of diabetic rats at the concentrations of 100 μM and 200 μM, whereas AMP hydrolysis was efficiently increased in all tested concentrations (1-200 μM) when compared with the diabetic group ($P< 0.05$) (Fig. 3). ATP, ADP and AMP hydrolysis in platelets of control rats were significantly increased in presence of 25, 50, 100 and 200 μM of caffeic acid when compared to the control group.

Experimental data demonstrated that platelets of diabetic rats incubated with gallic acid presented a significant increase in ATP and ADP hydrolysis in all tested concentrations, whereas AMP hydrolysis was significantly increased in the presence of 1, 25, 100 and 200 μM of gallic acid compared to the diabetic group ($P< 0.05$) (Fig. 3). When platelets of control rats were incubated with gallic acid, ADP and AMP hydrolysis was significantly increased in all tested concentrations, whereas ATP hydrolysis was increased only in the presence of 100 and 200 μM de gallic acid when compared to the control group ($P< 0.05$).

As illustrated in Fig.4, when platelets of diabetic rats were incubated with quercetin, a significant decrease in ATP, ADP and AMP hydrolysis in all tested concentrations of this flavonoid were observed in comparison to the diabetic group. ATP, ADP and AMP hydrolysis in platelets of control rats was also significantly decreased in all tested concentrations of quercetin when compared to the control group ($P< 0.05$).

In relation to the flavonoid rutin, a significant decrease in the ATP hydrolysis in platelet of diabetic rats at the concentration of 200 μM was observed, whereas ADP and AMP hydrolysis was significantly decreased in the presence of the concentrations 50, 100 and 200 μM of rutin when compared to the diabetic group ($P< 0.05$) (Fig.4). ATP hydrolysis was significantly decreased when platelets of the control rats were incubated with 200 μM of quercetin, whereas ADP and AMP hydrolysis presented a significant decrease at the concentrations of 100 and 200 μM when compared to the control group ($P< 0.05$).

The combination of resveratrol, caffeic acid, quercetin, and rutin produced a significant increase in the hydrolysis of ATP, ADP, and AMP in platelets of diabetic rats in all tested concentrations when compared to the diabetic group (Fig. 5).

Similarly, this same combination of polyphenols also produced a significant increase in ATP, ADP and AMP hydrolysis in platelets of the control group (*P< 0.05*).

3.6. Effects of polyphenols in vitro on the ADA activity

The effects of resveratrol, caffeic acid, gallic acid, quercetin, and rutin on the ADA activity are presented in Table 4. As can be observed, the ADA activity in platelets of the diabetic group was significantly increased when compared to the control group (*P< 0.05*).

When platelets of diabetic rats were incubated with resveratrol a significant decrease in the ADA activity was observed in all tested concentrations when compared to the diabetic group (*P< 0.05*). The ADA activity in platelets of control rats in the presence of 25, 50, 100 and 200 μM of resveratrol was significantly inhibited when compared to the control group (*P< 0.05*).

Caffeic acid significantly decreased the ADA activity in platelets of diabetic rats at the concentrations of 25, 50, 100 and 200 μM when compared to the diabetic group (*P< 0.05*). When platelets of control rats were incubated with 100 and 200 μM of caffeic acid, a significant decrease in the ADA activity was observed in comparison to the control group (*P< 0.05*).

Gallic acid also significantly decreased the ADA activity in platelets of diabetic rats in the concentrations of 50, 100 and 200 μM when compared to the diabetic group (*P< 0.05*). However, when platelets of control rats were incubated with gallic acid, no significant difference in the ADA activity was observed in comparison to the control group (*P< 0.05*).

The ADA activity was significantly decreased when platelets of diabetic rats were incubated with 25, 50, 100 and 200 μM of quercetin compared to the diabetic group (*P< 0.05*). In platelets of control rats, the ADA activity was significantly inhibited in the concentrations of 50, 100 and 200 μM of quercetin when compared to the control group (*P< 0.05)*.

In addition, when platelets of diabetic rats were incubated with 50, 100 and 200 μM of rutin, the ADA activity was significantly decreased when compared to the

diabetic group *(P< 0.05)*. However, no significant differences in the ADA activity in platelets of rats in the presence of all tested concentrations of rutin were observed in comparison to the control group.

3.7. Effects of polyphenols in vitro on platelet aggregation

Table 5 presents the results obtained for *in vitro* to the platelet aggregation. The platelet aggregation in samples of the diabetic group was significantly increased when compared to the control group *(P< 0.05).* However, resveratrol, caffeic acid, gallic acid, rutin, and quercetin significantly decreased the platelet aggregation in samples of diabetic rats in the concentrations of 25, 100 and 200 μM when compared to the diabetic group *(P<0.05*). In samples of control rats, the platelet aggregation rats was significantly decreased in the concentrations of 100 and 200 μM of resveratrol, caffeic acid, gallic acid, rutin, and quercetin when compared to the control group *(P<0.05).*

4. Discussion

Atherothrombotic complications are the main cause of morbidity and mortality in patients with diabetes mellitus. Among factors contributing to the prothrombotic condition, present in the diabetic state, platelet hyperreactivity plays a pivotal role [2-5, 39]. In this context, several experimental and clinical studies have indicated that polyphenols found in the grape and their derived products are able to modulate several pathways involved in the platelet activation and in the consequent thrombus growth, exerting thus important cardioprotective effects [21, 40, 41]. In view of this, the effects of RW and GJ *ex vivo* and the effects of grape polyphenols *in vitro* on the activity of enzymes that hydrolyze adenine nucleotides and nucleosides in platelets, as well as platelet aggregation in STZ-induced diabetic rats were investigated in the present study.

Platelets are an important source of purine signaling molecules for blood, such as ATP and ADP, which have been implicated to play relevant roles in haemostatic, thrombotic and inflammatory processes [7, 42]. Once released, the biological effect

of extracellular nucleotides is tightly regulated by the action of the enzyme cascade located in the platelet surface constituted by ectonucleotidases and ADA [12]. These ecto-enzymes modulate the responses mediated by nucleotides/nucleosides within the vascular system and may potentially be altered in pathological states [43]. In line with this, we observed a significant increase in NTPDase, E-NPP and 5'-nucleotidase activities in platelets from STZ-induced diabetic rats (Figs. 1A-C and 2A). These results agree with studies of our laboratory that have reported an increase in ATP, ADP and AMP hydrolysis in platelets from diabetic type 2 and diabetic type 2/hypertensive patients [32], as well as in platelets from diabetic rats experimentally induced with alloxan [44] and STZ [26], demonstrating the important role for these enzymes in the hyperglycemic state. In fact, Lunkes et al. [45] found an increase in NTPDase and 5'-nucleotidase activities directly proportional to the increase in glucose and fructose concentrations *in vitro*, indicating the participation of high glucose levels as a probable interference factor in the ectonucleotidase activities.

It is important to note that the regulation of extracellular ADP and adenosine concentration is critical due to their physiological effects in the vascular system [7, 42]. ADP is known to induce changes in platelet shape and aggregation [8], whereas adenosine is a molecule with a variety of cardiovascular protective effects such as vasodilatation and inhibition of platelet aggregation [11]. In this scenario and considering our results, we can suggest that the increase in NTPDase, E-NPP and 5'-nucleotidase activities in platelets from diabetic rats may be related to an important compensatory organic response of the ectonucleotidase way that could occur to terminate the function of extracellular ADP, including its pro-aggregant effects, as well as to increase the extracellular adenosine production, an important cardioprotective molecule.

On the other hand, it is important to point out that despite the increase of ATP, ADP and AMP hydrolysis contributing to an increase of adenosine production, this study also found an elevation of the ADA activity in platelets of diabetic rats (Fig. 2B). ADA is an important enzyme that degrades adenosine into inosine, tightly regulating local extracellular concentrations of adenosine [16]. Therefore, a rise in the activity of this enzyme may lead to increased adenosine deamination, causing a reduction of the levels of this nucleoside in the circulation. Consequently, this situation may produce a favorable scenario for the development of vascular diseases in diabetic

state, since adenosine has an important role in the prevention of platelet aggregation and atherothrombotic complications [11, 46].

In the present study, when diabetic rats received RW and GJ, a more accentuated increase in NTPDase, E-NPP and 5′-nucleotidase activities in platelets was observed (Fig. 1A-C and 2B) indicating that the consumption of both beverages derived from grape interferes with purinergic signaling. This consequent increase in the ectonucleotidases activities reflects an increased degradation of ATP, ADP, and AMP resulting in an increment in the adenosine formation. In this sense, we may suggest that the moderate consume of RW and GJ can have an antiaggregant effect, limiting the bioavailability of ADP, the main agonist to platelet aggregation. Moreover, it also promotes the production of adenosine, an antiaggregant and vasodilating agent [11], contributing to the control of hemostasis in diabetic state.

In relation to these results, it is important to point out that although both RW and GJ increased ATP, ADP and AMP hydrolysis in diabetic rats, we observed an increase significantly higher in the nucleotide hydrolysis in diabetic rats treated with RW when compared to those treated with GJ (Fig. 1A-C). It is important to note that the most pronounced effects observed in rats that received RW may be associated with the presence of ethanol. In fact, Dias et al. [47] found that lower doses of ethanol, similar to that found in RW, increased NTPDase and 5’-nucleotidase activities in platelets, showing a stimulatory response of ethanol in the nucleotide hydrolysis. In addition, Schmatz et al. [25] demonstrated that the treatment with resveratrol, an important polyphenol of RW and GJ, also increased the ectonucleotidases activities in platelets of diabetic rats. Thus, we can suggest that the ethanol and the resveratrol can act synergistically [48-49] in the modulation of ectonucleotidases pathway, potentially the effects of RW on adenine nucleotide hydrolysis, while in the GJ the activation can be related only to the effects of the polyphenols.

Another important aspect to be discussed is that the treatment with RW and GJ prevented the increase in the ADA activity in platelets of diabetic rats (Fig. 2B). Studies have shown that an inhibition of the ADA activity can increase the concentration of adenosine in the extracellular medium and to potentiate the effects of this nucleoside on their cell receptors [50, 51]. Based on these findings, we may

suggest that RW and GJ are able to preserve adenosine levels in the circulation, which act upon platelet adenosine receptors and can inhibit platelet aggregation and promote vasodilatation, exerting an important protective role in the prevention of the development and progression of vascular complications caused by the hyperglycemic state. In fact, studies have shown that polyphenolic compounds present in RW and GJ can inhibit the process of thrombus formation [19-24]. Thus, these results support the hypothesis that one of the ways by which RW and GJ exert cardioprotective actions may be mediated by an increase in the adenosine levels and an amplification of the effect of this nucleoside via adenosine receptors, since both beverages derivate of grape have demonstrated to inhibit ADA activity.

A relevant datum of this study is that ADP-induced platelet aggregation was significantly increased in diabetic rats (Table 3). Corroborating with these results, several studies have reported that platelets from diabetic patients and animals show a hypersensitivity to physiological pro-aggregant agents and an enhanced activation state, which can be observed in an early stage in the disease course and may precede the development of atherothrombotic complications in the diabetes [2-5, 51]. Furthermore, these platelets are more prone to form spontaneous microaggregates with ADP receptor involvement [53]. In addition, it is important to note that the increase in the ADP-induced platelet aggregation observed in this study may be associated with a decrease in the adenosine levels caused by an increase in the ADA activity in diabetic rats, since the adenosine is one of its most potent inhibitors of platelet aggregation and consequently of pro-thrombotic conditions.

On the other hand, there is accumulating evidence that the hyperglycemia contributes to greater reactivity and aggregability of platelets, particularly through the generation of reactive oxygen species (ROS) and by the glycation of platelet membrane proteins [2, 54, 55]. In this study, we found an increase in the glucose levels in STZ- induced diabetic rats, accompanied by an elevation in the glycated hemoglobin levels, which may be an indicative that the platelet proteins and vascular wall protein can also be suffering non-enzymatic glycosylation (Table 2). In hyperglycemic states, glucose non-enzymatically binds to different proteins in the platelet membrane, causing abnormalities in the function and damage in their structure and conformation, as well as alterations of membrane lipid dynamics, contributing to the platelet hyperfunction in diabetic state [55, 56]. In addition, high

levels of glycated hemoglobin have been strongly associated with the risk of cardiovascular disease in diabetics and in the general population [57]. Thus, based on these evidences, we can infer that the hyperglycemia and consequent events such as the oxidative stress and increased non-enzymatic glycation of platelet proteins can be associated with increased in ADP- mediated platelet aggregation found in diabetic rats.

Of great importance, our results demonstrated that the treatment with RW and GJ was able to prevent the increase in the platelet aggregation in diabetic rats, confirming the antiplatelet effects attributed to the moderate consumption of these beverages (Table 3) [30, 58, 59]. These results are consistent with studies that have demonstrated that both RW and GJ can decrease platelet aggregation in human and experimental models, by reducing plasma thromboxane B2 concentration and the concentration of ADP and thrombin available for platelet aggregation [58, 59]. In this context, it is important to point out that the ectonucleotidases via modulation by RW and GJ, associated with the prevention in the increase of the ADA activity may be one potential mechanism by which these beverages inhibit ADP- induced platelet aggregation in diabetic rats. In fact, the increase in the ADP hydrolysis can lead to a decrease in the levels of this pro-aggregant nucleotide and to increase adenosine levels, a powerful inhibitor of platelet aggregation. Thus, we propose for the first time, that this way can partly explain the inhibitory properties of platelet aggregation and vasodilator attributed to RW and GJ in the diabetes.

The treatment with RW and GJ was able to prevent the increase in the glycated hemoglobin levels in diabetic rats, suggesting that the moderate consumption of these beverages may prevent non-enzymatic glycation of platelet proteins and consequently functional and structural damages that contribute to the platelet hyperaggregability and formation of thrombus in diabetic state (Table 2). In fact, it has been found that polyphenols can inhibit the glycation and autoxidation of glucose, preventing the initiation and propagation of protein modification [60, 61]. Moreover, several studies have indicated that the oxidative stress that accompanies diabetes can induce endothelial dysfunction and promote major platelet reactivity through direct effects on platelets, such as increase of lipid peroxidation and depletion of antioxidant defense systems [52, 54]. However, it is well known that the main polyphenols present in RW and GJ are powerful antioxidants, protecting many

tissues and cells, including platelets, of damages caused by oxidative stress [62]. In addition, it has been shown that the resveratrol exerted a potent antioxidant effect on the generation of different ROS in activated platelets [63]. Based on these findings, we can suggest that the strong antioxidant properties of RW and GJ may contribute to the prevention of an increase in the platelet aggregation found in diabetic rats treated with both beverages derived from grape.

The results of our study, compared favorably with several studies, demonstrated the protective effects of moderate RW and GJ consumption in reducing risk factors associated with several degenerative diseases including diabetes and cardiovascular diseases [18, 23, 61, 66-68]. These protective effects in the vascular system might be primarily attributable to combined, additive or perhaps synergistic effects of the various components of the complex mixture of bioactive compounds present in RW including ethanol, resveratrol, flavonols, flavan-3-ols, anthocyanins, phenolic acids as well as their metabolites formed either in the tissues or in the colon by the microflora [31, 68]. Initially, the cardioprotective effects of RW were attributed only to its ethanol content, which in lower concentration has antiplatelet effects [48, 49, 69]. However, studies indicated that wine might confer benefits beyond those of other alcoholic beverages, indicating that nonalcoholic factors in wine may also play a protective role [49, 70, 71]. Supporting this hypothesis GJ also presents inhibitory properties of platelet aggregation, suggesting that grape-derived polyphenols and not only ethanol, may contribute to the apparent antithrombotic effect of RW (Table 3). However, in our study the effects of RW on the platelet aggregation and ectonucleotidase activities were more pronounced than those obtained to GJ. Similarly, Pace-Asciak et al. [59] found that platelet aggregation was strongly inhibited by RW and moderately inhibited by GJ. Interestingly, it has been postulated that alcohol increases the intestinal absorption and consequently the bioavailability of the polyphenols from RW [72-75]. Moreover, the ethanol is able to prevent the precipitation of the polyphenolic tannins in the digestive tract being considered a natural stabilizing agent for polyphenols in RW [73]. In line with this, we can suggest that the potentiating of the antithrombotic effects of RW found in this study can be a consequence either of synergism between the ethanol and polyphenolic compounds of wine and /or of major absorption and biodisponibility of polyphenols in diabetic rats that received RW compared with GJ.

Reinforcing this line of reasoning, the next set of experiments was performed in order to verify the effects of the main polyphenols of RW and GJ, such as resveratrol, caffeic acid, gallic acid, quercetin, rutin on NTPDase, 5- nucleotidase and ADA activities, and platelet aggregation in platelets from diabetic rats under *in vitro* conditions.

Resveratrol, gallic acid, and caffeic acid tested *in vitro* increased the hydrolysis of ATP, ADP, and AMP in platelets of diabetic rats (Fig. 3). Previously, the quercetin and rutin, the latter known as a glycoside derivative, showed contrasting *in vitro* effects when compared to the polyphenols above, presenting a decrease in ATP, ADP and AMP hydrolysis in the platelets of diabetic rats (Fig. 4). Similar results were found by Spier et al. [76], who observed that resveratrol increased the hydrolysis of ATP and ADP in rat serum, while the quercetin and rutin decreased the NTPDase and 5'-nucleotidase activities. From these results, we may consider that these polyphenols act via different mechanisms on the ectonucleotidases. As mentioned above, in our study the treatment with RW and GJ promoted activation in NTPDase and 5'-nucleotidase in diabetic rats (Fig. 1). Thus, although all polyphenols tested are present in both beverages from grape, the effects of activation of resveratrol, gallic acid and caffeic acid on the ectonucleotidases activities seem to predominate on the effects of inhibition of quercitin and rutin. In fact, when we tested the effects *in vitro* the combination of resveratrol, caffeic acid, quercetin and rutin was observed an increase in ATP, ADP and AMP hydrolysis, confirming that the effects of the non-flavonoids resveratrol and caffeic acid are prevalent on the ectonucleotidases activities (Fig. 5). Interestingly, Pignatelli et al. [31] have showed that there is a synergy among three phenols resveratrol, caffeic acid, and catechin, which ensures biological activities such as inhibition of oxidative stress and of platelet aggregation, despite their low plasma concentrations following chronic moderate wine consumption. Moreover, it has been shown that quercetin interferes with the sulphation and glucuronidation of resveratrol in the liver, thereby increasing its bioavailability [77, 78]. Based on these findings we can infer that the resveratrol and caffeic acid can be acting synergistically in the increase of hydrolysis of nucleotides, while quercetin, despite the opposite effects on ectonucleotidases, can contribute indirectly to the prevalent effects of resveratrol through the increasing bioavailability

of this compound. Further studies in the understanding of synergic effects of the polyphenols of RW and are currently under investigation by our group.

On the other hand, we observed that, *in vitro,* resveratrol, gallic acid, caffeic acid, quercetin, and rutin decreased significantly the ADA activity in the platelets of diabetic rats (Table 4). These results are consistent with those found in the treatment *ex vivo* with RW and GJ in platelets of diabetic rats, suggesting that these compounds can act in a combined and additive way in order to increase the levels of adenosine in the circulation playing an important cardioprotective role in the diabetic state.

In addition, all polyphenolic compounds of grape tested *in vitro* decreased the ADP-induced platelet aggregation in diabetic rats, suggesting that the synergic effects of these polyphenols can contribute to the decrease of platelet aggregation in diabetic rats treated with RW and GJ (Table 5). These results are in accordance with several studies that have demonstrated that the resveratrol, quercetin, caffeic acid, gallic acid, and rutin have inhibitory effects on platelet hiperaggregation in the status diabetic [21, 79, 80, 81]. Among the several mechanisms proposed, it is possible to infer that the resveratrol, gallic acid, and caffeic acid may reduce platelet aggregation by increasing NTPDase and 5'-nucleotidase activities, decreasing ADP levels, and increasing adenosine levels. However, rutin and quercetin should reduce the platelet aggregation by a mechanism different from ectonucleotidase via, since these flavonoids inhibit the hydrolysis of ADP, the main platelet agonist. Interestingly, Wright et al. [40] demonstrated that platelets themselves take part in the flavonoid metabolism. Quercetin and its plasmatic metabolite 4′-0-methyl quercetin (tamarixetin) are internalized by platelets and further metabolized by the addition of sulphate or glucuronide groups. Thus, formed compounds inhibit platelet activation by antagonizing surface receptors (especially estrogen receptors and thromboxane A2 receptors) [40, 79].

It is important to point out that in the present study the moderate intake of RW and GJ per se also promoted an increase in NTPDase, E-NPP and 5′-nucleotidase activities, and did not alter the ADA activity (Fig. 1 and 2 B). In addition, the effects *in vitro* of the non-flavonoids resveratrol, gallic acid, and caffeic acid and of the flavonoids rutin and quercetin on NTPdase, 5'-nucleotidase and ADA activities and

platelet aggregation in control rats were similar to the effects observed in the platelets of diabetic rats, indicating that the polyphenols from RW and GJ can exert their protective effects in both pathological and healthy conditions (Fig. 3 and 4). These results, partly explain the beneficial effects to the health globally attributed to the regular and moderate consumption of RW and GJ in the prevention of the development and progression of vascular complications [18-24, 80].

In conclusion, the results found in the present study demonstrate alterations in the platelet aggregation as well as in the adenine nucleotide and nucleoside hydrolysis in platelets of STZ-induced diabetic rats, which might reinforce the abnormal hemostasis caused by the diabetic state. In addition, both RW and GJ, as well as the polyphenols present in these beverages modulated the hydrolysis of adenine nucleotides and nucleosides in platelets and consequently reduced the platelet aggregation in diabetic rats. Thus, we propose by first time that the modulation of ecto-enzyme activities of platelets can be one of the mechanisms by which the RW and GJ can prevent and reduce the platelet abnormality and consequently vascular complications in diabetic state. Furthermore, these results are very important from the clinical point of view because they reinforce the several benefits to the health, in special to the diabetes, of the intake of two glasses of RW and GJ per day, which can provide about 40% of the total antioxidant polyphenols present in a healthy diet [82], as well as a number of polyphenols, such as resveratrol that are virtually absent from commonly consumed fruit and vegetables.

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References

[1] American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2010; 33:62-69.

[2] Morel O, Kessler L, Ohlmann P, Bareiss P. Diabetes and the platelet: toward new therapeutic paradigms for diabetic atherothrombosis. Atherosclerosis. 2010; 212(2):367-376.

[3] Stratmann B, Tschoepe D. Pathobiology and cell interactions of platelets in diabetes. Diab Vasc Dis Res. 2005; 2(1):16-23.

[4] Haouari, M.E., Rosado, J.A., 2008. Platelet signalling abnormalities in patients with type 2 diabetes mellitus: a review. Blood Cells, Molecules, and Diseases 41 (1), 119–123.

[5] Arjomand H, Roukoz B, Surabhi SK, Cohen M. Platelets and antiplatelet therapy in patients with diabetes mellitus. J Invas Cardiol. 2003;15: 264-269.

[6] Di Virgilio F, Chiozzi P, Ferrari D, Falzoni S, Sanz JM, Morelli A, Torboli M, Bolognesi G, Baricordi OR. Nucleotide receptors: an emerging family of regulatory molecules in blood cells. Blood. 2001; 97(3):587-600.

[7] Zimmermann H. Nucleotides and CD39: principal modulatory players in hemostasis and thrombosis. Nature. 1999; 5:987-988.

[8] Rozalski M, Nocun M, Watala C. Adenosine diphosphate receptors on blood platelets: potential new targets for antiplatelet therapy. Acta Biochim Pol. 2005; 52(2):411-415.

[9] Birk AV, Broekman MJ, Gladek EM, Robertson HD, Drosopoulos JH, Marcus AJ, Szeto HH. Role of extracellular ATP metabolism in regulation of platelet reactivity. J Lab Clin Med. 2002; 140(3):166-175.

[10] Anfossi G, Russo I, Massucco P, Mattiello L, Cavalot F, Balbo A, Trovati M. Adenosine increases human platelet levels of cGMP through nitric oxide: possible role in its antiaggregating effect. Thromb Res. 2002; 105(1):71-78.

[11] Borowiec A, Lechward K, Tkacz-Stachowska K, Składanowski AC. Adenosine as a metabolic regulator of tissue function: production of adenosine by cytoplasmic 5'-nucleotidases. Acta Biochim Pol. 2006; 53(2):269-78.

[12] Yegutkin G. Nucleotide and nucleoside converting ectoenzymes: important modulators of purinergic signaling cascade. Biochim Biophys Acta. 2008; 1783:673-694.

[13] Robson S, Sevigny J, Zimmermann H. The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. Purinergic Signal. 2006; 2:409-430.

[14] Stefan C, Jansen S, Bollen M. Modulation of purinergic signaling by NPP-type ectophosphodiesterases. Purinergic Signal. 2006; 2:361-370.

[15] Colgan S, Etlzsching H, Eckle T, Thompson L. Physiological roles for ecto-50-nucleotidase (CD73). Purinergic Signal. 2006; 2:351-360.

[16] Franco R, Casado V, Ciruela F, Saura C, Mallol J, Canela E, Luis C Cell surface adenosine deaminase: much more that an ectoenzyme. Prog Neurobiol. 1997; 52:283-294.

[17] Zimmermann H, Mishra S, Shukla V, Langer D, Gampe K, Grimm I, Delic J, Braun N. Ecto-nucleotidases, molecular properties and functional impact. An R Acad Nac Farm. 2007; 73:537-566.

[18] Pérez-Jiménez J, Saura-Calixto F. Grape products and cardiovascular disease risk factors. Nutr Res Rev. 2008, 21(2):158-73.

[19] Bertelli. A.A.E. Wine, research and cardiovascular disease: Instructions for use. Atherosclerosis. 2007; 195:242-247.

[20] Dohadwala MM, Vita JA. Grapes and cardiovascular disease. J Nutr. 2009; 139(9):1788-1793.

[21] Gresele P, Cerletti C, Guglielmini G, Pignatelli P, de Gaetano G, Violi F. Effects of resveratrol and other wine polyphenols on vascular function: an update. J Nutr Biochem. 2011; 22(3):201-11.

[22] Cooper KA, Chopra M, Thurnham DI. Wine polyphenols and promotion of cardiac health. Nutr Res Rev. 2004; 17(1):111-30.

[23] Leifert WR, Abeywardena MY. Cardioprotective actions of grape polyphenols. Nutr Res. 2008; 28(11):729-37.

[24] Dell'Agli M, Busciala A, Bosisio E. Vascular effects of wine polyphenols. Cardiovasc Res. 2004; 63(4):593-602.

[25] Schmatz R, Schetinger MR, Spanevello RM, Mazzanti CM, Stefanello N, Maldonado PA, Gutierres J, Corrêa Mde C, Girotto E, Moretto MB, Morsch VM. Effects of resveratrol on nucleotide degrading enzymes in streptozotocin-induced diabetic rats. Life Sci. 2009; 84(11-12):345-50.

[26] Schmatz R, Mazzanti CM, Spanevello R, Stefanello N, Gutierres J, Corrêa M, da Rosa MM, Rubin MA, Chitolina Schetinger MR, Morsch VM. Resveratrol prevents memory deficits and the increase in acetylcholinesterase activity in streptozotocin-induced diabetic rats. Eur J Pharmacol. 2009; 610(1-3):42-48.

[27] Schmatz R, Mazzanti CM, Spanevello R, Stefanello N, Gutierres J, Maldonado PA, Corrêa M, da Rosa CS, Becker L, Bagatini M, Gonçalves JF, Jaques Jdos S, Schetinger MR, Morsch VM. Ectonucleotidase and acetylcholinesterase activities in synaptosomes from the cerebral cortex of streptozotocin-induced diabetic rats and treated with resveratrol. Brain Res Bull. 2009; 80(6):371-6.

[28] Schmatz R, Perreira LB, Stefanello N, Mazzanti C, Spanevello R, Gutierres J, Bagatini M, Martins CC, Abdalla FH, Daci da Silva Serres J, Zanini D, Vieira JM, Cardoso AM, Schetinger MR, Morsch VM. Effects of resveratrol on biomarkers of oxidative stress and on the activity of delta aminolevulinic acid dehydratase in liver and kidney of streptozotocin-induced diabetic rats. Biochimie. 2011.

[29] Machado MM, Boligon AA, Athayde ML, Rocha MI, Leria JP, Bello C, Cruz IB. Determination of polyphenol content and antioxidant capacity of no-alcoholic red grape products (Vitis labrusca) from conventional and organic crops. Quím Nova. 2011; 34:798-803.

[30] Gresele P, Pignatelli P, Guglielmini G, Carnevale R, Mezzasoma AM, Ghiselli A, Momi S, Violi F.Resveratrol, at concentrations attainable with moderate wine consumption, stimulates human platelet nitric oxide production. J Nutr. 2008; 138(9):1602-1608.

[31] Pignatelli P, Ghiselli A, Buchetti B, Carnevale R, Natella F, Germano G, Fimognari F, Di Santo S, Lenti L, Violi F. Polyphenols synergistically inhibit oxidative stress in subjects given red and white wine. Atherosclerosis. 2006; 188(1):77-83.

[32] Lunkes G, Lunkes D, Stefanello F, Morsch A, Morsch V, Mazzanti C, Schetinger M. Enzymes that hydrolyze adenine nucleotides in diabetes and associated pathologies. Thromb Res. 2003; 109:189-194.

[33] Chan K, Delfert D, Junger D. A direct colorimetric assay for Ca2?-ATPase activity. Anal Biochem. 1986; 157:375-378.

[34] Fürstenau C, Trentin D, Barreto-Chaves M, Sarkis J. Ectonucleotide pyrophosphatase/phosphodiesterase as a part of multiple system for nucleotide hydrolysis by platelets from rats: kinetic characterization and biochemical proprieties. Platelets. 2006; 17:84-91

[35] Guisti G, Galanti B. Colorimetric method. In: Bergemeyer HU (ed) Methods of enzymatic analysis. Verlag Chemie, Weinheim. 1984; pp 315-323.

[36] Born GVR. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature. 1962; 194:927-929.

[37] Borriello A, Cucciolla V, Della Ragione F, Galletti P. Nutr Metab Cardiovasc Dis. Dietary polyphenols: focus on resveratrol, a promising agent in the prevention of cardiovascular diseases and control of glucose homeostasis. 2010; 20(8):618-25.

[38] Iacopini P, Baldi M, Storchi P, Sebastiani L. Catechin, epicatechin, quercetin, rutin and resveratrol in red grape: Content, in vitro antioxidant activity and interactions. J Food Compost Anal. 2008; 21(8):589-598.

[39] Ferroni P, Basili S, Falco A, Davý G. Platelet activation in type 2 diabetes mellitus. J Thromb Haemost. 2004; 2:1282-1291.

[40] Wright B, Gibson T, Spencer J, Lovegrove JA, Gibbins JM. Platelet-mediated metabolism of the common dietary flavonoid, quercetin. PLoS One. 2010; 5: e9673.

[41] Shanmuganayagam D, Warner TF, Krueger CG. Concord grape juice attenuates platelet aggregation, serum cholesterol and development of atheroma in hypercholesterolemic rabbits. Atherosclerosis. 2007; 190:135-142.

[42] Burnstock, G. Potential therapeutic targets in the rapidly expanding field of purinergic signalling. Clin Med. 2002; 2:45-53.

[43] Schetinger MR, Morsch VM, Bonan CD, Wyse AT. NTPDase and 5'-nucleotidase activities in physiological and disease conditions: new perspectives for human health. Biofactors. 2007; 31(2):77-98.

[44] Lunkes GI, Lunkes DS, Morsch VM, Mazzanti CM, Morsch AL, Miron VR, Schetinger MR. NTPDase and 5'-nucleotidase activities in rats with alloxan-induced diabetes. Diabetes Res Clin Pract. 2004; 65(1):1-6.

[45] Lunkes GI, Lunkes DS, Leal D, Araújo Mdo C, Corrêa M, Becker L, Rosa CS, Morsch VM, Schetinger MR. Effect of high glucose levels in human platelet NTPDase and 5'-nucleotidase activities. Diabetes Res Clin Pract. 2008; 81(3):351-7.

[46] Birk AV, Bubman D, Broekman MJ, Robertson HD, Drosopoulos JH, Marcus AJ, Szeto HH. Role of a novel soluble nucleotide phospho-hydrolase from sheep plasma in inhibition of platelet reactivity: hemostasis, thrombosis, and vascular biology. J Lab Clin Med. 2002; 139(2):116-124.

[47] Dias GR, Schetinger MR, Spanevello R, Mazzanti CM, Schmatz R, Loro VL, Morsch VM. Hormetic acute response and chronic effect of ethanol on adenine nucleotide hydrolysis in rat platelets. Arch Toxicol. 2009; 83(3):263-269.

[48] Renaud S, de Lorgeril M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet. 1992; 339:1523-1526.

[49] Di Castelnuovo A, Rotondo S, Iacoviello L, Donati MB, de Gaetano G. Meta-analysis of wine and beer consumption in relation to vascular risk. Circulation. 2002; 105:2836-2844.

[50] Melzig MF. Inhibition of adenosine deaminase activity of aortic endothelial cells by selected flavonoids. Planta Med. 1996; 62(1):20-1.

[51] Koch HP, Jäger W, Groh U, Plank G. In vitro inhibition of adenosine deaminase by flavonoids and related compounds. New insight into the mechanism of action of plant phenolics. Methods Find Exp Clin Pharmacol. 1992; 14(6):413-417.

[52] Schäfer A and Bauersachs J. Endothelial dysfunction, impaired endogenous platelet inhibition and platelet activation in diabetes and atherosclerosis. Curr Vasc Pharmacol. 2008; 6: 52-60.

[53] Matsuno H, Tokuda H, Ishisaki A, Zhou Y, Kitajima Y and Kozawa O. P2Y12 receptors play a significant role in the development of platelet microaggregation in patients with diabetes. J Clin Endocrinol Metab. 2005; 90: 920-927.

[54] Freedman JE. Oxidative stress and platelets. Arterioscler Thromb Vasc Biol. 2008; 28:11-16.

[55] Hasegawa Y, Suehiro A, Higasa S, Namba M and Kakishita E. Enhancing effect of advanced glycation end products on serotonin-induced platelet aggregation in patients with diabetes mellitus. Thromb Res. 2002; 107:319-323.

[56] Yamagishi S, Nakamura K, Imaizumi T. Advanced glycation end products (AGEs) and diabetic vascular complications. Curr Diabetes Rev. 2005; 1:93-106.

[57] Selvin E, Steffes MW, Zhu H, Matsushita K, Wagenknecht L, Pankow J, Coresh J, Brancati FL. Glycated hemoglobin, diabetes, and cardiovascular risk in nondiabetic adults. N Engl J Med. 2010; 362(9):800-811.

[58] Freedman JE, Parker C III, Li L. Select flavonoids and whole juice from purple grapes inhibit platelet function and enhance nitric oxide release. Circulation. 2001; 103:2792-2798.

[59] Pace-Asciak CR, Rounova O, Hahn SE, Diamandis EP, Goldberg DM. Wines and grape juices as modulators of platelet aggregation in healthy human subjects. Clin Chim Acta. 1996; 246:163-182.

[60] Wu CH, Yen GC. Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts. J Agric Food Chem. 2005; 53(8):3167-3173.

[61] Montilla P, Barcos M, Muñoz MC, Muñoz-Castañeda JR, Bujalance I, Túnez I. Protective effect of Montilla-Moriles appellation red wine on oxidative stress induced by streptozotocin in the rat. J Nutr Biochem. 2004; 15(11):688-93.

[62] Rodrigo R, Miranda A, Vergara L. Modulation of endogenous antioxidant system by wine polyphenols in human disease. Clin Chim Acta. 2011; 412(6):410-424.

[63] Olas B, Wachowicz B. Resveratrol, a phenolic antioxidant with effects on blood platelet functions. Platelets. 2005 16(5):251-60.

[66] Huang PH, Tsai HY, Wang CH, Chen YH, Chen JS, Lin FY, Lin CP, Wu TC, Sata M, Chen JW, Lin SJ. Moderate intake of red wine improves ischemia-induced neovascularization in diabetic mice-roles of endothelial progenitor cells and nitric oxide. Atherosclerosis. 2010; 212(2):426-435.

[67] Tozzi Ciancarelli MG, Di Massimo C, De Amicis D, Ciancarelli I, Carolei A. Moderate consumption of red wine and human platelet responsiveness. Thromb Res. 2011; 128(2):124-129.

[68] Lippi G, Franchini M, Favaloro EJ, Targher G. Moderate red wine consumption and cardiovascular disease risk: beyond the "French paradox". Semin Thromb Hemost. 2010; 36(1):59-70.

[69] Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov. 2006; 5:493-506.

[70] Gronbaek M, Becker U, Johansen D, Gottschau A, Schnohr P, Hein HO, Jensen G, Sorensen TI. Type of alcohol consumed and mortality from all causes, coronary heart disease, and cancer. Ann Intern Med. 2000; 133:411-419.

[71] Klatsky AL, Friedman GD, Armstrong MA, Kipp H. Wine, liquor, beer, and mortality. Am J Epidemiol. 2003; 158:585-595.

[72] Dragoni S, Gee J, Bennett R, Valoti M, Sgaragli G. Red wine alcohol promotes quercetin absorption and directs its metabolism towards isorhamnetin and tamarixetin in rat intestine in vitro. Br J Pharmacol. 2006; 147(7):765-771.

[73] Serafini, M., Maiani, G. & Ferro-Luzzi AF. Effect of ethanol on red wine tannin-protein (BSA) interactions. J. Agric. Food Chem. 1997; 45: 3148-3151.

[74] Ruf JC, Berger JL, Renaud S. Platelet rebound effect of alcohol withdrawal and wine drinking in rats. Arterioscler Thromb Vasc Biol. 1995; 15:140-114.

[75] Auger C, Caporiccio B, Landrault N, Teissedre PL, Laurent C, Cros G, Besançon P, Rouanet JM. Red wine phenolic compounds reduce plasma lipids and apolipoprotein B and prevent early aortic atherosclerosis in hypercholesterolemic golden Syrian hamsters (Mesocricetus auratus). J Nutr. 2002; 132(6):1207-1213.

[76] Spier AP, Bavaresco CS, Wyse ATS, Carvalho D, Sarkis JJF. Effects of resveratrol and purple grape juice on nucleotide hydrolysis by adult rat serum. Food Chem. 2007; 103(2) 565-571.

[77] De Santi C, Pietrabissa A, Spisni R, Mosca F, Pacifici GM. Sulphation of resveratrol, a natural product present in grapes and wine, in the human liver and duodenum. Xenobiotica. 2000; 30:609-617.

[78] De Santi C, Pietrabissa A, Mosca F, Pacifici GM. Glucuronidation of resveratrol, a natural product present in grape and wine, in the human liver. Xenobiotica. 2000; 30: 1047-1054.

[79] Guerrero JA, Navarro-Nuñez L, Lozano ML, Martínez C, Vicente V. Flavonoids inhibit the platelet TxA2 signalling pathway and antagonize TxA2 receptors (TP) in platelets and smooth muscle cells. 2007; Br J Clin Pharmacol. 64: 133-144.

[80] Olas B, Wachowicz B, Saluk-Juszczak J, Zieliński T. Effect of resveratrol, a natural polyphenolic compound, on platelet activation induced by endotoxin or thrombin. Thromb Res. 2002; 107(3-4):141-145.

[81] Soleas GJ, Diamandis EP, Goldberg DM. Wine as a biological fluid: history, production, and role in disease prevention. J Clin Lab Anal. 1997; 11(5):287-313.

TABLES

Table 1. Concentrations of phenolic compounds present in the Tannat red wine and Bordo grape juice.

Compounds	Concentrations	
	Red wine	**Grape Juice**
Total polyphenols (mg/mL)	4.15 ± 0.03	3.10± 0.03
Flavonoids (mg/mL)	0.257 ± 0.002	0.249 ± 0.002
Condensed tannins (mg/mL)	0.312 ± 0.004	0.215 ± 0.005
Resveratrol (mg/L)	4.12 ± 0.12	3.95 ± 0.01
Quercetin (mg/L)	1.35 ± 0.31	8.95 ± 0.09
Rutin (mg/L)	1.21 ± 0.18	3.75 ± 0.03
Gallic acid (mg/L)	9.97 ± 1.17	81.07 ± 2.03
Caffeic acid (mg/L)	6.49 ± 0.71	30.28 ± 2.00

Results are expressed as mean ± S.E. of three determinations.

Table 2. Effects of moderate consumption of red wine and grape juice on body weight and blood glucose, glycated hemoglobin levels and platelets number in STZ-induced diabetic rats. Values are expressed as mean ± S.D. Groups with different letters are statistically different (^{a,b,c} *P<0.05*, n=10). ANOVA-Duncan's Test.

Groups	Body weight (g)	Glucose (mg/dL)	Glycated hemoglobin (mg/dL)	Platelets (n/mm^3)
Control/saline	316 ± 8.1^{a}	74.7± 2.4^{a}	4.83± 0.8^{a}	633.062±104.4^{a}
Control/GJ	303 ± 9.4^{a}	80.2± 6.0^{a}	3.95± 0.4^{a}	701.687±80.4^{a}
Control/RW	289 ± 4.6^{a}	74.2± 2.1^{a}	4.15± 0.4^{a}	809.142±95.4^{a}
Diabetic /saline	169 ±15.4^{b}	415.2± 75.1^{b}	8.64± 1.6^{b}	712.800±102.4^{a}
Diabetic/GJ	173 ± 7.4^{b}	394.1± 40.5^{b}	6.25± 2.4^{ab}	656.167±101.4^{a}
Diabetic/RW	192± 11.4^{b}	212.6± 30.1^{c}	5.42± 2.4^{a}	545.750±91.4^{a}

Table 3. Effects of moderate consumption of red wine and grape juice on ADP-induced platelet aggregation in STZ-induced diabetic rats. The results are expressed as percentage of aggregation (mean±S.D). Groups with different letters are statistically different (^{a,b,c} *P<0.05*, n=10). ANOVA-Duncan's Test.

Groups	Agonist	
	ADP 5.0 µM	**ADP 10.0 µM**
Control/saline	31.4± 8.5^{a}	39.8± 3.7^{a}
Control/GJ	33.0± 3.7^{b}	36.1± 4.7^{a}
Control/RW	25.2± 5.2^{b}	28.5± 2.5^{b}
Diabetic /saline	55.8± 5.5^{c}	65.0± 4.4^{c}
Diabetic/GJ	37.3±5.7^{a}	42.0± 3.2^{a}
Diabetic/RW	34.6±4.3^{a}	41.4 ±2.7^{a}

Table 4. Effects *in vitro* of resveratrol, caffeic acid, gallic acid, quercetin and rutin on ADA activity in platelets of diabetic (DT) and control (CT) rats. The results of ADA activity were expressed in units per liter (U/L) (mean±S.D). Groups with different letters are statistically different (^{a,b,c} *P<0.05*, n=5). ANOVA-Duncan's Test.

Concentra-tions of phenols μM)	Platelets	Resveratrol	Caffeic acid	Gallic acid	Quercetin	Rutin
0	CT	3.79±0.5 ^{a}	3.25±0.5 ^{a}	2.4±0.4 ^{a}	3.89±0.9^{a}	2.30±0.4 ^{a}
	DT	7.60±1.5^{c}	6.69±1.5^{c}	5.3±0.2 ^{b}	6.70±1.1^{b}	4.48±0.1^{b}
1	CT	3.23±0.8^{a}	3.64±0.8^{a}	2.5±0.4 ^{a}	3.23±0.2^{a}	2.25±0.4 ^{a}
	DT	4.25±1.1^{a}	5.85±1.1^{c}	4.9±0.3 ^{b}	5.54±0.4^{b}	4.68 ±0.3 ^{b}
25	CT	2.55±0.4^{b}	3.55±0.4^{a}	3.1±0.6 ^{a}	3.95±0.6^{a}	1.43±0.4 ^{a}
	DT	3.74±0.6 ^{a}	4.04±0.6 ^{a}	4.1±0.4^{ab}	4.25±0.9^{a}	3.73±0.2 ^{b}
50	CT	2.61±0.5 ^{b}	3.22±0.5^{a}	2.9±0.1 ^{a}	2.61±0.2^{c}	1.89±0.4 ^{a}
	DT	3.45±0.9 ^{b}	3.36±0.9 ^{a}	3.0±0.2 ^{a}	4.15±0.8^{a}	2.42±0.5 ^{a}
100	CT	2.43±0.5 ^{b}	2.67±0.5 ^{b}	3.6±0.4 ^{a}	2.62±0.5^{c}	1.95±0.4 ^{a}
	DT	2.56±0.5^{ab}	2.96±0.5^{a}	2.8±0.2 ^{a}	3.13±0.5^{a}	1.70±0.1 ^{a}
200	CT	1.98±0.5^{b}	2.46±0.5^{b}	2.5±0.4 ^{a}	2.27±0.7^{c}	2.12±0.4 ^{a}
	DT	2.06±0.5^{b}	2.86±0.5^{ab}	2.6±0.4 ^{a}	2.75±0.3^{c}	1.93±0.4 ^{a}

Table 5. Effects *in vitro* of resveratrol, caffeic acid, gallic acid, quercetin and rutin on platelet aggregation using 10.0 μM ADP as agonist in STZ-induced diabetic rats. The results are expressed as percentage of aggregation (mean±S.D). Groups with different letters are statistically different ($^{a,b,\ c,\ d}$ *P<0.05*, n=5). ANOVA-Duncan's Test.

Concentrations of plenols (μM)	**Samples**	**Resveratrol**	**Caffeic acid**	**Gallic acid**	**Quercetin**	**Rutin**
0	**CT**	42.3±6.3^{a}	42.3±6.3^{a}	42.3±6.3^{a}	42.3±6.3^{a}	42.3±6.3^{a}
	DT	75.1±8.4^{b}	75.1±8.4^{b}	75.4±8.4^{b}	75.4±8.4^{b}	75.4±8.4^{b}
25	**CT**	38.6±2.6^{a}	44.7±6.2^{a}	39.8±6.3^{a}	40.2±8.2^{a}	45.1±9.3^{a}
	DT	55.4±6.9^{c}	62.9±9.5^{bc}	58.3±7.4^{c}	61.6±7.8^{bc}	55.3±6.3^{c}
100	**CT**	28.7±1.9^{d}	39.6±4.3^{a}	31.6±8.1^{d}	36.5±4.5^{ad}	40.7±9.3^{a}
	DT	43.6±4.8^{a}	51.7±8.7^{c}	46.9±7.6^{a}	47.3±6.7^{a}	51.9±6.4^{c}
200	**CT**	24.5±6.4^{d}	31.3±8.7^{d}	28.7±3.5^{d}	32.1±4.9^{ad}	34.5±7.8^{d}
	DT	38.6 ±9.1^{a}	41.4±5.4^{a}	39.4±4.7^{a}	35.8±5.1^{a}	45.6±6.3^{a}

LEGENDS

Fig.1. Effects of moderate consumption of red wine and grape juice on NTPDase activity using ATP (A) and ADP (B) as substrate and on 5'-nucleotidase activity using AMP(C) as substrate in STZ-induced diabetic rats. Bars represent means ± S.D. Groups with different letters are statistically different (P< 0.05; n=10). ANOVA-Duncan's Test.

Fig. 2. Effects of moderate consumption of red wine and grape juice on E-NPP (A) and ADA (B) activities in STZ-induced diabetic rats. Bars represent means ± S.D. Groups with different letters are statistically different (P< 0.05; n=10). ANOVA-Duncan's Test.

Fig. 3. Effects *in vitro* of resveratrol, caffeic acid and gallic acid on ATP, ADP and AMP hydrolysis in platelets of diabetic and control rats. Bars represent means ± S.D. Groups with different letters are statistically different (P< 0.05; n=5). ANOVA-Duncan's Test.

Fig. 4. Effects *in vitro* of quercetin and rutin on ATP, ADP and AMP hydrolysis in platelets of diabetic and control rats. Bars represent means ± S.D. Groups with different letters are statistically different (P< 0.05; n=5). ANOVA-Duncan's Test.

Fig. 5. Effects *in vitro* of combination of resveratrol, caffeic acid, quercetin and rutin on ATP, ADP and AMP hydrolysis in platelets of diabetic and control rats. Final concentration of 20 μM was resulting of combinations of forth phenols in the concentration of 5 μM each. Final concentration of 100 μM was resulting of combinations of forth phenols in the concentration of 25 μM each. Final concentration of 200 μM was resulting of combinations of forth compounds in the concentration of 50 μM each. Bars represent means ± S.D. Groups with different letters are statistically different (P< 0.05; n=5). ANOVA-Duncan's Test.

FIGURES

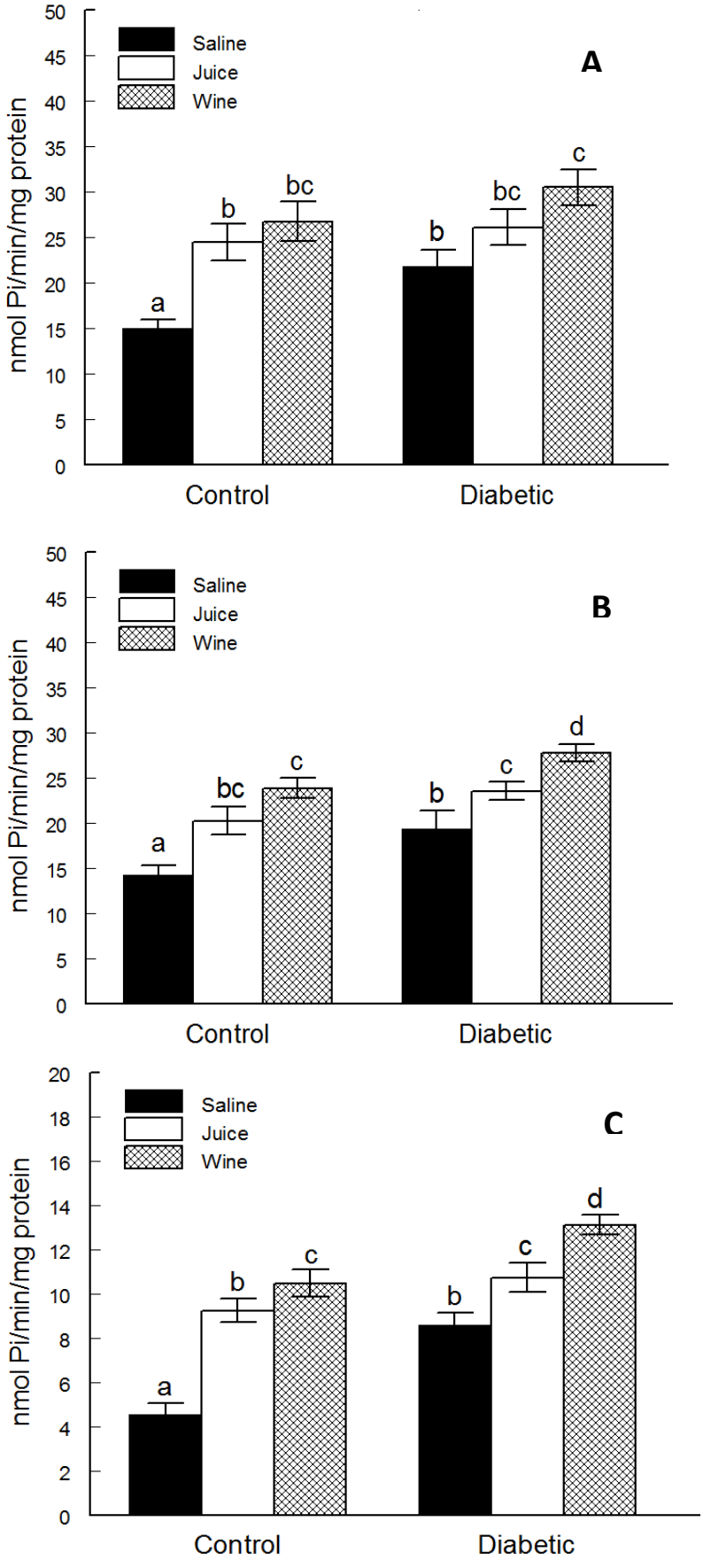

Fig. 1.

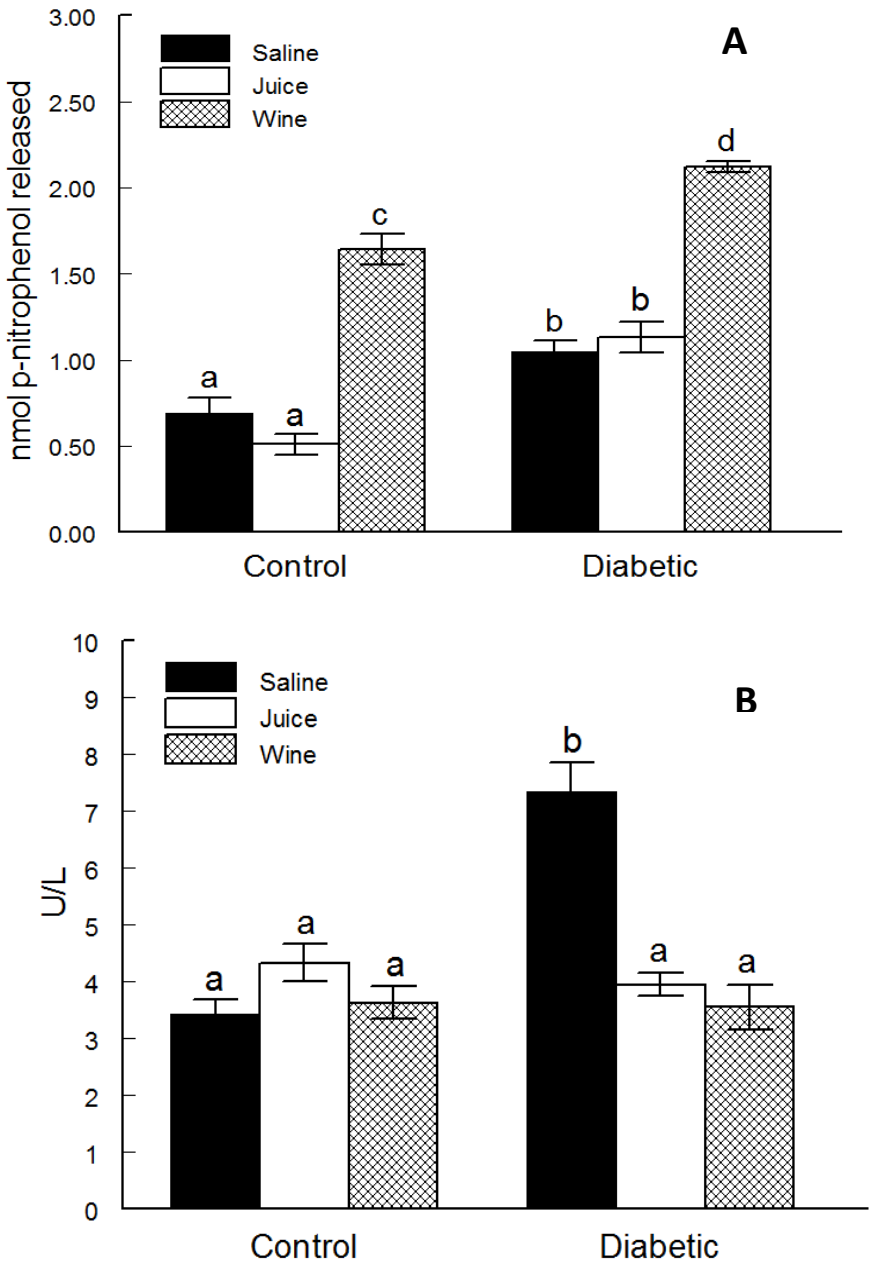
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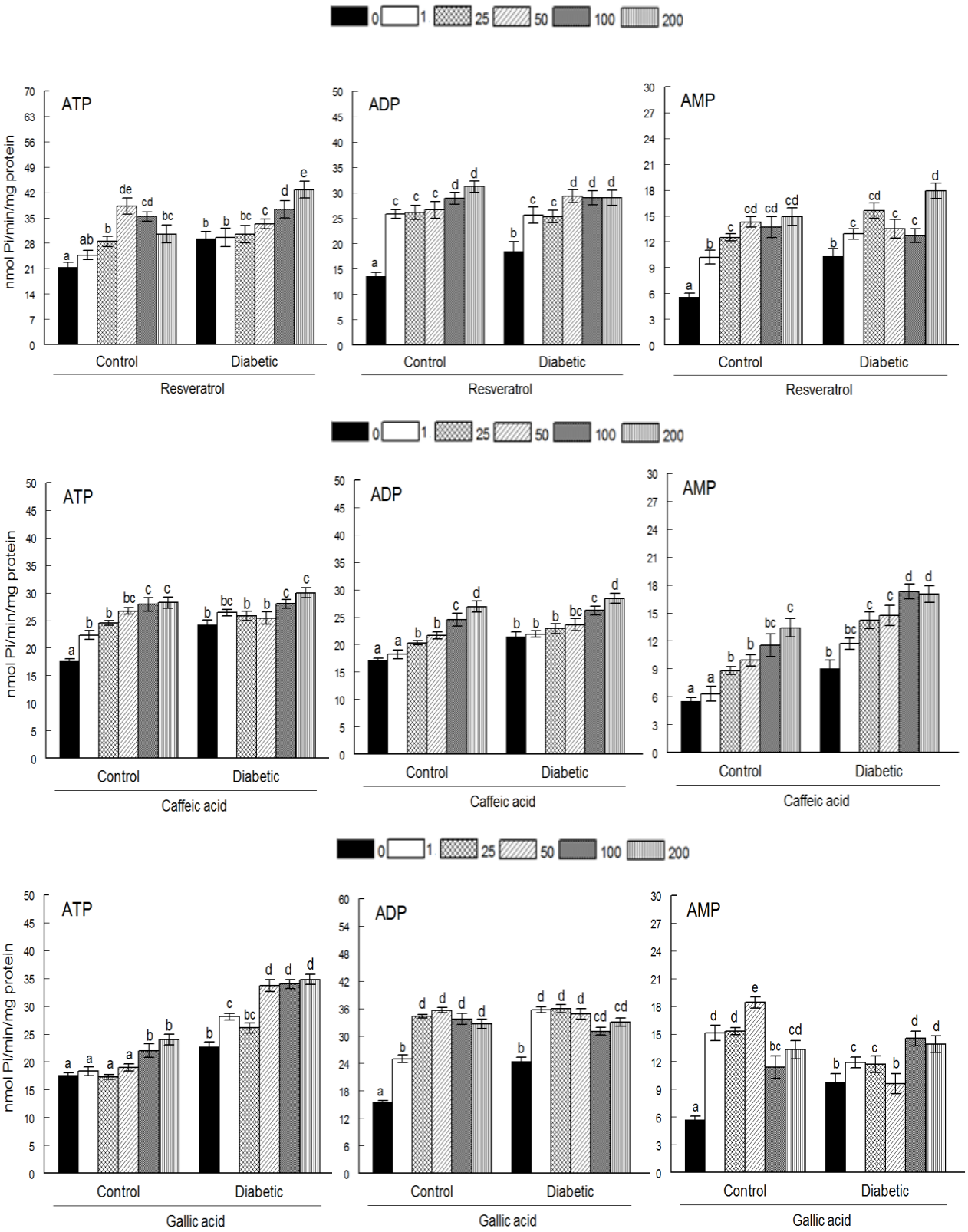
Fig. 2.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Diabetic
Resveratrol
Caffeic acid
Gallic acid

Fig. 3.

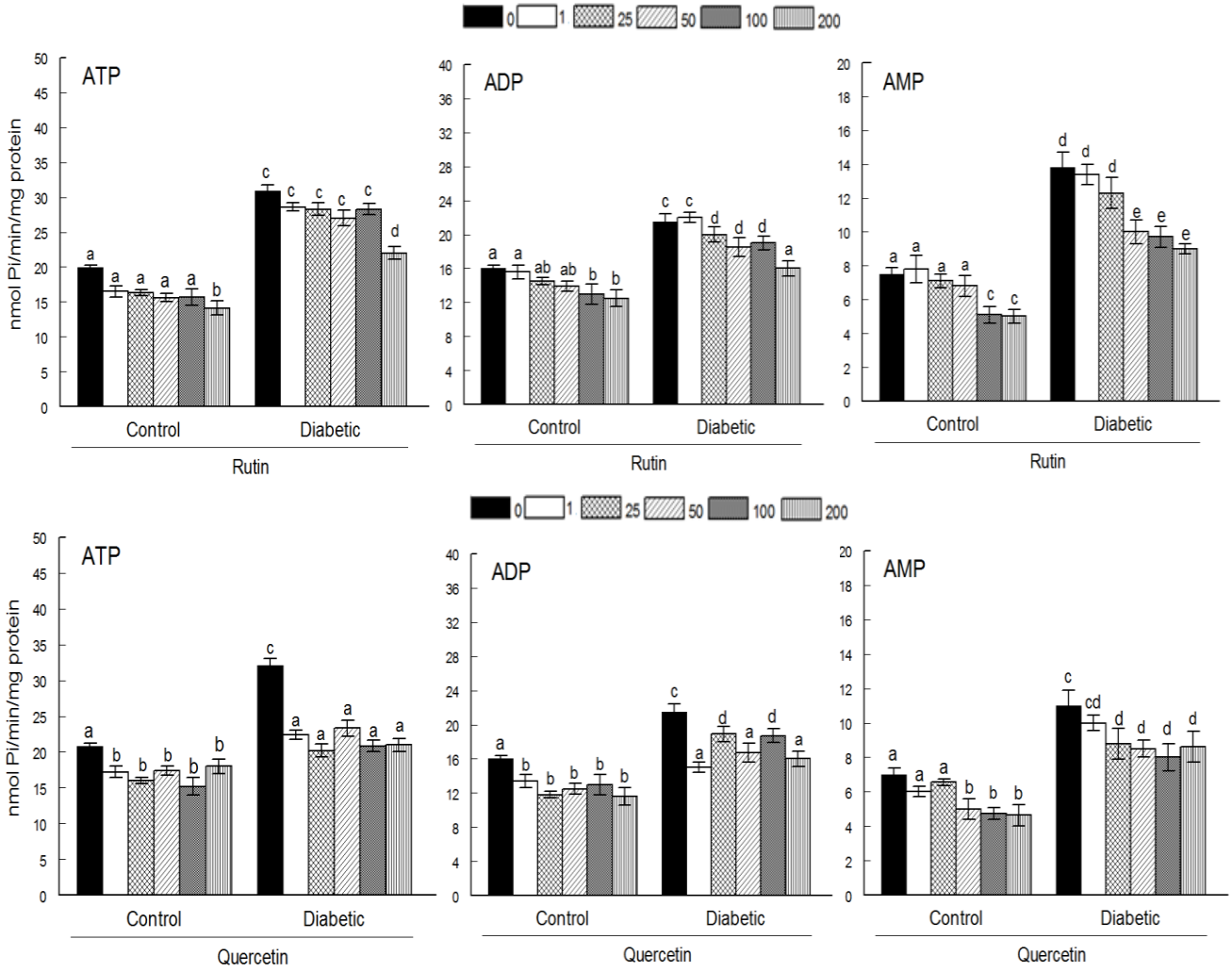
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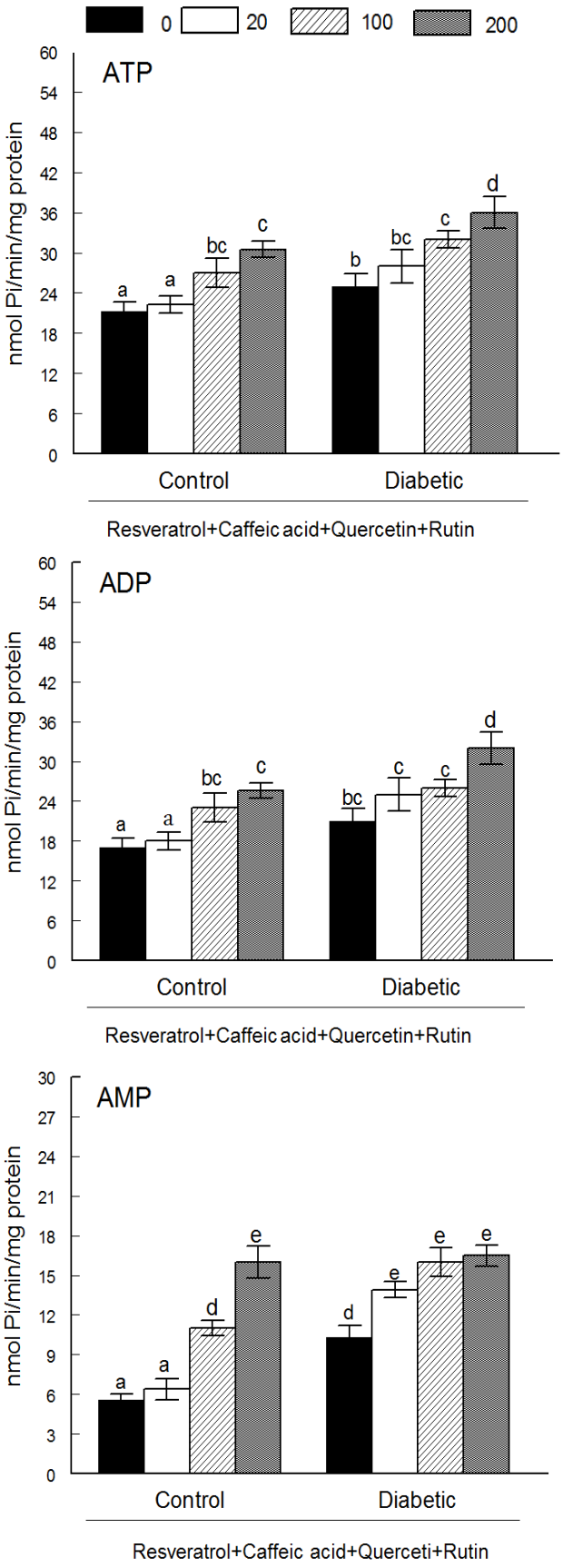
Fig. 4.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Diabetic
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ADP
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Control
Diabetic
Resveratrol+Caffeic acid+Quercetin+Rutin
AMP
nmol Pi/min/mg protein
Control
Diabetic
Resveratrol+Caffeic acid+Querceti+Rutin

Fig. 5.

4. DISCUSSÃO

Atualmente, a procura por compostos naturais ou sintéticos efetivos no tratamento do DM tem sido intensa. Compostos com propriedades antioxidantes e hipoglicêmicas têm sido usados com relativo sucesso no controle da hiperglicemia e do estresse oxidativo no diabetes. Dentre os vários compostos testados os polifenóis da uva, em especial o resveratrol, tem demonstrado inúmeras propriedades biológicas, destacando-se a atividade antioxidante (KUMAR et al. 2007), antiinflamatória (ZHU et al., 2008), cardioprotetora (GRESELE et al. 2007) e neuroprotetora (KING et al., 2006). Desta forma, considerando os efeitos promissores deste composto na prevenção e no tratamento de complicações diabéticas, em um primeiro momento neste estudo foi investigado os efeitos do tratamento com resveratrol (10 e 20 mg/kg) sobre os biomarcadores de estresse oxidativo em fígado e rins de ratos diabéticos induzidos por STZ (Artigo 1).

Os resultados encontrados neste estudo indicam um aumento significativo na peroxidação lipídica em fígado e rins de ratos diabéticos. Estes resultados estão de acordo com vários outros que relatam um aumento nos níveis de TBARS em rins, fígado, soro e eritrócitos de animais com diabetes experimental (YILMAZ et al., 2004; LIKIDLILID et al., 2010) . Neste contexto, pode-se sugerir que a exposição a altos níveis de glicose em animais diabéticos pode elevar a geração de EROs através de mecanismos como a glicação não-enzimática de proteínas e auto-oxidação da glicose. Como conseqüência, as EROs geradas em excesso interagem com os ácidos graxos poliinsaturados da membrana provocando danos na integridade estrutural e funcional dos tecidos hepático e renal, como evidenciado pelo aumento na deterioração oxidativa dos lipídeos da membrana celular no estado diabético.

Além disso, o aumento da peroxidação lipídica em fígado e rins, encontrado neste estudo, pode ser devido ao aumento do estresse oxidativo resultante do decréscimo nos sistemas de defesa antioxidante celular (SANTINI et al., 1997). As enzimas antioxidantes SOD, CAT e GSH-Px formam a primeira linha de defesa contra EROs no organismo. A diminuição na atividade destas enzimas pode levar a um acúmulo do ânion $O_2^{\bullet -}$ e consequentemente do H_2O_2, que através da reação de

Fenton pode gerar radicais $OH^{\bullet}$ altamente reativos, resultando na iniciação e propagação da peroxidação lipídica celular (MARITIM et al., 2003; PALSAMY et al., 2010). Neste contexto, no presente estudo foi observada uma diminuição na atividade da SOD e da CAT em fígado e rins de ratos diabéticos associada a um aumento concomitante da peroxidação lipídica nestes tecidos. A redução na atividade da SOD e CAT observada neste estudo pode ser devido à inativação causada pelo acúmulo de radicais livres e/ ou pela glicação não-enzimática destas proteínas, a qual tem sido amplamente relatada no estado diabético (YAMAGISHI et al., 2005).

Por outro lado, o tratamento com resveratrol preveniu o aumento dos níveis de TBARS e o decréscimo na atividade da SOD e da CAT em fígado e rins de ratos diabéticos, confirmando o papel deste polifenol na inativação de radicais livres e na defesa antioxidante. Estes resultados são consistentes com a redução do estresse oxidativo encontrada em outros estudos, onde o tratamento com resveratrol preservou a atividade de enzimas antioxidantes e impediu o aumento de peróxidos lipídicos nos tecidos e células sanguíneas de animais diabéticos (PANDEY & RIZVI, 2009; PALSAMY & SUBRAMANIAN, 2011). Além disso, dados da literatura tem demonstrado que o resveratrol é capaz de regular a expressão do mRNA das enzimas antioxidantes (LEONARD et al., 2003; CAO & LI, 2004). Com base nestes resultados, pode-se sugerir que o resveratrol é capaz de modular a atividade da SOD e da CAT, o que pode ser muito importante, pois aumenta a capacidade de remoção de EROs no fígado e nos rins proporcionando maior proteção contra o dano oxidativo induzido pelo diabetes nestes tecidos. É importante notar, que a reativação da SOD promovida pelo resveratrol pode acelerar a dismutação do $O_2^{\bullet-}$ para H_2O_2, o qual é rapidamente removido pela CAT protegendo o fígado e os rins de ratos diabéticos contra os radicais hidroxila e, conseqüentemente, impedindo a peroxidação lipídica destes tecidos (LEE et al., 2011).

Juntamente com a diminuição na atividade das enzimas antioxidantes, neste estudo também se observou um decréscimo nos níveis de tióis não-protéicos (NPSH) e de vitamina C no fígado e rim de ratos diabéticos, corroborando com outros estudos que relatam uma redução nas defesas antioxidantes não-enzimáticas no diabetes (ARULSELVAN & SUBRAMANIAN, 2007; KADE et al., 2009). A diminuição nos níveis de NPSH e vitamina C em fígado e rim de ratos diabéticos pode ser atribuída a um aumento da utilização desses antioxidantes na remoção de

EROs (SHARMA et al., 2000). Além disso, a diminuição nos níveis de vitamina C pode estar associada à diminuição nos níveis de GSH, já que este tripeptídeo é necessário para regenerar o ácido ascórbico (LI et al., 2001). No entanto, o tratamento com resveratrol restaurou os níveis de NPSH e de vitamina C em ratos diabéticos. Similarmente, em um estudo realizado por PALSAMY et al. (2010) o resveratrol preveniu a diminuição nos níveis de GSH no fígado e no pâncreas de ratos diabéticos. Estudos têm demonstrado que os polifenóis podem aumentar a atividade da enzima γ-glutamilcisteínil sintetase responsável pela síntese da GSH e simultaneamente aumentar o nível intracelular deste tripeptídeo (RODRIGO et al., 2011). Neste contexto, pode-se sugerir que a prevenção do decréscimo no conteúdo de NPSH no fígado e rim de ratos diabéticos encontrado neste estudo pode ser em parte responsável pela diminuição na formação de EROS e consequentemente pela redução do estresse oxidativo observado em animais tratados com resveratrol.

Por outro lado, a enzima δ-ALA-D é altamente sensível à presença de uma variedade de elementos pró-oxidantes, capazes de oxidar os grupos -SH desta enzima e inibir a sua atividade (NOGUEIRA et al., 2003; BRITO et al., 2007). A inibição da δ-ALA-D pode prejudicar a biossíntese de heme e resultar no acúmulo do ácido aminolevulínico que, sob condições fisiológicas, pode ter efeitos pró-oxidantes que contribuem para o estresse oxidativo (BECHARA, 1996). No presente estudo, a atividade da δ-ALA-D foi inibida nos tecidos hepático e renal de ratos diabéticos. Estes resultados estão de acordo com dados encontrados tanto em humanos quanto no diabetes experimental, onde a inibição da δ-ALA-D é descrita e tem sido relacionada principalmente aos altos níveis de glicose e a produção excessiva de EROs (SOUZA et al., 2007, KADE et al., 2009). Assim, pode-se sugerir que a inibição da δ-ALA-D em fígado e rins de ratos diabéticos pode ser uma consequência tanto da glicação do resíduo de lisina no sítio ativo da δ-ALA-D, quanto da oxidação dos resíduos de cisteína da enzima pelas EROs (CABALLERO et al., 1998; FOLMER et al., 2003). Além disso, a diminuição da atividade da δ-ALA-D encontrada neste estudo pode estar associada à redução das defesas antioxidantes nos tecidos hepático e renal de ratos diabéticos, em especial no conteúdo de NPSH, o qual é responsável pela prevenção da oxidação dos grupos-SH essenciais para a atividade dessa enzima.

O tratamento com resveratrol preveniu a inibição da δ-ALA-D em fígado e rim de ratos diabéticos, sugerindo que este composto pode impedir a oxidação de

grupos -SH localizados no sítio ativo da δ-ALA-D e, conseqüentemente, a sua inibição. Pode-se sugerir que a prevenção do decréscimo no conteúdo de NPSH, bem como a diminuição do estresse oxidativo em ratos diabéticos tratados com resveratrol pode estar associado à prevenção no decréscimo da atividade da δ-ALA-D. Além disso, estes resultados fortalecem a hipótese que a inibição da δ-ALA-D não está somente relacionada ao desenvolvimento de hiperglicemia, destacando a importância de antioxidantes como o resveratrol para minimizar os efeitos deletérios do diabetes na atividade desta importante enzima.

Neste estudo também foi determinado à atividade das aminotransferases séricas, as quais têm sido consideradas indicadores sensíveis ao dano hepático (MOLANDER et al., 1955). Os danos nos hepatócitos alteram a função de transporte e permeabilidade da membrana, levando ao vazamento de enzimas das células (ELIZABETH & HARRIS, 2005). Portanto, a liberação acentuada da AST e ALT do citosol dos hepatócitos para a circulação e o aumento da γ-GT, uma enzima de membrana, indicam danos severos às membranas do tecido hepático durante o diabetes. Assim, o aumento das atividades da AST, ALT e γ-GT encontrada neste estudo pode ser interpretado como resultado da destruição das células do fígado ou alterações na permeabilidade da membrana, indicando que houve um dano hepatocelular grave nos ratos diabéticos. Contudo, a administração do resveratrol preveniu o aumento da atividade dessas enzimas, demonstrando o efeito protetor deste polifenol contra danos hepáticos induzidos pelo estado diabético. Estes resultados estão de acordo com os resultados encontrados em estudos com resveratrol e também com outros antioxidantes como a rutina, o ácido caféico e a quercetina (JANBAZ et al., 2004; PALSAMY et al., 2010; FERNANDEZ et al., 2010). Pode-se atribuir estes efeitos protetores do resveratrol à capacidade deste polifenol para conservar a integridade da membrana das organelas celulares no estado diabético. De fato, neste estudo o resveratrol foi capaz de impedir a peroxidação lipídica no tecido hepático.

Além dos danos hepáticos, no presente estudo, ratos diabéticos também apresentaram danos renais que foram evidenciados pela elevação da uréia e creatinina sérica, que são consideradas marcadores importantes da disfunção renal (SHARMA et al., 2006). O resveratrol impediu o aumento nos níveis de uréia e creatinina em ratos diabéticos. Estes resultados sugerem que o resveratrol possui um potencial para atenuar a lesão renal causada pelo estado hiperglicêmico e isso

pode estar associado diretamente com a capacidade antioxidante deste polifenol, principalmente protegendo os rins contra danos oxidativos, como evidenciado neste e em outros estudos (HASSAN-KHABBAR et al., 2008).

Assim, os resultados deste primeiro estudo demonstraram que o tratamento com resveratrol pode fornecer proteção eficaz contra o dano oxidativo em fígado e rins de ratos diabéticos, uma vez que este composto foi capaz de melhorar o sistema de defesa antioxidante enzimático e não-enzimático e prevenir a peroxidação lipídica nestes tecidos. Estes resultados podem ser atribuídos tanto a remoção direta de EROs pelo resveratrol quanto a uma possível modulação das enzimas antioxidantes, exercida por este composto. Além disso, estes resultados podem contribuir para uma melhor compreensão do papel hepatoprotetor e renoprotetor do resveratrol, enfatizando a influência deste antioxidante e outros polifenóis da dieta para a saúde humana, possivelmente prevenindo complicações hepáticas e renais associadas com o DM.

Além das complicações hepáticas e renais, o diabetes está associado com importantes complicações vasculares, as quais têm sido consideradas como a principal causa de morbidade e mortalidade em pacientes com esta endocrinopatia. Entre os fatores que contribuem para a condição pró-trombótica, presente no estado diabético, a hiperreatividade plaquetária desempenha um papel central (STRATMANN & TSCHOEPE, 2005; MOREL et al., 2011). Neste contexto, vários estudos experimentais e clínicos têm indicado que os polifenóis encontrados na uva, principalmente o resveratrol, e seus derivados como o vinho tinto e o suco são capazes de modular várias vias envolvidas na ativação plaquetária e no conseqüente crescimento de trombos, exercendo, assim, importantes efeitos cardioprotetores (SHANMUGANAYAGAM et al., 2007; GRESELE et al., 2011).

Dessa forma, considerando que as plaquetas possuem uma cascata de ecto-enzimas responsável pela hidrólise de nucleotídeos de adenina, a qual desempenha um importante papel nos mecanismos de tromboregulação e alterações na sua atividade tem sido observada em várias patologias incluindo o diabetes testou-se, em um segundo momento, os efeitos do tratamento com resveratrol nestas enzimas em plaquetas de ratos diabéticos (Artigo 2). Tendo em vista os resultados promissores obtidos com o tratamento com resveratrol em um terceiro momento testou-se os efeitos da suplementação com vinho tinto e suco de uva sobre estas enzimas, bem como a agregação plaquetária em ratos diabéticos (Manuscrito 1).

Os resultados obtidos tanto no artigo 2 quanto no manuscrito 1 demonstraram um aumento na atividade das enzimas NTPDase, E-NPP e 5'-nucleotidase em plaquetas de ratos diabéticos. Estes resultados estão de acordo com estudos prévios do nosso grupo de pesquisa que também encontraram uma aumento na atividade da NTPDase e da 5'-nucleotidase em plaquetas de ratos diabéticos experimentalmente induzidos com aloxano, bem como em pacientes com diabetes tipo 2, demonstrando o importante papel destas enzimas no estado hiperglicêmico (LUNKES et al., 2003, 2004). Lunkes et al. (2008), encontraram um aumento na atividade da NTPDase e 5'-nucleotidase diretamente proporcional ao aumento nas concentrações de glicose e frutose *in vitro*, indicando a participação de altos níveis de glicose como um provável fator de interferência na atividade das ectonucleotidases.

É importante ressaltar que a regulação da concentração de ADP e de adenosina extracelular é de grande importância devido aos efeitos fisiológicos destas moléculas no sistema vascular (BURNSTOCK et al., 2002). O ADP é conhecido por induzir mudanças na forma e na agregação das plaquetas (ROZALSKI et al., 2005), enquanto a adenosina possui uma variedade de efeitos cardioprotetores, como vasodilatação e inibição da agregação plaquetária (BOROWIEC et al., 2006). Neste contexto e considerando estes resultados, o aumento na atividade das ectonucleotidases em plaquetas de ratos diabéticos pode ser relatado como uma resposta orgânica compensatória do organismo a fim de evitar processos trombóticos pela depleção do ADP e aumento na produção de adenosina.

Por outro lado, é importante ressaltar que, apesar do aumento na hidrólise do ATP, ADP e AMP contribuindo para o aumento da produção de adenosina, encontrou-se uma elevação na atividade da ADA em plaquetas de ratos diabéticos. A ADA é uma importante enzima que degrada a adenosina para inosina, regulando estritamente as concentrações extracelulares deste nucleosídeo (FRANCO et al., 1997). Portanto, um aumento na atividade desta enzima pode levar à rápida desaminação da adenosina, causando uma redução nos níveis deste nucleosídeo na circulação. Conseqüentemente, esta situação pode produzir um cenário favorável para o desenvolvimento de doenças vasculares no estado diabético, já que a adenosina tem um papel importante na prevenção de processos trombóticos (BOROWIEC et al., 2006).

Neste estudo, o tratamento com resveratrol (10 e 20 mg/kg) aumentou a atividade da NTPDase, E-NPP e 5’-nucleotidase em plaquetas de ratos diabéticos (Artigo 2). Estes resultados demonstram que a modulação destas enzimas causadas pelo resveratrol tem um efeito benéfico no estado diabético, já que o aumento na hidrólise do ADP protege as plaquetas da agregação excessiva, contribuindo para o controle da hemostase e prevenindo condições pró-trombóticas no diabetes. Outro aspecto a ser discutido é que a atividade da ADA em ratos diabéticos tratados com resveratrol foi igual à encontrada no grupo controle. Assim, pode-se sugerir que o tratamento com resveratrol é capaz de manter um alto nível de adenosina no ambiente extracelular, o qual promove vasodilatação e tem um importante papel protetor sobre condições patofisiológicas causadas pelo estado hiperglicêmico.

Similarmente aos resultados obtidos no tratamento com resveratrol, quando ratos diabéticos receberam suco de uva e vinho tinto também foi observado um aumento atividade da NTPDase, E-NPP e 5'-nucleotidase em plaquetas, indicando que o consumo de ambas as bebidas derivados da uva interfere com a sinalização purinérgica (Manuscrito 1). Este aumento na atividade das ectonucleotidases reflete uma maior degradação do ATP, ADP e AMP, resultando em um aumento na formação da adenosina. Neste sentido, pode-se sugerir que o consumo moderado de vinho tinto e suco de uva pode ter um efeito antiagregante, limitando a biodisponibilidade do ADP, o principal agonista da agregação plaquetária e promovendo a produção da adenosina, um agente vasodilatador e antiagregante, contribuindo para o controle da hemostasia no estado diabético.

Em relação a estes resultados, é importante ressaltar que, embora tanto o suco de uva quanto o vinho tinto tenham aumentado a hidrólise do ATP, ADP e AMP em ratos diabéticos, observou-se um aumento significativamente maior na hidrólise de nucleotídeos em ratos diabéticos tratados com vinho tinto, quando comparado com aqueles tratados com suco de uva. É importante notar que os efeitos mais pronunciados observados em ratos que receberam vinho tinto podem estar associados com a presença do etanol. Dias et al. (2007) encontraram, em seu estudo, que doses mais baixas de etanol, similar aquelas encontradas no vinho tinto, aumentaram a atividade da NTPDase e 5'-nucleotidase em plaquetas, indicando um efeito estimulante do etanol na hidrólise dos nucleotídeos de adenina. Além disso, estes resultados estão de acordo com aqueles obtidos para o tratamento com resveratrol, que também provocou um aumento a atividade das ectonucleotidases

em plaquetas de ratos diabéticos. Assim, pode-se sugerir que o etanol e o resveratrol podem agir sinergicamente (RENAUD & LORGERIL, 1992; DI CASTELNUOVO et al., 2002) na modulação da via de ectonucleotidases, potencializando os efeitos do vinho tinto sobre a hidrólise dos nucleotídeos de adenina, enquanto que no suco de uva a ativação pode estar relacionada somente aos efeitos do resveratrol.

Outro aspecto importante a ser discutido é que o tratamento com vinho tinto e suco de uva preveniu o aumento da atividade da ADA em plaquetas de ratos diabéticos. Baseado nestes resultados pode-se sugerir que o suco de uva e o vinho tinto preservam os níveis de adenosina na circulação, os quais agem sobre receptores de adenosina das plaquetas podendo inibir a agregação plaquetária e promover a vasodilatação, prevenindo complicações vasculares causadas pelo estado hiperglicêmico (KOCH et al., 1992; MELZIG,1996). De fato, estudos têm mostrado que compostos polifenólicos presentes no vinho tinto e suco de uva podem inibir o processo de formação de trombos (COOPER et al., 2004; BERTELLI, 2007; DOHADWALA & VITA, 2009). Assim, estes resultados apóiam a hipótese de que uma das maneiras pela qual o suco de uva e o vinho tinto exercem ação cardioprotetora pode ser através do aumento nos níveis de adenosina, já que ambas as bebidas derivadas da uva têm demonstrado inibir a atividade da ADA.

Um dado relevante deste estudo é que a agregação plaquetária induzida pelo ADP foi significativamente maior em ratos diabéticos. Corroborando com esses resultados, diversos estudos têm relatado que as plaquetas de pacientes e animais diabéticos mostram uma hipersensibilidade para agentes pró-agregantes fisiológicos como ADP, colágeno e tromboxano (MATSUNO et al, 2005; SCHÄFER & BAUERSACHS, 2008; HAOUARI & ROSADO, 2008). Contudo, a suplementação com vinho tinto e suco de uva preveniu o aumento na agregação plaquetária em ratos diabéticos. Estes resultados são consistentes com vários estudos que demonstraram que tanto o vinho tinto quanto o suco de uva podem reduzir a agregação plaquetária, através da redução da concentração plasmática de tromboxano, ADP e trombina disponíveis para a agregação plaquetária (PACE-ASCIAK et al., 1996; FREEDMAN et al., 2001; GRESELE et al., 2008). Neste contexto, é importante ressaltar que o aumento na hidrólise do ADP pelo suco de uva e pelo vinho tinto, associados com a prevenção do aumento da atividade da

ADA pode ser um mecanismo potencial pelo qual estas bebidas inibem a agregação plaquetária induzida pelo ADP em ratos diabéticos.

Por outro lado, há evidências de que a hiperglicemia contribui para uma maior reatividade e agregabilidade plaquetária, particularmente através da geração de EROs e pela glicação de proteínas da membrana das plaquetas (FREEDMAN et al,. 2008; HASEGAWA et al., 2008). Neste estudo, observou-se um aumento nos níveis de hemoglobina glicada, que pode ser um indicativo de que as proteínas das plaquetas também estão sofrendo glicosilação não-enzimática. Interessantemente, o tratamento com vinho tinto e suco de uva preveniu o aumento nos níveis de hemoglobina glicosilada em ratos diabéticos, sugerindo que o consumo moderado dessas bebidas pode impedir a glicação não-enzimática das proteínas das plaquetas e, consequentemente, danos funcionais e estruturais que contribuem para hiperagregabilidade plaquetária e formação de trombos no estado diabético (MONTILLA et al., 2004; WU & YEN, 2005).

Além disso, vários estudos têm indicado que o estresse oxidativo que acompanha o diabetes pode induzir disfunção endotelial e aumentar a reatividade plaquetária através de efeitos diretos sobre as plaquetas, como o aumento da peroxidação lipídica e a depleção dos sistemas de defesa antioxidante (SCHÄFER & BAUERSACHS, 2008; FREEDMAN et al,. 2008). No entanto, os polifenóis presentes no vinho tinto e no suco de uva são potentes antioxidantes, protegendo muitos tecidos e células, incluindo as plaquetas, dos danos causados pelo estresse oxidativo (OLAS & WACHOWICZ, 2005; RODRIGO et al., 2011). Com base nesses resultados pode-se sugerir que as fortes propriedades antioxidantes do vinho tinto e do suco de uva podem contribuir para a prevenção do aumento da agregação plaquetária encontrada em ratos diabéticos tratados com ambas as bebidas derivadas de uva.

Os resultados deste estudo estão de acordo com vários trabalhos demonstrando os efeitos protetores do moderado consumo do vinho tinto e do suco de uva na redução de fatores de risco associados com várias doenças degenerativas, incluindo o diabetes e doenças cardiovasculares (LEIFERT & ABEYWARDENA, 2008; SZKUDELSKA & SZKUDELSKI, 2010; GRESELE et al., 2011; FOTI et al., 2011). Inicialmente, os efeitos cardioprotetores de vinho tinto foram atribuídos apenas ao etanol, que em baixas concentrações tem efeitos antiagregantes (GRONBAEK et al. 2000). No entanto, estudos indicam que o vinho

apresenta benefícios superiores aos observados em outras bebidas alcoólicas, indicando que fatores não-alcoólicos do vinho podem também desempenhar um papel protetor (KLATSKY et al., 2003) Fortalecendo esta hipótese o suco de uva também apresentou propriedades inibidoras da agregação plaquetária, o que sugere que os polifenóis da uva, e não somente o etanol, podem contribuir para os efeitos antitrombóticos do vinho.

No entanto, neste estudo os efeitos do vinho tinto sobre a agregação plaquetária e atividade das ectonucleotidases foram mais pronunciados quando comparados para os efeitos do suco de uva. Notadamente, tem sido reportado que o álcool aumenta a absorção intestinal e, conseqüentemente, a biodisponibilidade dos polifenóis do vinho tinto, além de evitar a precipitação destes compostos no trato digestivo (SERAFINI et al., 1997; DRAGONI et al., 2006). Neste contexto, pode-se sugerir que a potencialização dos efeitos antitrombóticos do vinho tinto encontrada neste estudo pode ser conseqüência tanto do sinergismo entre os vários compostos polifenólicos do vinho com o etanol e/ou a maior absorção e biodisponibilidade dos polifenóis em ratos diabéticos que receberam vinho tinto em comparação com o suco de uva.

Reforçando essa linha de raciocínio, o próximo conjunto de experimentos foi realizada a fim de verificar os efeitos dos principais polifenóis do vinho tinto e do suco de uva, como o resveratrol, ácido caféico, ácido gálico, quercetina, rutina sobre a atividade da NTPDase, 5 - nucleotidase e da ADA e agregação plaquetária em de ratos diabéticos sob condições *in vitro*.

Quando testados *in vitro,* o resveratrol, ácido gálico e o ácido caféico aumentaram a hidrólise do ATP, ADP e AMP, enquanto a quercetina e a rutina decresceram a hidrólise destes nucleotídeos em plaquetas de ratos diabéticos. A partir desses resultados, pode-se considerar que estes polifenóis atuam através de mecanismos diferentes sobre as ectonucleotidases. Como mencionado acima, neste estudo o tratamento *ex vivo* com vinho tinto e suco de uva aumentou a atividade da NTPDase e 5'-nucleotidase em ratos diabéticos. Assim, embora todos os polifenóis testados estejam presentes em ambas as bebidas da uva, os efeitos de ativação do resveratrol, ácido gálico e ácido caféico sobre as ectonucleotidases parecem prevalecer sobre os efeitos inibitórios da quercetina e rutina. De fato, quando os efeitos *in vitro* da combinação do resveratrol, ácido caféico, quercetina e rutina foram testados observou-se um aumento na hidrólise do ATP, ADP e AMP, confirmando

que os efeitos do resveratrol e ácido cafeíco são predominantes sobre a atividade das ectonucleotidases.

De maneira muito interessante, estudos têm demonstrado que o ácido cafeíco, a catequina e o resveratrol atuam de forma sinérgica, o que garante a atividade biológica destes compostos, como a inibição do estresse oxidativo e da agregação plaquetária, apesar das baixas concentrações plasmáticas destes polifenóis após o consumo moderado de vinho tinto (PIGNATELLI et al., 2006). Além disso, estudos indicam que a quercetina interfere com a sulfatação e glicuronidação do resveratrol no fígado, aumentando sua biodisponibilidade (DE SANTI et al., 2000) Baseado nestes resultados é possível sugerir que o resveratrol e o ácido caféico possam estar agindo sinergicamente no aumento da hidrólise dos nucleotídeos, enquanto que a quercetina, apesar dos efeitos opostos sobre as ectonucleotidases, pode contribuir indiretamente para os efeitos do resveratrol, através do aumento da biodisponibilidade deste composto.

Por outro lado, quando testados *in vitro,* o resveratrol, o ácido gálico, o ácido caféico, a quercetina e a rutina inibiram a atividade da ADA em plaquetas de ratos diabéticos. Estes resultados são consistentes com os encontrados no tratamento *ex vivo* com suco de uva e vinho tinto em plaquetas de ratos diabéticos, sugerindo que estes compostos podem atuar de forma combinada, a fim de aumentar os níveis de adenosina na circulação, desempenhando um importante papel cardioprotetor no estado diabético.

Além disso, todos os compostos polifenólicos da uva testados *in vitro* neste estudo diminuíram a agregação plaquetária induzida pelo ADP em ratos diabéticos, sugerindo que os efeitos sinérgicos destes polifenóis podem contribuir para a diminuição da agregação plaquetária em ratos diabéticos tratados com vinho tinto e suco de uva. Estes resultados estão de acordo com vários estudos que demonstram que o resveratrol, a quercetina, o ácido caféico, o ácido gálico e a rutina têm efeitos inibitórios sobre a agregação plaquetária no estado diabético (OLAS et al., 2002; GUERRERO et al., 2007; GRESELE et al., 2011). Entre os vários mecanismos propostos, é possível inferir, com base nos resultados encontrados neste estudo, que o resveratrol, o ácido gálico e o ácido caféico podem reduzir a agregação plaquetária, através do aumento da atividade da NTPDase e da 5'-nucleotidase, reduzindo os níveis de ADP e aumentando os níveis de adenosina. Contudo, a rutina e a quercetina devem reduzir a agregação plaquetária através de um mecanismo

diferente da via das ectonucleotidases, uma vez que estes flavonóides inibiram a hidrólise do ADP, o principal agonista da agregação plaquetária. Wright et al. (2009) demonstraram que as plaquetas podem participar do metabolismo dos flavonóides. Em especial, a quercetina e seu metabólito plasmático 4'-0-metil quercetina, são internalizados pelas plaquetas e metabolizados pela adição de grupo sulfatos e os compostos formados podem inibir a ativação plaquetária por antagonizar receptores de superfície como os receptores de estrógeno e tromboxano A2 (GUERRERO et al., 2007; WRIGHT et al., 2009).

Finalmente, os resultados obtidos neste estudo demonstram que, a modulação da atividade das ecto-enzimas nas plaquetas pode ser um dos mecanismos pelo qual o resveratrol, o vinho tinto e o suco de uva podem prevenir e reduzir anormalidades plaquetárias e, consequentemente, complicações vasculares no estado diabético. Além disso, o tratamento com resveratrol pode fornecer proteção eficaz contra os danos oxidativos no fígado e nos rins de ratos diabéticos, suportando as propriedades antioxidantes atribuídas a este polifenol. Em conjunto, estes resultados são muito importantes do ponto de vista clínico, porque reforçam os diversos benefícios para a saúde, em especial para a prevenção de complicações diabéticas, que podem ser obtidos através da ingestão de dois copos de vinho tinto ou suco de uva por dia, o que pode fornecer cerca de 40% dos polifenóis antioxidantes totais presentes em uma dieta saudável, bem como uma série de polifenóis, altamente benéficos como o resveratrol.

5. CONCLUSÕES

- O decréscimo na atividade da SOD, CAT e dos níveis de vitamina C e NPSH acompanhado pelo aumento nos níveis de TBARS em fígado e rins de ratos diabéticos sustenta a ocorrência do estresse oxidativo nesta patologia. No entanto, o tratamento com resveratrol forneceu proteção eficaz contra os danos oxidativos no fígado e no rim de ratos diabéticos, uma vez que este composto preveniu decréscimo no sistema de defesa antioxidante enzimático e não-enzimático e preveniu a peroxidação lipídica nestes tecidos.

- A atividade da δ-ALA-D hepática e renal foi inibida em ratos diabéticos. O tratamento com resveratrol preveniu esta inibição enfatizando a importância de compostos antioxidantes para minimizar os efeitos deletérios do diabetes na atividade desta importante enzima.

- A atividade das enzimas NTPDase, E-NPP, 5'-nucleotidase e ADA aumentou em ratos diabéticos. Isto indica que a adenosina produzida pela hidrólise dos nucleotídeos de adenina é rapidamente consumida pela ADA não exercendo seus efeitos cardioprotetores. O tratamento com resveratrol, vinho tinto e suco de uva potencializou a ativação da NTPDase, E-NPP, 5'-nucleotidase e preveniu o aumento da atividade da ADA indicando que tanto o resveratrol quanto os produtos derivados da uva são capazes de modular a hidrólise dos nucleotídeos de adenina, o que pode contribuir para o controle dos mecanismos de tromboregulação no diabetes.

- A suplementação com suco de uva e vinho tinto preveniu o aumento da agregação plaquetária em ratos diabéticos sugerindo que estes produtos têm um importante papel na prevenção de processos trombóticos no estado diabético.

- Quando testados *in vitro,* o resveratrol, o ácido gálico e o ácido caféico aumentaram a hidrólise do ATP, ADP e AMP, enquanto a quercetina e a rutina

decresceram a hidrólise destes nucleotídeos em plaquetas de ratos diabéticos, sugerindo que estes polifenóis podem estar atuando por mecanismos diferentes sobre as enzimas, provavelmente devido a sua diferença estrutural.

REFERÊNCIAS

ABE, L.T. et al. Phenolic compounds and antioxidant activity of Vitislabrusca and Vitisvinifera cultivars.**Ciência e Tecnolologia dos Alimentos**, v. 27, n. 2, p. 394-400, 2007.

ABRAHAM, K.A. Acute red wine consumption elevates plasma insulin and decreases plasma glucose in women during an oral glucose tolerance test. **InternationalJournal of Diabetes and Metabolism**, v. 18, p. 95-98, 2010.

AGARWAL, B.; BAUR, J.A. Resveratrol and life extension.**Annals of the New York Academy of Sciences**, v.1215, p.138-43, 2011.

AMERICAN DIABETES ASSOCIATION.Diagnosis and classification of diabetes mellitus.**Diabetes Care**,v. 33, p. 62-69, 2010.

ANEKONDA, T.S. Resveratrol - A boon for treating Alzheimer's disease? **Brain Research Reviews**, v. 52, p. 316-326, 2006.

ANFOSSI, G. et al. Adenosine increases human platelets levels of cGMP through nitric oxide possible role in its antiaggregating effect. **Thrombosis Research**, v. 105, p. 71-78, 2002.

ANGIOLILLO, D. J. et al. Impact of platelet reactivity on cardiovascular outcomes in patients with type 2 diabetes mellitus and coronary artery disease.**Journal of the American College of Cardiology**, v. 50, n. 16, p. 1541-1547, 2007.

ARAUJO, M. et al. Enzymes that hydrolyze adenine nucleotides in platelets from breast cancer patients. **BiochimicaetBiophysicaActa**, v. 1740, p. 421-426, 2005.

ARULSELVAN, P.; SUBRAMANIAN, S.P. Beneficial effects of Murrayakoenigii leaves on antioxidant defense system and ultra structural changes of pancreatic b-cells in experimental diabetes in rats. **Chemico-Biological Interactions**, v. 165, p. 155-164, 2007.

ASENSI, M. et al. Inhibition of cancer growth by resveratrol is related to its low bioavailability. **Free Radical Biology & Medicine**, v. 33, p. 387-398, 2002.

ATES, O. et al. Central nervous system protection by resveratrol in streptozotocin-induced diabetic rats.**Journal Clinical Neuroscience**, v. 14, p. 256-260, 2005.

ATKINSON, B. et al. Ecto-nucleotidases on the CD39/NTPDase family modulate platelet activation and thrombus formation: potential as therapeutic targets. **Blood Cells, Molecules and Diseases**, v.36, p. 217- 222, 2006.

ATKINSON, M. A.; EISENBARTH, G. S. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. **The Lancet**, v. 358, p. 221-229, 2001.

BAGATINI, M. D. et al. Enzymes that hydrolyze adenine nucleotides in patients with ischemic heart disease. **Clinica Chimica Acta**, v. 412, p. 159-64, 2011.

BARREIROS, A.; DAVID, J. Estresse oxidativo: relação entre geração de espécies reativas e defesa do organismo. **Química Nova**, v. 29, p. 113-123, 2006.

BAUR, J.A.; SINCLAIR, D.A. Therapeutic potential of resveratrol: the in vivo evidence. Nature reviews. **Drug discovery**, v. 5, p. 493-506, 2006.

BECHARA, E. J. Oxidative stress in acute intermittent porphyria and lead poisoning may be triggered by 5-aminolevulinic acid. **Brazilian Journal of Medical and Biological Research**, v. 29, p. 841-851, 1996.

BECHARA, E. J. Oxidative stress in acute intermittent porphyria and lead poisoning may be triggered by 5-aminolevulinic acid. **Brazilian Journal of Medical and Biological Research**, v. 29, p. 841-851, 1996.

BECKER L.V. et al. Activities of enzymes that hydrolyze adenine nucleotides in platelets from patients with rheumatoid arthritis.**Clinical Biochemistry**, v. 43, p. 1096-1100, 2010.

BERTELLI, A. A. E. Wine, research and cardiovascular disease: Instructions for use. **Atherosclerosis**, v. 195, p. 242-247, 2007.

BERTELLI. A. A. E. et. al. Wine, research and cardiovascular disease: Instructions for use. **Atherosclerosis**, v.195, p. 242-247, 2007.

BIRK, A. V. et al. Role of extracellular ATP metabolism in regulation of platelet reactivity. **The Journal of Laboratory and Clinical Medicine**, v. 140, p. 166-175, 2002.

BITAR, M.; WEINER, M. Diabetes-induced metabolic alterations in heme synthesis and degradation and various heme-containing enzymes in female rats.**Diabetes**, v. 33, p. 37-44, 1994

BOROWIEC, A. et al. Adenosine as a metabolic regulator of tissue function: production of adenosine by cytoplasmic 5'-nucleotidases. **ActaBiochimicaPolonica**, v. 53, n. 2, 269-278, 2006.

BORRIELLO, V. et al. Dietary polyphenols: Focus on resveratrol, a promising agent in the prevention of cardiovascular diseases and control of glucose homeostasis. **Nutrition, Metabolism & Cardiovascular Diseases**, v. 20, p.618-625, 2010.

BRADAMANTE, S. et al. Resveratrol provides late-phase cardioprotection by means of a nitric oxide and adenosine-mediated mechanism. **European Journal Pharmacology**, v. 465, p.115-123, 2004.

BRAVO, L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. **Nutrition Reviews**, v. 56, p. 317-333, 1998.

BREKSA, A. P. et al. Antioxidant activity and phenolic content of 16 raisin grape (*Vitisvinifera* L.) cultivars and selections.**Food Chemistry**, v. 121, 740-745, 2010.

BRIONES, A. M.; TOUYZ, R. M. Oxidative Stress and Hypertension: Current Concepts. **Current Hypertension Reports**, v. 12, p. 135–142, 2010.

BRITO, V.B. et al. Long-term sucrose and glucose consumption decreases the delta-aminolevulinatedehydratase activity in mice. **Nutrition**, v. 23 p. 818-826, 2007.

BRITO, V.B. et al. Long-term sucrose and glucose consumption decreases the delta-aminolevulinatedehydratase activity in mice. **Nutrition**, v. 23, p. 818-826, 2007.

BRITO; V. B. et al. Long-term sucrose and glucose consumption decreases the delta-aminolevulinatedehydratase activity in mice.**Nutrition**, v. 23, p. 818-826, 2007.

BRUNDEGE, J. M.; DUNWIDDIE T. V. Role of adenosine as a modulator of synaptic activity in the central nervous system.**Advances in Pharmacology**, v. 39, p. 353-391, 1997.

BURNSTOCK, G. Historical review: ATP as a neurotransmitter. **Trends in Pharmacological Science**, v. 27, p. 166-176, 2006.

BURNSTOCK, G. Potential therapeutic targets in the rapidly expanding field of purinergicsignalling.**Clinical Medicine**, v. 2, p. 45-53, 2002.

CAO, Z.; LI, Y. Potent induction of cellular antioxidants and phase 2 enzymes by resveratrol in cardiomyocytes: protection against oxidative and electrophilic injury. **EuropeanJournalofPharmacology**, v. 489, p 39-48, 2004.

CASTELNUOVO, A. D. et al. Meta-analysis of wine and beer consumption in relation to vascular risk. **Circulation**, v. 105, p. 2836-2844, 2002.

CERBONE, A. M. et al. Diabetes, vascular complications and antiplatelet therapy: open problems. **Acta Diabetologica**, v. 46, n. 4, p. 253-61, 2009.

CHEN, C.Y. et al. Resveratrol upregulates hemeoxygenase-1 expression via activation of NF-E2-related factor 2 in PC12 cells. **Biochemical and Biophysical Research Communications**, v. 331, p. 993-1000, 2005.

CHERUBINI, A. et al. Potential markers of oxidative stress in stroke.**Free Radical in Biology & Medicine**, v. 39, p. 841-852, 2005.

CNOP, M. et al. Mechanisms of: Pancreatic ß-Cell Death in Type 1 and Type 2 Diabetes. **Diabetes**, v. 54, p.97-107, 2005.

COLGAN, S. et al. Physiological roles for ecto-5'-nucleotidase (CD73). **PurinergicSignalling**, v. 2, p. 351-360, 2006.

COLWELL, J. A.; NESTO, R. W.The platelet in diabetes.**Diabetes Care**, v. 26, p. 2181- 2188, 2003.

COOPER, K. A.; CHOPRA, M.; THURNHAM, D. I. Wine polyphenols and promotion of cardiac health.**Nutrition Research Reviews**, v. 17, n. 1, 111-130, 2004.

CRITCHLEY, J. A. et al. Management of nephropathy in patients with type 2 diabetes. **Chinese Medical Journal**, v. 115, p. 129-135, 2002.

DAL-ROS, S. et al. Chronic intake of red wine polyphenols by young rats prevents aging-induced endothelial dysfunction and decline in physical performance: Role of NADPH oxidase. **Biochemical and Biophysical Research Communications**, v. 404, p. 743-749, 2011.

DA SILVA, A. C. et al. Oxidative stress and delta-ALA-D activity in chronic renal failure patients. **Biomed Pharmacother**, v. 61, n. 2-3, p. 180-185, 2007.

DELL'AGLI, M.; BUSCIALA, A.; BOSISIO, E. Vascular effects of wine polyphenols.**Cardiovascular Research**, v. 63, n. 4, p. 593-602, 2004.

DE SANTI, C. et al. Glucuronidation of resveratrol, a natural product present in grape and wine, in the human liver. **Xenobiotica**, v. 30, p. 1047-1054, 2000.

DE SANTI, C. et al. Sulphation of resveratrol, a natural product present in grapes and wine, in the human liver and duodenum. **Xenobiotica**, v. 30, p. 609-617, 2000.

DIAS, G. R. et al. Hormetic acute response and chronic effect of ethanol on adenine nucleotide hydrolysis in rat platelets.**Archives of Toxicology**, v. 83, n. 3, p. 263-269, 2009.

DI VIRGILIO, F. Purinergicsignalling in the immune syste: A brief update. **PurinergicSignalling**, v. 3, p. 1-3, 2007.

DIXON, R. A. Natural products and plant disease resistance.**Nature**, v. 411, p. 843–847, 2001.

DMITRIEV, L. F.; TITOV, V. N. Lipid peroxidation in relation to ageing and the role of endogenous aldehydes in diabetes and other age-related diseases. **Ageing Research Reviews**, v. 9, p. 200-210, 2010.

DOHADWALA, M. M.; VITA, J. A. Grapes and cardiovascular disease.**Journal of Nutritional Science and Vitaminology**, v. 139, n. 9, p. 1788-1793, 2009.

DONG, Z. Molecular mechanism of the chemopreventive effect of resveratrol. **Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis**, v. 523-524, p.145-150, 2003.

DONNE, I. D. et al. Protein carbonyl groups as biomarkers of oxidative stress. **ClinicaChimicaActa**, v. 329, p. 23-38, 2003.

DORÉ, S. Unique Properties of Polyphenol Stilbenes in the Brain: More than Direct Antioxidant Actions Gene/Protein Regulatory Activity. **Neurosignals**, v. 14, p. 61-70, 2005.

DRAGONI, S. et al. Red wine alcohol promotes quercetin absorption and directs its metabolism towards isorhamnetin and tamarixetin in rat intestine in vitro. **British Journal of Clinical Pharmacology**, v.147, p.765-771, 2006.

DRÖGE, W. Free radicals in the physiological control of cell function.**Physiological Reviews**, v. 82, p. 47-95, 2002.

DUDLEY, J. I. et al. Does white wine qualify for French paradox? Comparison of the cardioprotective effects of red and white wines and their constituents: resveratrol, tyrosol, and hydroxytyrosol. **Journal of Agricultural and Food Chemistry**, v. 56, p. 9362-9373, 2008.

ELIZABETH, H.; HARRIS, M. D. Elevated liver function tests in type 2 diabetes, Clin.**Diabetes**, v. 23, p. 115-119, 2005.

ENJYOJI, K. etal.Target disruption of CD39/ATP diphosphohydrolase result in disordered hemostasis and thromboregulation. **Nature Medicine**, v.5, p. 1010-1017, 1999.

El-MOHSEN, M. A. et al. Distribution of [3H]transresveratrol in rat tissues following oral administration. **The British Journal of Nutrition**, v. 96, p. 62-70, 2006.

FABRIS, S. et al. Antioxidant properties of resveratrol and piceid on lipid peroxidation in micelles and monolamellar liposomes.**Biophysical Chemistry**, v. 135, p. 76-83, 2008.

FEIJÓO, O.; MORENO, A.; FALQUÉ, E. Content of trans- and cis-resveratrol in Galician white and red wines. **Journal of Food Composition and Analysis**, v. 21, n. 8, p. 608-613, 2008.

FERNANDES, A. A. et al. Influence of rutin treatment on biochemical alterations in experimental diabetes. **Biomedicine & Pharmacotherapy**, v. 64, p. 214-219, 2010.

FERNANDEZ-CUARTERO, B. et al. Delta aminolevulinatedehydratase (ALA-D) activity in human and experimental diabetes mellitus.**The International Journal of Biochemistry & Cell Biology**, v. 31, p. 479-488, 1999.

FIDAN, A. F.; DÜNDAR, Y. The effects of Yucca schidigera and Quillajasaponaria on DNA damage, protein oxidation, lipid peroxidation, and some biochemical parameters in streptozotocin-induced diabetic rats. **The Journal of Diabetic Complications**, v. 22, p. 348-356, 2008.

FOTI CUZZOLA, V. et al. Role of Resveratrol and its Analogues in the Treatment of Neurodegenerative Diseases: Focus on Recent Discoveries. **CNS & Neurological Disorders - Drug Targets**. 2011

FRANCO, R. et al. Cell surface adenosine deaminase much more than an ectoenzyme. **Progress in Neurobiology**, v. 52, p. 283-294, 1997.

FREEDMAN, J. E.; PARKER, C.; LI, L. Select flavonoids and whole juice from purple grapes inhibit platelet function and enhance nitric oxide release. **Circulation**, v. 103, p. 2792-2798, 2001.

FREEDMAN, J. E. Oxidative stress and platelets. **Arteriosclerosis, Thrombosis, and Vascular Biology**, v. 28, p. 11-16, 2008.

FRÉMONT L. Biological effects of resveratrol. **Life Sciences**, v. 66, p. 663-673, 2000.

FU, W. J. et al. Changes of the tubular markers in type 2 diabetes mellitus with glomerular hyperfiltration. **Diabetes Research and Clinical Practice**, 2011.

GARRO, J. C. et al. Glucose effect on haem metabolism.**Molecular Aspects of Medicine**, v. 11, p. 28, 1990.

GAYLE, R. B. et al. Inhibition of platelet function by recombinant soluble ecto-ADPase/CD39. **The Journal of Clinical Investigation**, v.101, n. 9, p.1851-1859, 1998.

GIACCO, F.; BROWNLEE, M. Oxidative stress and diabetic complications.**Circulation Research**, v. 107, p. 1058-1070, 2010.

GIBSON, K. D.; NEUBERGER, A.; SCOTT, J. J.The purification and properties of delta-aminolevulinic acid dehydratase.**International Journal of Biochemistry and Molecular Biology**, v. 61, p. 618-629, 1955.

GIEHL, M. R. et al. Eficácia dos flavonóides da uva,vinho tinto e suco de uva tinto na prevenção e no tratamento secundário da aterosclerose. **The Journals of Gerontology.Series A, Biological Sciences and Medical Sciences**, v. 17, n. 3, p. 145-155, 2007.

GILL, P.S.; WILCOX, C.S. NADPH oxidases in the kidney.**Antioxidants & Redox Signaling**, v.8, p. 1597-1607, 2006.

GIUSTARINI, D. et al. Low molecular mass thiols, disulfides and protein mixed disulfides in rat tissues: Influence of sample manipulation, oxidative stress and ageing. **Mechanisms of Ageing and Development**, v. 132, p. 141-148, 2011.

GODING, J. W.; GROBBEN, B.; SLEGERS, H. Physiological and pathophysiological functions of the ectonucleotidepyrophosphatase/phosphodiesterase family.**Biochim. Biophys Acta**, v. 1638, p. 1-19, 2003.

GONZALEZ-PARAMAS, A. M. et al. Flavanol Content and Antioxidant Activity in Winery Byproducts. **Journal of Agricultural and Food Chemistry**, v. 52, p. 234-238, 2004.

GONÇALVES, T. L. et al. Involvement of oxidative stress in the pre-malignant and malignant states of cervical cancer in women. **Clinical Biochemistry**, v. 38, p. 1071-1075, 2005.

GRANT, P. J. Diabetes mellitus as a prothrombotic condition.**Journal of internal Medicine**, v. 262, n. 2, p. 157-172, 2007.

GRESELE, P. et al. Effects of resveratrol and other wine polyphenols on vascular function: an update. **The Journal of Nutritional Biochemistry**, v. 22, n. 3, p. 201-211, 2011.

GRONBAEK, M. et al. Type of alcohol consumed and mortality from all causes, coronary heart disease, and cancer. **American Journal of Epidemiology**, v. 133, p. 411-419, 2000.

GUERRERO, J. A. et al. Flavonoids inhibit the platelet TxA2 signalling pathway and antagonize TxA2 receptors (TP) in platelets and smooth muscle cells. **British Journal of Clinical Pharmacology**, v. 64, p. 133-144, 2007.

GUL, M. et al. Cellular and Clinical implications of glutathione.**Indian Journal of Experimental Biology**, v. 38, p. 625-634, 2000.

GRESELE, P. et al. Effects of resveratrol and other wine polyphenols on vascular function: an update.**The Journal of Nutritional Biochemistry**,v. 22, n. 3, 201-211,2011.

HALLIWELL, B.; GUTTERIDGE, J.M. **Free Radicals in biology and medicine**.3ª Ed. Oxford, New York, p. 543, 2000.

HALLIWELL, B. The antioxidant paradox.**The Lancet**, v. 355, p. 1179-1180, 2000.

HAOUARI, M. E.; ROSADO, J. A. Platelet signalling abnormalities in patients with type 2 diabetes mellitus: a review. **Blood Cells, Molecules, and Diseases**, v. 41, n. 1, p. 119-123, 2008.

HASEGAWA, Y. et al. Enhancing effect of advanced glycation end products on serotonin-induced platelet aggregation in patients with diabetes mellitus. **Thrombosis Research**, v. 107, p. 319-323, 2002.

HASSAN-KHABBAR, S. et al. Postischemic treatment by trans-resveratrol in rat liver ischemia-reperfusion: a possible strategy in liver surgery. Liver Transplantation and Surgery :**Official Publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society**, v. 14, p. 451-459, 2008.

HE, J. et al. Isolation and Structural Characterization of New Anthocyanin-Derived Yellow Pigments in Aged Red Wines.**Journal of Agricultural and Food Chemistry**, v. 54, p. 9598-9603, 2006.

HERR, R. R. et al. Isolation and characterization of streptozotocin.**Antibiotics Annual**, v. 7, p. 1959-1960, 1960.

HOUNSOM, L. et al. A lipoic acid- gamma linolenic acid conjugate is effective against multiple indices of experimental diabetic neuropathy. **Diabetologia**, v. 41, p. 50-57, 1998.

HUANG, P. H. et al. Moderate intake of red wine improves ischemia-induced neovascularization in diabetic mice-roles of endothelial progenitor cells and nitric oxide. **Atherosclerosis**, v. 212, n. 2, p. 426-435, 2010.

ILLES, P., RIBEIRO, J. A. Neuronal P2 receptors of the central nervous system. **Current Topics in Medicinal Chemistry**, v. 4, p. 831-838, 2004.

IMHOF, A. et al. Overall alcohol intake, beer, wine, and system markers of inflammation in western Europe: results from three MONICA samples (Augsburg, Glasgow, Lille). **European Heart Journal**, v. 25, p. 2092-2100. 2004.

IRITI, M. FAORO, F. Bioactivity of grape chemicals for human health.**Natural Product Communications**, v. 4, p. 611-634, 2009.

ISLAS-ANDRADE, S.; MONSALVE, M.C.R; PEÑA, J.E. Streptozotocin and Alloxan in Experimental Diabetes: Comparison of the Two Models in Rats. **ActaHistochemicaandCytochemica**, v. 33, p. 201-208, 2000.

JANBAZ, K. H.; SAEED, S. A.; GILANI, A. H. Studies on the protective effects of caffeic acid and quercetin on chemical-induced hepatotoxicity in rodents, **Phytomedicine**, v. 11, p. 424-430, 2004.

JANNIN, B. etal.Transport of resveratrol, a cancer chemopreventive agent, to cellular targets: plasmaticproteinbinding and cell uptake.**Biochemical Pharmacology**, v. 68, p. 1113-1118, 2004.

JIN, L. et al. Antioxidant and pancreas-protective effect of aucubin on rats with streptozotocin-induced diabetes.**European Journal of Pharmacology**, v. 582, p. 162-167, 2008.

KADE, I. J. Effect of oral administration of diphenyldiselenide on antioxidant status, and activity of delta aminolevulinic acid dehydratase and isoforms of lactate dehydrogenase, in streptozotocin-induced diabetic rats.**Cell biology and toxicology**, v. 25, p. 415-424, 2009.

KARUNA, R. et al. Protective effects of Phyllanthusamarus aqueous extract against renal oxidative stress in Streptozotocin -induced diabetic rats. Indian Journal of Pharmacology, v. 43, n. 4, p. 414-8, 2011.

KASHYAP, M. K. et al. Different antioxidant status, total antioxidant power and free radical in essential hypertension.**Molecular and Cellular Biochemistry**, v. 277, p. 89-99, 2005.

KAWASHIMA, Y.; NAGASAWA, T.; NINOMIYA, H. Contribution of 5'-nucleotidase to the inhibition of platelet aggregation by human endothelial cells.**Blood**, v. 96, n. 6, p. 2157-2162, 2000.

KEYS, A. et al.The diet and 15-year death rate death rate in the seven countries study.**American Journal of Epidemiology**, v.124, p. 903-915, 1986.

KIM, Y.A. et al. Resveratrol inhibits inducible nitric oxide synthase and cyclooxygenase-2 expression in beta-amyloid-treated C6 glioma cells. **International Journal of Molecular Medicine**, v. 17, p.1069-75, 2006.

KINALSKI, M. et al. Lipid peroxidation and scavenging enzyme activity in streptozotocin-induced diabetes.**ActaDiabetologica**, v. 37, p. 179-183, 2000.

KING, R. E.; BOMSER, J. A.; MIN, D. B. Bioactivity of Resveratrol.**Comprehensive Reviews in Food Science and Food Safety**, v.5, p. 65 - 70, 2006.

KINUGAWA, T. et al. Catabolism of adenine nucleotides favors adenosine production following exercise in patients with chronic heart failure. **Journal of Cardiac Failure**, v. 12, p. 720-705, 2006.

KLATSKY, A. L. et al. Wine, liquor, beer, and mortality.**American Journal of Epidemiology**, v. 158, p. 585-95, 2003.

KOCH, H. P. et al. In vitro inhibition of adenosine deaminase by flavonoids and related compounds.New insight into the mechanism of action of plant phenolics.**Methods and Findings in Experimental and Clinical Pharmacology**, v. 14, n. 6, p. 413-417, 1992.

KUMAR, A.; KAUNDAL, R.K.; IYER, S.; SHARMA, S.S. Effects of resveratrol on nerve functions, oxidative stress and DNA fragmentation in experimental diabetic neuropathy.**Life Sciences**, v. 80, n. 13, p. 1236-1244, 2007.

KURTUL, N. et al. Adenosine deaminase activity in the serum of type 2 diabetic patients.**ActaMedica**, p. 47, v. 33-35, 2004.

LEE, R. G. et al. **Wintrole, Hematologia Clínica**. v.I/II. São Paulo: Manole, 1998, p.2623.

LEE, S. M. et al. Prevention and treatment of diabetes with resveratrol in a non-obese mouse model of type 1 diabetes. **Diabetologia**, v. 54, p. 1136-1146, 2011.

LEIFERT, W.R.; ABEYWARDENA, M.Y. Cardioprotective actions of grape polyphenols.**Nutrition Research**, v. 28, p. 729-737, 2008.

LEONARD, S. S. et al. Resveratrol scavenges reactive oxygen species and effects radical-induced cellular responses. **Biochemical and Biophysical Research Communications**, v. 309, p. 1017-1026, 2003.

LI, X.; QU, Z. C.; MAY, J. M. GSH is required to recycle ascorbic acid in cultured liver cell lines.**Antioxidants & RedoxSignaling**, v. 3 p. 1089-1097, 2001.

LI, Y. et al. Resveratrol-induced cell inhibition of growth and apoptosis in MCF7 human breast cancer cells are associated with modulation of phosphorylated akt and caspase-9. **Applied Biochemistry and Biotechnology**, v. 135, p.181-192, 2006.

LIKIDLILID, A. et al. Lipid peroxidation and antioxidant enzyme activities in erythrocytes of type 2 diabetic patients. **Journal of the Medical Association of Thailand**, v. 93, p. 682-693, 2010.

LIPPI, G. et al. Moderate red wine consumption and cardiovascular disease risk: beyond the "French paradox". **Seminars in Thrombosis and Hemostasis**, v. 36, p. 59-70, 2010.

LONG, A. N.; DAGOGO-JACK, S. Comorbidities of diabetes and hypertension: mechanisms and approach to target organ protection. **Journal of Clinical Hypertension,** v.13, p. 244-51, 2011.

LOPEZ-VELEZ, M.; MARTÍNEZ-MARTÍNEZ, F.; DEL VALLE-RIBES, C.The study of phenolic compounds as natural antioxidants in wine.**Critical Reviews in Food Science and Nutrition**, v. 43, p. 233-244, 2003.

LUNKES, G.I. et al. Effect of high glucose levels in human NTPDase and 5'-nucleotidase activities. **Diabetes Research and Clinical Practice**, v. 81, p. 1-6, 2008.

LUNKES, G.I. et al. Enzymes that hydrolyze adenine nucleotides in diabetes and associated pathologies. **Thrombosis Research**, v. 109, p. 189-194, 2003.

LUNKES, G. I. et al. NTPDase and 5'-nucleotidase activities in rats with alloxan-iduced diabetes.**Diabetes Research and Clinical Practice**, v. 65, p. 1-6, 2004.

MACHADO, M. M. et al. Determination of polyphenol content and antioxidant capacity of no-alcoholic red grape products (Vitislabrusca) from conventional and organic crops. **Química Nova**, v. 34, p. 798-803, 2011.

MADDUX, B. A.; GOLDFINE, L. D. Membrane glycoprotein PC-1 inhibition of insulin receptor function occurs via direct interaction with the receptor subunit. **Diabetes**, v. 49, p.13-19, 2000.

MADDUX, B. A. et al. Membrande glycoprotein PC-1and insulin resistance in non-insulin-dependent diabetes mellitus.**Diabetes**, v. 373, p. 95-102, 1995.

MALDONADO, P. et al. Ectonucleotidepyrophosphatase/phosphodiesterase (E-NPP) and adenosine deaminase (ADA) activities in patients with uterine cervix neoplasia.**Clinical Biochemistry**, v. 41, p. 400-406, 2008.

MALDONADO P. A. Nucleotide degrading enzymes in platelets from uterine cervical neoplasia patients treated with conization or radiotherapy. **BiomedPharmacother**, v. 64, p. 499-504, 2010.

MAMEDE, M. E. O.; PASTORE, G. M. Compostos Fenólicos do Vinho: Estrutura e Ação Antioxidante. **Centro de Pesquisa e Processamento de Alimentos**, v. 22, n. 2, p. 233-252, 2004.

MANSOURI, E. et al. Effects of grape seed proanthocyanidin extract on oxidative stress induced by diabetes in rat kidney. **Iranian Biomedical Journal**, v. 15, p. 100-106, 2011.

MARCUS, A. J. Heterologous cell-cell interactions: thromboregulation, cerebroprotection and cardioprotection by CD39 (NTPDase1). **Journal of Thrombosis and Haemostasis**, v. 1, p. 2497-2509, 2003.

MARCUS, A. J. Role of CD39 (NTPDase-1) in thromboregulation, cerebroprotection, and cardioprotection.**Seminars in Thrombosis and Hemostasis**, v. 31, p. 234-246, 2005.

MARIC-BILKAN, C.; FLYNN, E. R.; CHADE, A. R. Microvascular disease precedes the decline in renal function in the streptozotocin-induced diabetic rat. American **Journal of Physiology.Renal Physiology**. 2011.

MARITIM, A. C.; SANDRES, R. A. Watkins J.B, Diabetes, oxidative stress, and antioxidants: a review. **Journal of Biochemical and Molecular Toxicology**, v. 17, p. 24-39, 2003.

MASELLA, R. et al. Novel mechanisms of natural antioxidant compounds in biological systems: Involvement of glutathione and glutathionerelated enzymes. **Journal of Nutritional Biochemistry**, v.16, p. 577-586, 2005.

MATSUNO, H. et al. P2Y12 receptors play a significant role in the development of platelet microaggregation in patients with diabetes. **The Journal of Clinical Endocrinology and Metabolism**, v. 90, p. 920-927, 2005.

MAYN, S. T. Antioxidant nutrients and chronic disease: use of biomarkers of exposure and oxidative stress status in epidemiologic research. **The Journal of Nutrition**, v. 133, p. 933-940, 2003.

MELZIG, M. F. Inhibition of adenosine deaminase activity of aortic endothelial cells by selected flavonoids. **PlantaMedica**, v. 62, n. 1, p. 20-1, 1996.

MICHNO, A. et al. Alterations of adenine nucleotide metabolism and function of bloodplatelets in patients with diabetes.**Diabetes**, v. 56, n. 2, p. 462-467, 2007.

MINISTÉRIO DA SAÚDE. **Cadernos de Atenção Básica. Diabetes mellitus. Normas e Manuais Técnicos**. Brasília, v. 16, p. 64, 2009.

MODUN, D. et al. The increase in human plasma antioxidant capacity after red wine consumption is due to both plasma urate and wine polyphenols. **Atherosclerosis**, v. 197, n. 1, p. 250-256, 2010.

MOKNI, M. et al. Effect of resveratrol on antioxidant enzyme activities in the brain of healthy rat. **Neurochemical Research**, v. 32, p. 981-987, 2007.

MOLANDER, D. W.; WROBLEWSKI, F.; LADUE, J. S. Serum glutamic oxalacetic transaminase as an index of hepatocellular integrity.**The Journal of Laboratory and Clinical Medicine**, v. 46, p. 831-839, 1955.

MONTILLA, P. et al. Protective effect of Montilla-Moriles appellation red wine on oxidative stress induced by streptozotocin in the rat.**The Journal of Nutritional Biochemistry**, v. 15, n. 11, p. 688-693, 2004.

MORDES, J.P.; ROSSINI, A.A. Animal models of diabetes.**The American Journal of Medicine**, v.70, p. 353-360, 1981.

MOREL, O. et al. Diabetes and the platelet: toward new therapeutic paradigms for diabetic atherothrombosis. **Atherosclerosis**, v. 212, n. 2, p. 367-376, 2010.

MUDALIAR, S. Intense management of diabetes mellitus: role of glucose control and antiplatelet agents. **Journal of Clinical Pharmacology**, v. 44, n. 4, p. 414-22, 2004.

McCORD, J.M.; FRIDOVICH, I. Superoxide dismutase: an enzymatic function for erythrocuprein (hemocuprein). **Journal Biological Chemistry**, v. 244, p. 6049-6055, 1969.

NADEAU, K. J.; REUSCH, J. E. Cardiovascular function/dysfunction in adolescents with type 1 diabetes.**Current Diabetes Reports**, v. 1, p. 185-92, 2011.

NAGAOKA, T. et al. Cyclic nucleotide phosphodiesterase 3 expression in vivo: evidence for tissue-specific expression of phosphodiesterase 3A or 3B mRNA and activity in the aorta and adipose tissue of atherosclerosis-prone insulin-resistant rats. **Diabetes**, v. 47, p. 1135-1144, 1998.

NAPOLI, R. ET AL. Red wine consumption improves insulin resistance but not endothelial function in type 2 diabetic patients. **Metabolism**, p. 54(3), v. 306-313, 2005.

NOGUEIRA, C. W. et al. 2,3-Dimercaptopropane-1-sulfonic acid and meso-2,3-dimercaptosuccinic acid increase mercury and cadmium-induced inhibition of delta-aminolevulinatedehydratase.**Toxicology**, v. 184, p. 85-95, 2003.

NORDBERG, J.; ARNER, E. S. J. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system.**Free Radical Biology & Medicine**, v. 31, n. 11, p. 1287–1312, 2001.

OLAS, B.; WACHOWIC, B. Resveratrol, a phenolic antioxidant with effects on blood platelet functions.**Platelets**, v. 16, p. 251-260, 2005.

OLAS, B. et al. Effect of resveratrol, a natural polyphenolic compound, on platelet activation induced by endotoxin or thrombin.**Thrombosis Research**, v. 107, n. 3/4, p. 141-145, 2002.

PACE-ASCIAK, C.R. et al. Wines and grape juices as modulators of platelet aggregation in healthy human subjects.**Clinica Chimica Acta**, v. 246, p.163-182, 1996.

PACE-ASCIAK, C. R. et al. Wines and grape juices as modulators of platelet aggregation in healthy human subjects. **ClinicaChimicaActa; International Journal of Clinical Chemistry**, v. 246, p. 163-182, 1996.

PALSAMY, P.; SIVAKUMAR, S.; SUBRAMANIAN, S. Resveratrol attenuates hyperglycemia mediated oxidative stress, proinflammatory cytokines and protects hepatocytes ultrastructure in streptozotocin-nicotinamide-induced experimental diabetic rats. **Chemico-biological Interactions**, v.186, p. 200-210, 2010.

PALSAMY, P.; SUBRAMANIAN S. Resveratrol protects diabetic kidney by attenuating hyperglycemia-mediated oxidative stress and renal inflammatory cytokines via Nrf2-Keap1 signaling. **BiochimicaetBiophysicaActa**, v.1812, p. 719-731, 2011.

PANDEY, K. B.; RIZVI, S. I. Protective effect of resveratrol on formation of membrane protein carbonyls and lipid peroxidation in erythrocytes subjected to oxidative stress.**AppliedPhysiology, Nutrition, andMetabolism**, v. 34, p. 1093-1097, 2009.

PAROLIN, M. B. Prevalência de infecção pelo vírus da hepatite C em pacientes com diabetes melito tipo 2. **Arquivos de Gastroenterologia**, v. 43, p. 77-80, 2006.

PIGNATELLI, P. et al. Polyphenols synergistically inhibit oxidative stress in subjects given red and white wine. **Atherosclerosis**, v. 188, n. 1, p. 77-83, 2006.

POLO, C. F. et al. Haem biosynthesis in streptozotocin-induced diabetic mice. **Molecular Aspects of Medicine**, v.11, p. 29, 2000.

POLO, C. et al. **Further studies on the non-enzymatic glycosilation of ALA-D, PBG-D and rhodenase**, Abstracts International Conference of Porphyrins, Porphyria and Photodynamic therapy, Melbourne, Australia.November (1993).

PRAKASH, M. S. Altered adenosine deaminase activity in type 2 diabetes mellitus.**Journal Indian Academy of Clinical Medicine**, v. 7, p.114-121, 2006.

QUIDEAU, S. Plant polyphenols: chemical properties, biological activities, and synthesis. **AngewandteChemie** (International ed. in English), v. 17, n. 50, p. 586-621, 2011.

RAINS, J. L., JAIN, S. K. Oxidative stress, insulin signaling, and diabetes. **Free Radical Biology & Medicine**, v. 50, p. 567-575, 2011.

RAMKUMAR, V. et al. Adenosine antioxidant enzymes and cytoprotection. **Trends in Pharmacological Sciences**, v. 16, p. 283-285, 1995.

RASHID, A. et al. Resveratrol Enhances Prostate Cancer Cell response to Ionizing Radiation.Modulation of the AMPK, Akt and mTOR Pathways.**Radiation Oncology**, v. 6, p. 144-160, 2011.

REMIJIN, J. Role of ADP receptor P2Y12 in platelet adhesion and thrombus formation in flowing blood. **Arteriosclerosis, Thrombosisand Vascular Biology**, v. 22, p. 686-691, 2002.

RENAUD, S.; DE LORGERIL, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease.**Lancet**, v. 339, p.1523-1526, 1992.

RIBEIRO, G. et al. N-acetylcysteine on oxidative damage in diabetic rats. **Drug and Chemical Toxicology**, v. 34, n. 4, p. 467-74, 2011.

ROBSON, S.; SÉVIGNY, J.; ZIMMERMANN, H. The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. **PurinergicSignalling**, v. 2, p. 409-430, 2006.

ROCHA, M. E. M. et al. Oxidative damage to ferritin by 5-aminolevulinic acid. **Archives of Biochemistry and Biophysics**, v. 409, p. 349-356, 2003.

RODRIGO, R.; MIRANDA, A.; VERGARA, L. Modulation of endogenous antioxidant system by wine polyphenols in human disease.**ClinicaChimicaActa; International Journal of Clinical Chemistry**, v. 412, n. 6, p. 410-424, 2011.

ROZALSKI, M.; NOCUN, M.; WATALA, C. Adenosine diphosphate receptors on blood platelets: potential new targets for antiplatelet therapy. **Acta BiochimicaPolonica**, v. 52, n. 2, p. 411-415, 2005.

RÜCKER, B. J. et al. E-NTPDases and ecto-5'-nucleotidase expression profile in rat heart left ventricle and the extracellular nucleotide hydrolysis by their nerve terminal endings. **Life Sciences**, v. 27, p. 477-486, 2008

SAIKO, P. et al. Resveratrol and its analogs: defense against cancer, coronary disease and neurodegenerative maladies or just a fad? **MutationResearchReview**, v. 658, p. 68-94, 2008.

SANTINI, S. A.; MARRA, G.; GIARDINA, B. Defective antioxidant defenses and enhanced susceptibility to lipid peroxidation in uncomplicated IDDM.**Diabetes**, v. 46, p. 1853-1858, 1997.

SASSA, S.; FUJITA, H.; KAPPAS, A. **Genetic and chemical influences on heme biosynthesis**. In: A. Kotyk, J. Skoda; V. Paces and V. Kostka (Eds.), Highlights of Modern Biochemistry VSP, Utrecht, v. 1, p. 329-338, 1989.

SCHAAN, B. D. et al. Relationship between cardiovascular dysfunction and hyperglycemia in streptozotocin-induced diabetes in rats. **Brazilian Journal of Medical and Biological Research**, v. 37, p. 1895-1902, 2004.

SCHETINGER, M. R. C. et al. ATP and ADP hydrolysis in fish, chicken and rat synaptosomes.**Comparative Biochemistry and Physiology B - Biochemistry and Molecular Biology**, v. 128, p. 731-741, 2001.

SCHETINGER, M. R. et al. NTPDase and 5'-nucleotidase activities in physiological and disease conditions: new perspectives for human health. **Biofactors**, v. 31, p. 77-98, 2007.

SCHMITT, C. A.; DIRSCH, V. M. Modulation of endothelial nitric oxide by plant-derived products.**Nitric Oxide**, v. 21, p. 77–91, 2009.

SCHÄFER, A.; BAUERSACHS J. Endothelial dysfunction, impaired endogenous platelet inhibition and platelet activation in diabetes and atherosclerosis. **Current Vascular Pharmacology**, v. 6, p. 52-60, 2008.

SERAFINI, M.; MAIANI, G.; FERRO-LUZZI, A.F. Effect of ethanol on red wine tannin-protein (BSA) interactions.**Journal of Agricultural and Food Chemistry**, v. 45, p. 3148-3151, 1997.

SHANMUGANAYAGAM, D.; WARNER, T. F.; KRUEGER, C. G. Concord grape juice attenuates platelet aggregation, serum cholesterol and development of atheroma in hypercholesterolemic rabbits. **Atherosclerosis**, v. 190, 135-142, 2007.

SHAO, J. et al. Hyperglycemia enhances PEPCK gene expression and hepatocellular glucose production via elevated liver activating protein/liver inhibitory protein ratio. **Diabetes**, v. 54, p. 976-984, 2005.

SHARMA, A. et al. Effect of glycemic control and vitamin E supplementation on total glutathione content in non-insulin-dependent diabetes mellitus. **AnnalsofNutrition&Metabolism**, v. 44, p. 11-13, 2000.

SHARMA, M.; GUPTA, Y. K. Chronic treatment with trans-resveratrol prevents intracerebroventricularstreptozotocin induced cognitive impairment and oxidative stress in rats. **Life Sciences**, v. 71, p. 2489-2498, 2002.

SHARMA, S. et al. Resveratrol a polyphenolicphytoalexin, attenuates Diabetic Nephropathy in rats.**Pharmacology**, v. 76, p. 69-75, 2006.

SHEMIN, D. 5-Aminolaevulinic acid dehydratase: structure, function, and mechanism. Philosophical Transactions of the Royal Society of London.Series B, **Biological Sciences**, v. 273, p. 109- 115, 1976.

SIGNORELLI, P.; GHIDONI, R. Resveratrol as an anticancer nutrient: molecular basis, open questions and promises. **JournalofNutritionalBiochemistry**, v. 16, p. 449-466, 2005.

SILVA, A.C. et al. Enzymes that hydrolyze nucleotides in chronic renal failure: Relationship between hemostatic defects and renal failure severity. **Biochimica Biophysica Acta**, v. 1741, p. 282-288, 2005.

SOLEAS, G. J.; DIAMANDIS, E. P; GOLDBERG, D. M. Wine as a biological fluid: history, production, and role in disease prevention. **Journal of Clinical Laboratory Analysis**, v. 11, p. 287-313, 1997.

SOLEAS, G.J; DIAMANDIS, E.P; GOLDBERG, D.M. Resveratrol: a molecule whose time has come? And gone? **Clinical Biochemistry**, v. 30(2), p. 91-113, 1997.

SOUSA NETO, J. A.; COSENZA, R. M. Efeitos do vinho no sistema cardiovascular. **Revista da Associação Médica de Minas Gerais**, v. 4, n. 3, p. 27-32, 1994.

SOUTO, A. A. et al. Determination of trans-Resveratrol Concentrations in Brazilian Red Wines by HPLC. **Journal of Food Composition and Analysis**, v. 14, p. 441-444, 2001.

SOUZA, J.B. et al. Delta-aminolevulinatedehydratase (delta-ALA-D) activity in diabetes and hypothyroidism.**Clinical Biochemistry**, v. 40, p. 321-325, 2007.

SPANEVELLO, R. M. et al. Activities of the enzymes that hydrolyze adenine nucleotides in platelets from multiple sclerosis patients. **Journal of Neurology**, v. 257, p. 24-30, 2010.

SPANIER, G. et al. Resveratrol reduces endothelial oxidative stress by modulating the gene expression of superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPx1) and NADPH oxidase subunit (Nox4). **Canadian Journal of Physiology and Pharmacology**, v. 60, p. 111-116, 2009.

STADTMAN, E. R. Role of oxidant species in aging. **Current Medicinal Chemistry**, v. 11, p. 1105-1112, 2004.

STAHL, W.; SIES, H. Antioxidant defense: vitamins E and C and carotenoids. **Diabetes**, v. 46, p. 14-18, 1997.

STARK, G. Functional consequences of membrane damage.**The Journal of Membrane Biology**, v. 205, p.1-16, 2005.

STEFAN, C.; JANSEN, S.; BOLLEN, M. Modulation of purinergicsignalling by NPP-type ectophosphodiesterases.**Purinergic Signalling**, v. 2, p. 361-370, 2006.

STEFAN, C.; JANSEN, S.; BOLLEN, M. NPP- type ectophosphodiesterases: unity and diversity. **Trends in Biochemical Sciences**, v. 30, p. 542-550, 2005.

STEVENS, M.J. Redox-based mechanisms in diabetes.**Antioxidants & Redox Signaling**, v. 7, p. 1483-1485, 2005.

STRATMANN, B.; TSCHOEPE, D. Pathobiology and cell interactions of platelets in diabetes.**Diabetes & Vascular Disease Research : Official Journal of the International Society of Diabetes and Vascular Disease**, v. 2, n. 1, p. 16-23, 2005.

SZEWCZUK, L.M. et al. Resveratrol is a Peroxidase-mediated Inactivator of COX-1 but Not COX-2**. Journal of Biological Chemistry**, v. 279, p. 22727-22737, 2004.

SZKUDELSKA, K.; SZKUDELSKI, T. Resveratrol, obesity and diabetes. **European Journal Pharmacology**, v. 635, p.1-8, 2010.

TAKAMA, Y. et al. Involvement of spontaneous nitric oxide production in the diabetogenic action of streptozotocin. **Pharmacology**, v. 50, p. 69-73, 1995.

TERKELTAUB, R. Physiologic and pathologic functions of the NPP nucleotide pyrophosphatase/phosphodiesterase family focusing on NPP1 in calcification. **Purinergic Signalling**, v. 2, p. 371-377, 2006.

THIRUNAVUKKARASU, M.; PENUMATHSA, S. V; KONERU, S. Resveratrol mediated cardioprotection in STZ-induced diabetic rat model. **Journal of Molecular and Cellular Cardiology**, v. 40, p. 881-887, 2006.

TOWNSEND, D. M.; TEW, K. D.; TAPIERO, H.The importance of glutathione in human disease.**Biomedicine Pharmacotherapy**, v. 57, p. 145-155, 2003.

TOZZI CIANCARELLI, M. G. et al. Moderate consumption of red wine and human platelet responsiveness. **Thrombosis Research**, v. 128, n. 2, p. 124-129, 2011.

TRICHOPOULOU, A. et al. Adherence to a Mediterranean diet and survival in a Greek population. **New England Journal of Medicine**, v. 348, p. 2599-2608, 2003.

TUNSTALL-PEDOE, H. et al. Contribution of trends in survival and coronary-events rates to changes in coronary heart disease mortality: 10-year results from WHO MONICA Project population. **Lancet**, v. 353, p.1547-1557, 1999.

VALKO, M. et al. Free radicals, metals and antioxidants in oxidative stress-induced cancer. **Chemical and Biological Interactions**, v. 160, p. 1-40, 2006.

VALKO, M. et al. Free radicals and antioxidants in normal physiological functions and human disease.**The International Journal of Biochemistry & Cell Biology**, v. 39, p. 44-84, 2007.

VENKOV, C. D. et al. Ethanol increases endothelial nitric oxide production through modulation of nitric oxide synthase expression. **Thrombosis and Haemostasis**, v. 81, p. 638-642, 1999.

VERICEL, E. et al. Diabetic Patients without Vascular Complications Display Enhanced Basal Platelet Activation and Decreased Antioxidant Status. **Diabetes**, v. 53, p.1046-1051, 2004.

VIEIRA, V. P. et al. Heparin and chondroitin sulfate inhibit adenine nucleotide hydrolysis in liver and kidney membrane enriched fractions. **International Journal of Biochemistry & Cell Biology**, v. 33, p. 1193-1201, 2001.

VILLACORTA, L.; AZZI, A.; ZINGG, J. M. Regulatory role of vitamins E and C on extracellular matrix components of the vascular system. **Molecular Aspects of Medicine**, v. 28, p. 507-537, 2007.

VITAGLIONE. P. et al. Bioavailability of trans-resveratrol from red wine in humans. **Molecular Nutrition & Food Research**, v. 49, p. 495-504, 2005.

VITRAC, X. et al. Determination of stilbenes (δ- viniferin, trans-astringin, trans-piceid, cis- and trans-resveratrol, viniferin) in Brazilian wines. **Journal of Agricultural and Food Chemistry**, v. 53, p. 5664-5669, 2005.

VOLLMAYER, P. et al. Hydrolysis of diadenosine polyphosphates by nucleotide pyrophosphatases/phosphodiesterases. **European Journal of Biochemistry / FEBS**, v. 270, p. 2971-2978, 2003.

WALLE, T. et al. High absorption and very low bioavailability of oral resveratrol in humans.**Drug Metabolism and Disposition**, v. 9, p. 1887-1893, 2004.

WANG, Q. et al. Resveratrol protects against neurotoxicity induced by kainic acid. **Neurochemical Research**, v. 29, p. 2105-2112, 2004.

WATERHOUSE, A. L. **Wine Phenolics**. Annals New York Academy of Sciences, v. 957, p. 21–36, 2002.

WIERNSPERGER, N.F. Oxidative stress as a therapeutic target in diabetes: revisiting the controversy. Diabetic Medicine: **A Journal of the British Diabetic Association**, v. 29, p. 579-585, 2003.

WORLDHEALTH ORGANIZATION (WHO) Diabetes. http://www.who.int./ncd/dia/databases.html. Acessado em outubro de 2011.

WRIGHT, B. et al. Platelet-mediated metabolism of the common dietary flavonoid, quercetin. **PLoS One**, v. 5, 9673, 2010.

WU, C. H.; YEN, G. C. Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycationendproducts. **Journal of Agricultural and Food Chemistry**, v. 53, n. 8, p. 3167-3173, 2005.

WU, K. K.; HUAN, Y. Streptozotocin-Induced Diabetic Models in Mice and Rats. **Current Protocols in Pharmacology**, v. 40, p. 1-5, 2008.

YADAV, M. et al. Biological and medicinal properties of grapes and their bioactive constituents: an update. **Journal of Medicinal Food**, v.12, p. 473-484, 2009.

YAMAGISHI, S.; NAKAMURA, K.; IMAIZUMI, T. Advanced glycation end products (AGEs) and diabetic vascular complications. **Current Diabetes Reviews**, v. 1, 93-106, 2005.

YILMAZ, H.R. et al. Protective effect of caffeic acid phenethyl ester (CAPE) on lipid peroxidation and antioxidant enzymes in diabetic rat liver. **Journal of Biochemical and Molecular Toxicology**, v.18, p. 234-238, 2004.

YU, C. et al. Human, rat, and mouse metabolism of resveratrol. **Pharmacology Research**, v. 19, p. 1907-1914, 2002.

YEGUTKIN, G. Nucleotide and nucleoside - converting ectoenzymes: important modulators of purinergicsignalling cascade. **Biochimica Biophysica Acta**, v. 1783, p. 673- 694, 2008.

ZIMERMANN, H. Ectonucleotidases: Some recent developments and a note on nomenclature. **Drug Development Research**, v. 52, p. 44-56, 2001.

ZIMMERMANN, H. Two novel families of ectonucleotidases: molecular structures, catalytic properties and a search for function. **Trends in Pharmacological Sciences**, v. 20, p. 231-236, 1999.

ZIMMERMANN, H. et al. Ecto-nucleotidases, molecular propertiesandfunctionalimpact. **Anales de La Real Academia Nacional de Farmacia**, v. 73, p. 537-566, 2007.