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EFEITO DO EXTRATO DE S*yzygium cumini* E ALTERAÇÕES PROVOCADAS PELA SÍNDROME METABÓLICA SOBRE PARÂMETROS BIOQUÍMICOS E INFLAMATÓRIOS

TESE DE DOUTORADO

Karine Santos De Bona

SANTA MARIA-RS
2013

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Karine Santos De Bona

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elaborada por
Karine Santos De Bona

como requisito parcial para obtenção do grau de
Doutor (a) em Farmacologia

COMISSÃO EXAMINADORA:

Maria Beatriz Moretto, Dra.
(Orientadora)

Carla Denise Bonan, Dra (PUCRS)

Ivana Beatrice Mânica da Cruz, Dra (UFSM)

Maria Rosa Chitolina Schetinger, Dra (UFSM)

Taís Cristina Unfer, Dra (UFSM)

Santa Maria, 04 de dezembro de 2013

“Que a felicidade não dependa do tempo, nem da paisagem, nem da sorte, nem do dinheiro...ser feliz sem motivo é a mais autêntica forma de felicidade.”

Carlos Drummond de Andrade

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Farmacologia
Universidade Federal de Santa Maria, RS, Brasil

EFEITO DO EXTRATO DE *Syzygium cumini* E ALTERAÇÕES PROVOCADAS PELA SÍNDROME METABÓLICA SOBRE PARÂMETROS BIOQUÍMICOS E INFLAMATÓRIOS

AUTORA: Karine Santos De Bona
ORIENTADORA: Maria Beatriz Moretto
Data e Local da Defesa: Santa Maria, 04 de dezembro de 2013.

A Síndrome Metabólica (SMet) é um transtorno complexo representado por um conjunto de fatores de risco cardiovascular, entre eles a disfunção endotelial, o estresse oxidativo e a inflamação. O *Syzygium cumini*, conhecido popularmente como jambolão, é uma planta que apresenta propriedades hipoglicêmicas, antiinflamatórias, antipiréticas, hipolipidêmicas e antioxidantes, além de ação antiviral e anticarcinogência. Considerando a importância da SMet no contexto social e econômico atual, a morbidade e as complicações consequentes desta situação clínica, o objetivo deste estudo foi avaliar parâmetros bioquímicos e inflamatórios em pacientes com SMet. Além disso, verificar o efeito do extrato aquoso de *Syzygium cumini* (ASc), bem como o seu mecanismo de ação sobre a atividade da enzima Adenosina desaminase (ADA) e outros parâmetros bioquímicos sob condições hiperglicêmicas e oxidativas, *in vitro.* Para o desenvolvimento da primeira etapa deste trabalho, foram utilizadas amostras de soro e sangue total de pacientes com diagnóstico de SMet (40 pessoas) antes de iniciarem um programa de atividades físicas, nos quais foram analisados parâmetros bioquímicos, inflamatórios e oxidativos. Os resultados demonstraram um aumento na atividade das enzimas ADA, acetilcolinesterase (AChE) e dipeptidil peptidase IV (DPP-IV) em linfócitos de pacientes com SMet. Ainda, nesses pacientes, observou-se um aumento na atividade das enzimas Butirilcolinesterase (BuChE) e gama-glutamiltransferase (GGT), e nos níveis de proteína C reativa (PCR) e óxido nítrico (NOx), bem como alterações nas defesas antioxidantes e algumas correlações entre os parâmetros analisados foram obtidos. Assim, esses resultados demonstraram que a SMet afeta a atividade da ADA e o sistema colinérgico refletindo o estado imune e inflamatório desses pacientes, além de alterar as defesas antioxidantes dos mesmos. Para as análises *in vitro*, primeiramente, foram utilizados eritrócitos (RBCs) de indivíduos saudáveis. O ASc foi capaz de prevenir o aumento na atividade da ADA causado pela exposição dos RBCs a condições hiperglicêmicas por 2 horas. Ainda, o ASc atuou sobre a atividade da ADA de maneira semelhante a cafeína e a insulina; por outro lado, o dipiridamol atenuou o efeito do ASc por antagonizar o efeito do mesmo ou por competição com o extrato. Assim,pode-se sugerir que o ASc influencia no metabolismo da adenosina, além de seu efeito estar relacionado a presença de compostos fenólicos e às propriedades antioxidantes atribuídas a essa planta. Na etapa seguinte da realização deste trabalho, observou-se um aumento na atividade da ADA e na lipoperoxidação, e redução da viabilidade celular após a exposição de linfócitos de indivíduos saudáveis ao 2,2'-azobis (aminodipropano) dihidrocloreto (AAPH) por 2 horas, *in vitro*. O ASc e ácido gálico foram capazes de reduzir a atividade da ADA, mas não alteraram a lipoperoxidação causada pelo AAPH. O ASc aumentou a viabilidade celular e reduziu a atividade da enzima Lactato desidrogenase (LDH). O ASc ao reduzir a atividade da ADA, pode estar aumentando os níveis de adenosina e colaborando para a manutenção dos efeitos benéficos provocados pela mesma, como ações antioxidantes, antiinflamatórias e antitrombóticas. Além disso, os resultados demonstraram o efeito citoprotetor evidenciado pelo extrato. Podemos concluir que as alterações encontradas nos pacientes com SMet estão relacionadas aos processos inflamatórios e oxidativos e podem favorecer medidas de prevenção e controle desta situação clínica. Também, os efeitos protetores demonstrados pelo ASc contribuem para o entendimento da ampla utilização desta planta e de seu valor terapêutico no tratamento de diversas patologias clínicas.

Palavras-chave: Adenosina desaminase; jambolão; inflamação; Síndrome Metabólica; *Syzygium cumini;* viabilidade celular*;*

ABSTRACT

PhD thesis
Graduation Program of Pharmacology
Federal University of Santa Maria, RS, Brazil

EFFECT OF *Syzygium cumini* EXTRACT AND CHANGES CAUSED BY METABOLIC SYNDROME ON BIOCHEMICAL AND INFLAMMATORY PARAMETERS

Author: Karine Santos De Bona
Advisor: Maria Beatriz Moretto
Place and date: Santa Maria, december 4, 2013.

Metabolic syndrome (MetS) is a complex disorder represented by a set of cardiovascular risk factors, including endothelial dysfunction, oxidative stress and inflammation. *Syzygium cumini* has hypoglycemic, anti-inflammatory, antipyretic, hypolipidemic and antioxidant properties, besides antiviral and anticarcinogenic action. Considering the importance of MetS in the current economic and social context, morbidity and complications following this pathology, the aim of this study was to assess biochemical and inflammatory parameters in patients with MetS. Moreover, to check the effect of aqueous extract of *Syzygium cumini* (ASc), as well as its mechanism of action on the activity of the enzyme adenosine deaminase (ADA) and other biochemical parameters under hyperglycemic and oxidative conditions *in vitro*. To develop the first step of this work, were obtained samples of serum and whole blood of patients diagnosed with MetS (n=40) before initiating a physical activity program, in which we analyzed biochemical, oxidative and inflammatory parameters. The results showed an increase in ADA, acetylcholinesterase (AChE) and dipeptidyl peptidase IV (DPP-IV) activities in lymphocytes of patients with MetS. Further, in these patients, we observed an increase in the activity of butyrylcholinesterase (BuChE) and ɣ- glutamyltransferase (ɣ-GT) and in C-reactive protein (hsCRP) and nitric oxide (NOx) levels, as well as disturbances in antioxidant defenses and some correlations between the parameters analyzed were obtained. Thus, these results demonstrated that MetS affects the purinergic and cholinergic systems reflecting the inflammatory and immune status of these patients, besides altering the antioxidant defenses of the same. For *in vitro* determinations, first, erythrocytes (RBCs) from healthy individuals were used. ASc was able to prevent the increase in ADA activity caused by exposure of RBCs to hyperglycemic conditions for 2 hours. Also, ASc acted on the activity of ADA similarly caffeine and insulin, on the other hand, dipyridamole attenuated the effect of ASc by antagonizing its the effect or by competition with the extract. Thus, it can be suggested that the ASc act by influencing the metabolism of adenosine, and its effect is related to the presence of phenolic compounds and the antioxidant properties attributed to this plant. In the next step of this work , we observed an increase in the ADA activity and lipid peroxidation and decreased cell viability after exposure of lymphocytes from healthy subjects to 2,2 ' -azobis -2- amidinopropane dihydrochloride (AAPH) by 2 hours *in vitro*. ASc and gallic acid were able to reduce the ADA activity, but did not alter the lipid peroxidation caused by AAPH . The ASc increased cell viability and reduced the activity of the enzyme lactate dehydrogenase (LDH). ASc, by reducing the activity of ADA, may be increasing adenosine levels and helping to maintain the beneficial effects caused by the same, such as antioxidant, anti-inflammatory and antithrombotic actions. Furthermore, the results demonstrate the cytoprotective effect evidenced by the extract. We conclude that the changes found in patients with MetS are related to inflammatory and oxidative processes and may favor the prevention and control of this clinical situation Also, the protective effects demonstrated by ASc contribute to the understanding of the wide use of this plant and its therapeutic value in the treatment of various clinical conditions.

Keywords: Adenosine deaminase; cellular viability; jambolão; inflammation; Metabolic Syndrome; *Syzygium cumini*;

LISTA DE ABREVIATURAS

AAPH - 2,2'-azobis-2-amidinopropane dihydrochloride
ASc- Extrato aquoso de *Syzygium cumini*
AChE- Acetilcolinesterase
ADA-Adenosina desaminase
AGLs- Ácidos graxos livres
AMP- Monofosfato de adenosina
ATP- Trifosfato de adenosina
BuChE- Butirilcolinesterase
ChAT- Colina acetiltransferase
ChT- Transportador de colina
CAT- Catalase
CRP-Proteína C Reativa
DM- Diabetes *mellitus*
DM2 – Diabetes *mellitus* tipo 2
DPPIV- Dipeptidil Peptidase IV
EROs- Espécies Reativas de Oxigênio
GGT – gama-glutamiltransferase
GPx- Glutationa Peroxidase
GSH- Glutationa
IDF- Federação Internacional de Diabetes
IL-6 – Interleucina 6
IL-1- Interleucina 1
LDH – Lactato desidrogenase
MTT - (brometo de [3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio])
nAChR- Receptores de Ach nicotínicos
NAG – N – acetil- β- glucosaminidase
NO - Óxido Nítrico
NOS – Óxido Nítrico Sintase
OMS- Organização Mundial da Saúde
Sc – *Syzygium cumini*
SMet – Síndrome Metabólica
SOD – Superóxido Dismutase
TBARS- Espécies Reativas ao ácido tiobarbitúrico
TNF-α – Fator de necrose tumoral α
TOTG- Teste oral de tolerância à glicose
VR- Vermelho Neutro
5'NT- Ecto- 5' Nucleotidase

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APRESENTAÇÃO

O presente trabalho consiste na apresentação dos resultados obtidos, para fins de defesa de Tese de Doutorado. Tais resultados estão apresentados na forma de dois artigos publicados, um artigo aceito para publicação e um manuscrito submetido a periódico científico.

Este trabalho encontra-se organizado da seguinte forma: a primeira parte refere-se à INTRODUÇÃO e OBJETIVOS, sendo seguida pelas seções ARTIGOS e MANUSCRITO, onde são apresentados os quatro trabalhos. As seções Materiais e Métodos, Resultados, Discussão e Referências são apresentadas nos próprios manuscritos, representando os resultados obtidos. Na sequência, os itens DISCUSSÃO e CONCLUSÃO apresentam uma síntese dos resultados e comentários gerais sobre os trabalhos apresentados.

Por fim, as REFERÊNCIAS BIBLIOGRÁFICAS utilizadas para as citações dos itens INTRODUÇÃO e DISCUSSÃO deste trabalho.

1.INTRODUÇÃO

1.1 Síndrome Metabólica

1.1.1 Conceito e Critérios de definição da Síndrome Metabólica

A Síndrome Metabólica (SMet) foi primeiramente descrita em 1920 por Kylin, um médico sueco, como alteração na pressão arterial sistêmica associada a hiperglicemia. Mais tarde, em 1947, foi observado que a adiposidade corporal, o fenótipo de obesidade, estava comumente associada a alterações metabólicas relacionadas com diabetes *mellitus* tipo 2 (DM2) e doenças cardiovasculares (CAMERON; SHAW; ZIMMET, 2004).

Em 1988, REAVEN introduziu o termo Síndrome X, com a resistência à insulina sendo um denominador comum para a síndrome. Em adição à Síndrome X, vários outras denominações foram propostas, entre eles, Síndrome DROP (dislipidemia, resistência à insulina, obesidade e alta pressão arterial) e Síndrome Plurimetabólica (AYYOBI; BRUNZELL, 2003).

A SMet representa uma situação clínica caracterizada por hipertensão arterial, alterações nos níveis lipídicos e glicêmicos, obesidade visceral e manifestações de disfunção endotelial. Sendo assim, a SMet está associada a aumento de risco de evento cardiovascular à longo prazo, assim como ao desenvolvimento de DM2 (DE FRONZO; FERRANNINI, 1991; ISOMAA et al., 2001; McNEILL et al., 2005).

O consenso final de SMet, entretanto, vem sendo amplamente discutido, tendo passado por várias definições baseadas em estudos de larga escala. Em 1998, de acordo com os critérios da Organização Mundial de Saúde (OMS), a SMet foi definida pelo critério de elevação da glicemia (ALBERTI; ZIMMET, 1998). Mais tarde o European Group for the study of Insulin Resistance (EGIR), modificou estes critérios solicitando a presença de hiperinsulinemia e diminuindo os níveis de glicose após teste oral de tolerância a glicose (TOTG) para ≥ 140 mg/dl (RIGO, 2007). Já a

Federação Internacional de Diabetes (IDF) definiu em 2004, como critérios para a SMet a obesidade central, tendo o valor da circunferência abdominal como marcador imprescindível para o diagnóstico.

De acordo com os critérios atualizados do NCEP/ATP III, *o Third Report of the National Cholesterol Education Expert Panel on Detection, Evaluation, and Treatment of high Blood Cholesterol in Adult Treatment Panel*, o diagnóstico desta síndrome é feito quando existe a presença de três ou mais fatores de risco associados, como: obesidade abdominal (medida pela circunferência abdominal), triglicerídeos, colesterol HDL e glicemia de jejum, além de pressão arterial elevada (GRUNDY et al., 2005) (tabela 1). Nesta definição foi incluída ainda a circunferência abdominal como marcador de obesidade central e a glicemia foi excluída como fator imprescindível passando apenas a servir como elemento diagnóstico.

Estes critérios são seguidos atualmente pela Sociedade Brasileira de Cardiologia estratificando os potenciais riscos e metas lipídicas para a prevenção e tratamento da aterosclerose (V DIRETRIZ BRASILEIRA SOBRE DISLIPIDEMIAS E PREVENÇÃO DA ATEROSCLEROSE, 2013).

Parâmetros	NCEP ATP3 2005	IDF 2005	EGIR 1999	WHO 1999 modificada
Exigido		Cintura ≥94cm(homens) ou ≥80cm(mulheres) *	Resistência à Insulina ou hiperinsulinemia de jejum no topo do percentil 25	Resistência à Insulina:HOMA no topo do percentil 25, glicemia de jejum ≥ 110 ou DM
Nº anormalidades	≥ 3 de:	E ≥ 2 de:	E ≥ 2 de:	E ≥ 2 de:
Glicose	≥100 mg/dl ou tratamento com droga para glicose elevada	≥100 mg/dl ou diagnóstico de DM	110 – 125 mg/dl	
Colesterol HDL	< 40(homens);< 50(mulheres) ou tratamento com droga para C-HDL baixo ◊	< 40(homens); < 50(mulheres) ou tratamento com droga para C-HDL baixo	< 40 mg/dl	< 35(homens); < 40(mulheres)
Triglicerídeos	≥150 mg/dl ou tratamento com droga para Triglicerídeos elevados ◊	≥150 mg/dl ou tratamento com droga para Triglicerídeos elevados	Ou ≥180 mg/dl ou tratamento com droga para dislipidemia	Ou ≥ 150 mg/dl
Obesidade	Cintura ≥102 cm(homens) ou ≥88 cm(mulheres) §		Cintura ≥94 cm(homens) ou ≥80 cm(mulheres)	Cintura ≥94 cm(homens) ou ≥88 cm(mulheres)
Hipertensão	≥130/85 mmHg ou tratamento com droga para HAS	≥130/85 mmHg ou tratamento com droga para HAS	≥140/90 mmHg ou tratamento com droga para HAS	≥140/90 mmHg ou tratamento com droga para HAS

Tabela 1: Critérios diagnósticos para a SMet
Fonte: Adaptado de MEIGS, 2006

1.1.2. Fisiopatologia da Síndrome Metabólica

Diversos eventos podem estar associados na gênese da SMet, contribuindo para a instalação de alterações características, não existindo um fator central responsável por estas anormalidades, mas fatores como predisposições genéticas, obesidade visceral, resistência à insulina, entre outros, podem estar associados.

Na fisiopatologia da SMet, o fluxo aumentado de ácidos graxos livres (AGLs) ao fígado resulta em diminuição da captação hepática de insulina, inibindo sua ligação ao receptor e sua degradação, causando hiperinsulinemia sistêmica. Além disso, o excesso de AGLs leva a uma maior produção de glicose e triglicerídeos no fígado e redução na degradação da apolipoproteína B100 (ApoB100), causando maior secreção hepática de VLDLs (lipoproteínas de densidade muito baixa) (WAJCHENBERG, 2000). Com isso, a lipase lipoprotéica reduz sua atividade e favorece um catabolismo mais lento dessas lipoproteínas e de quilomícrons, reduzindo os níveis de colesterol HDL. Além disso, as grandes VLDL sofrem hidrólise no fígado e são convertidas em partículas mais densas e aterogênicas de LDL (SVEDBERG et al., 1990;CARR; BRUNZELL, 2004;ECKEL;GRUNDY; ZIMMET, 2005). Os AGLs também reduzem a sensibilidade à insulina no músculo inibindo a captação de glicose e aumentando o acúmulo de triglicerídeos. A maior mobilização de AGLs na circulação contribui para maior ativação do sistema nervoso simpático e, por múltiplos mecanismos, determina também disfunção endotelial, aumento de cortisol, retenção de sódio e aumento da pressão arterial (PA) e hiperinsulinemia. Ocorre um aumento de interleucina 6 (IL-6) e fator de necrose tumoral alfa (TNF-α), fibrinogênio e do ativador do plasminogênio (PAI-1), resultando em um estado pró-trombótico, além do aumento da proteína C reativa-ultra sensível (ECKEL; GRUNDY; ZIMMET 2005) (Figura 1).

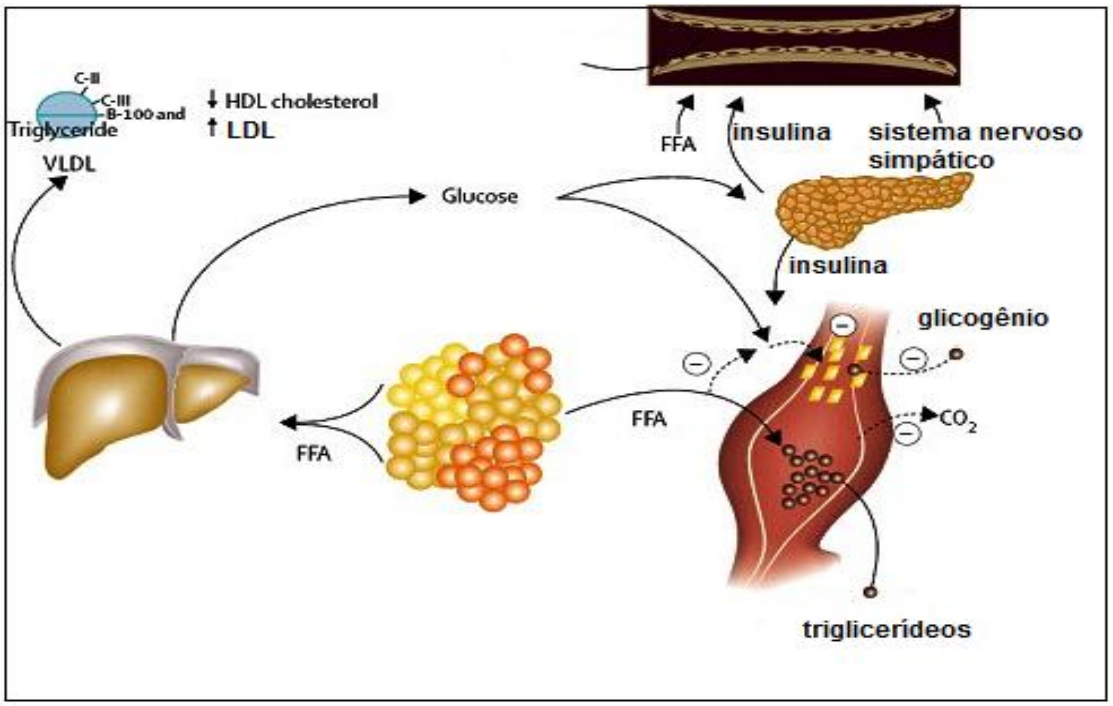

Figura 1: Fisiopatologia da SMet
Fonte: Adaptado de: www.thelancet.com

1.1.3 Prevalência da Síndrome Metabólica

A prevalência da SMet varia mundialmente, relacionando-se com a idade, etnicidade das populações estudadas e dos critérios estabelecidos para o seu diagnóstico (ECKEL ;GRUNDY; ZIMMET, 2005). Estima-se que ocorra entre 20 a 25% da população geral sendo esta prevalência ainda maior entre homens e mulheres idosos, chegando a 42% entre indivíduos com idade superior a 60 anos (ISOMAA et al., 2001; DUNSTAN et al., 2002; FORD; GINES; DIETZ, 2002). Segundo o NCEP/ATP III, são os norte americanos que apresentam maior prevalência de SMet, correspondendo a 60% de mulheres entre 45 e 49 anos de idade e 45% de homens nesta mesma faixa etária, sendo menos comum nos homens afromexicanos e mais prevalente em mulheres méxico-americanas (ECKEL; GRUNDY; ZIMMET 2005).

No Brasil, apesar da importância da SMet, os dados sobre as características epidemiológicas desta condição na população são fragmentados. Em estudo populacional realizado no Distrito Federal, utilizando os critérios da Federação

Internacional de Diabetes (2005), demonstrou-se uma prevalência da SMet em 32,0% da população, sem diferença entre sexos (DUTRA et al., 2012). Em outro estudo transversal, realizado com população de 378 idosos em Novo Hamburgo-RS, comparando os três principais critérios diagnósticos para a SMet, NCEP/ATPIII (2001), do NCEP/ATPIII revisado (2005) e da Federação Internacional de Diabetes (2005), a prevalência de SMet aumentou progressivamente, sendo que o aumento ocorreu em ambos os sexos, com maior prevalência entre as mulheres, com percentuais de 57,1%, 59,9% e 63,5% com os critérios do NCEP/ATPIII, NCEP/ATPIII revisado e da IDF, respectivamente (RIGO et al., 2009). Ainda, estudos realizados por SÁ e MOURA (2010) indicam que 22,7% da população adulta no Brasil apresenta pelo menos um dos fatores de risco para SMet e 14,2% dois ou mais fatores de risco.

Independente dos critérios para o diagnóstico da SMet e dos grupos étnicos pesquisados, a prevalência da SMet aumenta com o ganho de peso, principalmente na região abdominal, com o sedentarismo, em pessoas com histórico de diabetes na família, níveis elevados de gordura no sangue e pressão alta (SOCIEDADE BRASILEIRA DE ENDOCRINOLOGIA E METABOLOGIA, 2013).

1.1.4 Síndrome Metabólica e inflamação

A inflamação crônica sistêmica é uma característica comum da SMet, sendo que um aumento generalizado da expressão de citocinas derivadas do tecido adiposo pode ser um mecanismo plausível para a relação entre a inflamação e a SMet. Várias citocinas, quinases e fatores de transcrição têm sido implicados na resistência insulínica sistêmica e no estado inflamatório crônico aterogênico, fatores esses que contribuem para o DM2 e a aterosclerose (ESPOSITO; GIUGLIANO, 2004; SHAH; MEHTA; REILLY, 2008).

O possível elo entre SMet e inflamação é a resistência insulínica. Defeitos da ação da insulina nos tecidos- alvo (músculo, fígado e tecido adiposo) levam ao aumento do processo inflamatório crônico de baixa intensidade. Independentemente do agente iniciante, a relação entre resistência insulínica e processo inflamatório é bidirecional, ou seja, qualquer processo inflamatório crônico induz resistência

insulínica, e esta, por sua vez, acentua o processo inflamatório (DANDONA et al., 2007). Os resultados de vários estudos têm confirmado que as doenças crônicas são acompanhadas pelos processos inflamatórios e que a presença de inflamação pode preceder o futuro desenvolvimento destas doenças (FESTA et al., 2000; PEREZ, 2005; FRANCISCO; HERNÁNDEZ; SIMÓ, 2006; DARVALL et al., 2007; DANDONA et al., 2007).

Na prática clínica, alguns fatores inflamatórios, apesar de inespecíficos, como a proteína C reativa (CRP) e a interleucina- 6 (IL-6) têm demonstrado papel estabelecido preditivo e de prognóstico de grande relevância clínica em várias formas de doenças cardiovasculares (DA LUZ; LAURINDO, 2005).

A CRP é uma proteína de fase aguda, sintetizada pelo fígado e regulada por citocinas, predominantemente a interlecucina 6 (IL-6), o TNF-α e a IL-1. Embora o fígado seja a principal fonte de CRP, os adipócitos e o tecido arterial também a sintetizam (REXRODE et al., 2003; FRANCISCO; HERNÁNDEZ; SIMÓ, 2006). Seus níveis estão aumentados em resposta ao processo inflamatório agudo. Elevações modestas dos níveis de CRP estão também presentes em situações crônicas inflamatórias, como a aterosclerose e seus níveis aproximadamente triplicam na presença de risco de doenças vasculares periféricas (FRANCISCO; HERNÁNDEZ; SIMÓ, 2006; ABDELLAOUI; AL-KHAFFAF, 2007).

Além disso, estudos têm demonstrado que pessoas com SMet possuem valores séricos de CRP significativamente maiores que controles saudáveis e que esse marcador correlaciona-se com todos os componentes da SMet, incluindo glicemia de jejum, circunferência da cintura, triglicerídeos, colesterol HDL, pressão arterial sistólica e diastólica, e também com valores de índice de Massa Corporal (IMC), insulina, índice de sensibilidade à insulina, colesterol total e colesterol LDL (ISHIKAWA et al., 2007; DA CRUZ et al., 2013; MAKNI et al., 2013).

1.1.5 Síndrome Metabólica e disfunção endotelial

O termo “disfunção endotelial” refere-se a um desequilíbrio na produção endotelial de mediadores que regulam o tônus vascular, agregação plaquetária, coagulação e fibrinólise, sendo o tônus vascular o aspecto mais estudado. A

disfunção endotelial também é frequentemente referida como piora no relaxamento dependente do endotélio, causado pela perda da biodisponibilidade do óxido nítrico (NO), muito embora a produção de outras substâncias vasoativas derivadas do endotélio também possa estar alterada (MOMBOULI; VANHOUTE, 1999).

A disfunção endotelial está presente em diversas doenças metabólicas e/ou cardiovasculares, como na obesidade, intolerância à glicose, DM, hipertensão arterial e dislipidemia. Em todas essas condições ocorre resistência insulínica, a qual tem sido fortemente associada à disfunção endotelial (HSUEH; LYON; QUINONES, 2004).

O NO, embora seja estruturalmente uma molécula simples, desempenha um papel importante em quase todos os sistemas biológicos, sendo que existem três isoformas da enzima óxido nítrico sintase (NOS) que catalisam a reação de oxidação da L-arginina para produzir o NO: a neuronal (nNOS), indutível (iNOS) e endotelial (ENOS) (YOON et al., 2002; DUDZINSKI et al., 2006). O NO é um potente vasodilatador e inibe a agregração plaquetária e de leucócitos, a migração e a proliferação celular, eventos esses que estão envolvidos na patogênese da aterosclerose (DING et al., 2000). Uma inapropriada liberação desse mediador ou disponibilidade prejudicada de seu precursor L-arginina pode contribuir para o desenvolvimento de aterosclerose e para o aumento da tendência de formação de trombos nessa síndrome.

Embora a disfunção endotelial seja considerada uma importante questão em pacientes com SMet, os resultados de estudos sobre os níveis de NO no soro desses pacientes têm sido bastante contraditórios. KOWALSKI e colaboradores (2011) demonstraram que os níveis de NO estavam reduzidos em pacientes com SMet. Por outro lado, CAIMI e colaboradores (2013) mostraram concentrações elevadas de NO em pessoas com SMet e DM2. Ainda, um estudo realizado por LIN e colaboradores (2007) demonstrou que pacientes com obesidade severa e pessoas não obesas apresentaram níveis similares de NO no soro.

1.1.6 Síndrome Metabólica e estresse oxidativo

O termo estresse oxidativo é usado para descrever um número de reações químicas envolvidas na produção de radicais livres e outras moléculas reativas que podem potencialmente induzir injúria celular e, dessa forma, exercer um importante papel no processo aterogênico (HALLIWELL, 1996; HARRISON et al., 2003). É um grave desequilíbrio entre a geração de espécies reativas de oxigênio (EROs) e a proteção antioxidante em favor do primeiro, causando o excessivo dano oxidativo (HALLIWELL, 2011).

As células possuem uma série de defesas capazes de minimizar o efeito provocado pelo excesso das EROs, geradas pelo metabolismo aeróbio e organizada em diferentes níveis (FINKEL, 2003; SCANDALIOS, 2005). Estas defesas são comumente chamadas de defesas antioxidantes, e podem ser produzidas endogenamente ou adquiridas pela dieta. Estas estratégias de defesa incluem diferentes níveis de proteção, que podem ser resumidos em três formas principais de atuação: evitar a formação excessiva de EROs, a neutralização destas espécies reativas e a reparação de danos ocasionados por elas. Assim, o termo antioxidante pode ser considerado como qualquer substância que atrase, previna ou remova o dano oxidativo de uma molécula-alvo (HALLIWELL; GUTTERIDGE, 2007).

Os sistemas de defesa antioxidante são divididos em não-enzimático (SANE), como pequenas moléculas como vitaminas C e E, flavonoides, selênio, bilirrubina, ácido úrico e carotenoides, derivadas principalmente da dieta (WISEMAN; HALLIWELL, 1996; NORDBERG; ARNER, 2001; SCANDALIOS, 2005) e enzimático (SAE), como as enzimas Superóxido Dismutase (SOD), Catalase (CAT) e Glutationa Peroxidase (GPx).

O estresse oxidativo tem sido implicado na fisiopatologia da obesidade, hipertensão, disfunção endotelial e SMet (OHMORI et al., 2005; ABDILLA et al., 2007) e parece ser um dos elos para o desenvolvimento da resistência periférica a ação da insulina em pacientes obesos. O aumento da insulina, de ácidos graxos livres e dos níveis de glicose pode resultar em aumento na produção de EROs e consequentemente em estresse oxidativo (URAKAWA et al., 2003; MENON et al., 2004). Assim, uma produção descontrolada de radicais livres pode ser um dos mecanismos subjacentes ao desenvolvimento de co-morbidades em indivíduos com SMet.

1.2 Diabetes *mellitus*

O DM é uma desordem metabólica de etiologia múltipla, decorrente da diminuição da secreção de insulina e/ou da perda da capacidade desse hormônio de exercer adequadamente seus efeitos. A principal função da insulina é promover a entrada de glicose nas células do organismo dependentes de insulina, de modo que ela possa ser aproveitada para as diversas atividades celulares, portanto, a resistência à insulina ou a falta absoluta da mesma, resulta no comprometimento do controle metabólico da glicemia, culminando com um quadro de hiperglicemia que é considerado o fator de risco clássico para o desenvolvimento das chamadas complicações do diabetes (TIWARI; RAO, 2002).

A doença é altamente heterogênea, ou seja, causas genéticas diferentes, e talvez não genéticas, produzem essa condição clínica, que por isso é apresentada em algumas variedades comuns e incomuns (VOGEL; MOTULSKY, 2000). Suas principais manifestações incluem distúrbios metabólicos, que acarretam um estado crônico de hiperglicemia (RAMALHO, 1998), sede excessiva; aumento de apetite, fadiga, fraqueza, tonturas, perda de peso, e elevação do nível de glicose no sangue, o que resulta na excreção da glicose pela urina, sendo a principal causa de cegueira, retinopatia, nefropatia, neuropatia, amputação dos membros inferiores, distúrbios cardiovasculares, hipertensão e infarto. O DM está associado à reduzida expectativa de vida, significante morbidade e diminuição da qualidade de vida (CARVALHO; DINIZ; MUKHERJEE, 2005; WORLD HEALTH ORGANIZATION, 2006; AMERICAN DIABETES ASSOCIATION, 2009).

O número de adultos com diabetes em todo o mundo mais do que dobrou nos últimos 30 anos, chegando a um total de 371 milhões de pessoas. Segundo previsão anunciada em novembro de 2011 pela Federação Internacional de Diabetes, ligada à Organização Mundial da Saúde, até o ano 2030 esse número deverá chegar a 439 milhões de pessoas (FEDERAÇÃO INTERNACIONAL DE DIABETES, 2012). O Brasil ocupa a 4ª posição entre os países com maios prevalência de diabetes: 13,4 milhões de pessoas portadoras de diabetes, correspondendo a aproximadamente 6,5% da população entre 20 e 79 anos de idade (FEDERAÇÃO INTERNACIONAL DE DIABETES, 2012).

A classificação atual do DM toma como referência a etiopatogênese dos distúrbios glicêmicos, definidos de acordo com defeitos ou processos específicos, sendo que a grande maioria dos pacientes diabéticos pertence a uma das duas classes etiopatogênicas: Diabetes *mellitus* tipo 1 (DM1), que compreende cerca de 10% do total de casos e Diabetes *mellitus* tipo 2 (DM2), que compreende cerca de 90% do total de casos (MINISTÉRIO DA SAÚDE, 2006; AMERICAN DIABETES ASSOCIATION, 2012). Cerca de 80 a 90% das pessoas que apresentam o DM 2 possuem também SMet, podendo apresentar acidentes cardiovasculares, obesidade abdominal, resistência insulínica, tolerância alterada à glicose e hipertensão (LAAKSONEN; NISKANEN; LAKKA, 2004).

O diagnóstico do DM baseia-se na glicose plasmática de jejum (GROSS et al., 2002). O tratamento farmacológico do diabetes inclui agentes hipoglicemiantes orais e/ou injeções de insulina. Porém, a busca por produtos naturais tem aumentado em todo o mundo, sendo relatado o uso de, aproximadamente, 800 espécies de plantas para combater o diabetes (SAXENA; VIKRAM, 2004; PATIL et al., 2011). Além disso, de acordo com a Organização Mundial de Saúde (OMS), cerca de 90% da população dos países em desenvolvimento utilizam plantas e seus produtos como cuidados primários de saúde (WORLD HEALTH ORGANIZATION, 2002).

1.3 Sistema Purinérgico

O sistema purinérgico é caracterizado por ser uma via de sinalização importante em diversos tecidos, sendo considerado um sistema primitivo envolvido em muitos mecanismos neuronais e não neuronais e em eventos de curta e longa duração, incluindo resposta imune, inflamação, dor, agregação plaquetária, vasodilatação mediada pelo endotélio, proliferação e morte celular (BURNSTOCK; KNIGHT, 2004). A sinalização purinérgica envolve três principais componentes: 1) os nucleotídeos e nucleosídeos de adenina, 2) os receptores através dos quais eles exercem seus efeitos e 3) as ectoenzimas, responsáveis pelo controle dos níveis destas moléculas no meio extracelular (ATKINSON et al., 2006).

Os receptores purinérgicos são divididos em dois grandes grupos: receptores do tipo P1 e receptores do tipo P2. Os receptores P2 apresentam os subtipos P_{2X} e P_{2Y} que respondem ao ATP e/ou ADP. Os purinoceptores do tipo P1 possuem 4 subtipos: A1, A2A, A2B e A3 (FREDHOLM et al., 2001; BURNSTOCK, 2009). Os receptores do tipo A1 e A3 inibem a adenilato ciclase e são receptores de alta e baixa afinidade pela adenosina, respectivamente. Em contrapartida, os receptores A2A e A2B, de alta e baixa afinidade, respectivamente, ativam a adenilato ciclase. Os subtipos de receptores também atuam em outros sistemas efetores, entre eles os canais de cálcio e potássio, fosfolipase C, GMP cíclico e fosfodiesterases, atuando na modulação de diferentes funções celulares (FREDHOLM et al., 2000; BURNSTOCK, 2009) (Figura 2).

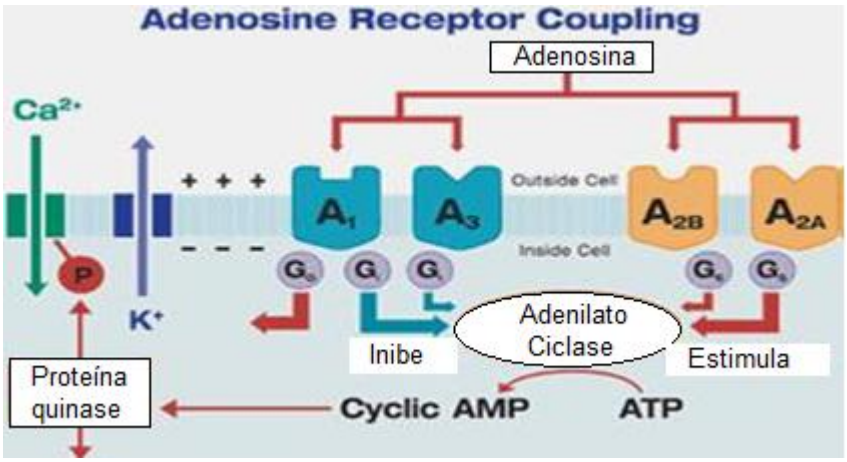

Figura 2: Receptores purinérgicos inibitórios (A1 e A3) e excitatórios (A2A e A2B) acoplados a diferentes tipos de proteína G, levando a efeitos intracelulares inerentes às respectivas vias desencadeadas.
Fonte: Adaptado de www.aderis.com/img/art_adenosine.gif.

1.3.1 Adenosina

A adenosina (Figura 3) é uma molécula sinalizadora endógena que regula numerosos processos fisiológicos e patológicos (FREDHOLM et al., 2007). Em situações homeostáticas, os níveis de adenosina extracelular permanecem em concentrações nanomolares (10 a 200 nM), já em situações de estresse metabólico ou dano celular os níveis de adenosina são elevados a concentrações micromolares (10 a 100 µM) (FREDHOLM, 2007).

No tecido cardíaco, a adenosina produz vasodilatação e diminuição da pressão cardíaca (SATO et al., 2005). Em outros sistemas apresenta diversas funções como modulação da liberação de neurotransmissores e de citocinas, inibição da lipólise, indução de broncoconstrição (BOUMA; VAN DEN WILDENBERG; BUURMAN, 1997; VAN DER GRAAF et al., 1999), além de exercer importante papel na modulação da ação da insulina em diferentes tecidos, por estimular a absorção de glicose provocada pela insulina (RUTKIEWICZ; GORSKI, 1990).

No meio intracelular, a produção de adenosina pode ocorrer a partir de 5´-AMP que, através da enzima 5'Nucleotidase (5'NT), é convertida em adenosina, sendo esta metabolizada em inosina pela enzima Adenosina desaminase (ADA, Figura 3) (LATINI; PEDATA, 2001). Outra via para síntese da adenosina é possibilitada pela hidrólise da S-adenosilhomocisteína pela S-adenosilhomocisteína hidrolase, embora estudos tenham demonstrado que esta via não é muito importante para a geração deste modulador, sendo considerada como principal via a que envolve a atividade da 5'NT (PAK et al., 1994). A fonte mais importante para o aumento dos níveis de adenosina extracelular durante situações de estresse metabólico é a liberação de ATP intracelular, seguido do catabolismo extracelular desse nucleotídeo, até a geração de adenosina por ação das ectonucleotidases (HASKÓ; PACHER, 2008).

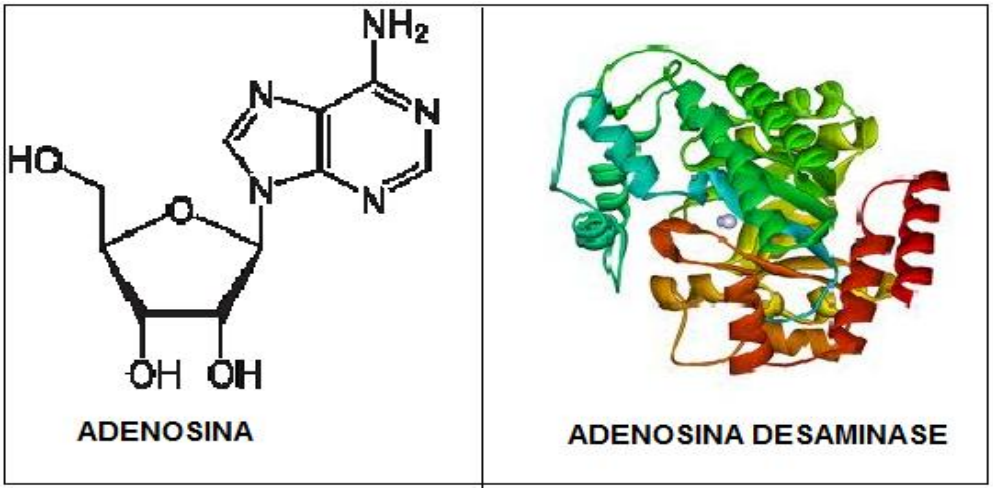

Figura 3: Estrutura da molécula de adenosina e da enzima adenosina desaminase
Fonte: Disponível em: http://pt.wikipedia.org/wiki/Adenosina_deaminase

1.3.2 Adenosina desaminase (ADA)

A Adenosina desaminase (ADA, Adenosina aminohidrolase, EC 3.5.4.4) é uma enzima chave no metabolismo das purinas, presente no citosol e na membrana celular (ecto-ADA), sendo assim objeto de considerável interesse devido ao seu papel na manutenção dos níveis de adenosina intra e extracelulares (FRANCO et al., 1997). É encontrada principalmente no sistema linfoide (timo, linfonodos e baço), podendo ser também encontrada em níveis menores nos eritrócitos (CRISTALLI et al., 2001; SBOURY et al., 2003).

A ADA é a enzima responsável por catalisar irreversivelmente a clivagem hidrolítica da adenosina à inosina e 2'-desoxiadenosina (dado) em 2'–desoxinosina (POSPISILOVA; FREBORT, 2007) (Figura 4). Assim, é provável que a ADA influencie nos níveis de adenosina promovendo proteção tecidual, através da regulação da resposta inflamatória e imune em tecidos lesionados (ABBRACCHIO; CERUTI, 2007).

Em humanos a ADA existe em pelo menos três formas moleculares: ADA1, ADA1 + CP (proteína de ligação) e ADA2, todas com características cinéticas distintas, o que faz com que desempenhem funções diferentes no organismo (SHAROYAN et al., 2006). A isoforma ADA1 é uma proteína monômera com massa molecular de aproximadamente 40 kDa. É principalmente localizada no citosol, sendo encontrada em todo organismo e também na superfície de macrófagos, linfócitos B e em alguns linfócitos T (TSUBOI et al., 1995). A isoforma ADA2 apresenta massa molecular de aproximadamente 100 kDa, sendo mais abundante no plasma, embora estudos sugiram que estas isoformas podem ser secretadas por monócitos ativados mediante processos inflamatórios (IWAKI-EGAWA; YAMAMOTO; WATANABE, 2006).

Extracelularmente, a ADA interage com proteínas de membrana como a dipeptidil peptidase IV (CD26/DPP-IV; EC 3.4.14.5) que exerce importante papel na modificação, processamento e/ou inativação de peptídeos, e em associação com a ADA pode estar diretamente envolvida na ativação de células T (MENTLEIN, 1999; PACHECO et al., 2005).

A deficiência congênita de ADA causa Imunodeficiência Severa Combinada, que é caracterizada pela ausência de linfócitos B e T funcionais. Por outro lado, um

aumento na atividade da enzima em diferentes fluídos biológicos (líquidos pleural, pericárdico, peritoneal, intra-articular e fluidos cerebroespinhais) tem sido utilizado para diagnóstico de tuberculose (CASTELO FILHO et al., 2004; ZARIC et al., 2008) peritonite infecciosa, mononucleose infecciosa, febre tifóide, sinusite, meningite e Síndrome de Imunodeficiência Adquirida (AIDS) (TITARENKO et al., 2006). Ainda, a partir de estudos realizados em nosso laboratório, observamos um aumento da atividade da ADA em soro e eritrócitos de pacientes hiperglicêmicos (BOPP et al., 2009), em plaquetas e membranas de eritrócitos de pacientes diabéticos (DE BONA et al., 2010 e 2011) e em linfócitos de pacientes com SMet (DE BONA et al., 2012). Por outro lado, outros estudos relataram que não houve diferença significativa na atividade da ADA em células cardíacas de ratos diabéticos (PODGORSKA et al., 2006)

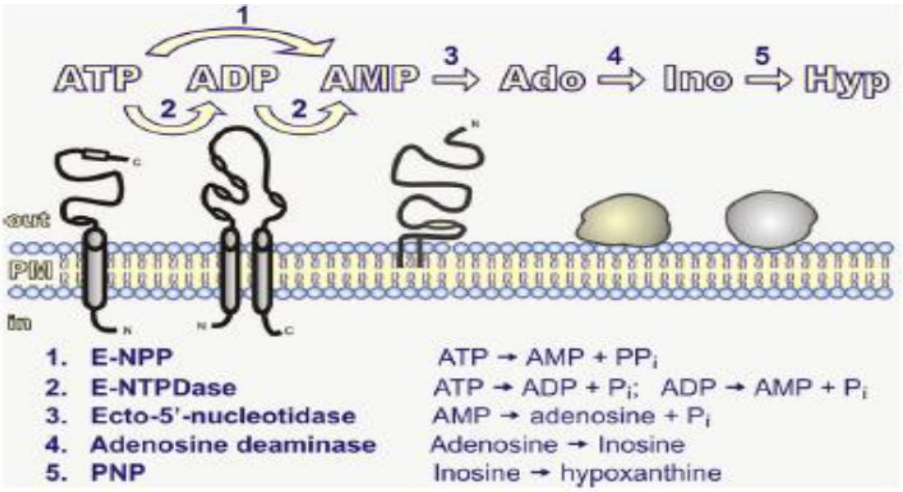

Figura 4: Cascata purinérgica enfatizando a desaminação da adenosina (Ado) em inosina (Ino) pela adenosina desaminase (4)
Fonte: Adaptado de YEGUTKIN, 2008.

1.3.3 Mediadores do metabolismo da adenosina

Apesar dos efeitos da adenosina já terem sido descritos há mais de 70 anos e da atividade de seus receptores já estar descrita desde meados dos anos 70, somente agora o potencial de fármacos que atuam em seu metabolismo está se tornando claro. Considerando que os níveis de adenosina são determinados por uma interação complexa de enzimas e transportadores, vários estudos investigaram

os componentes que contribuem para a determinação da concentração de adenosina extracelular (ZHANG; FRANKLIN; MURRAY, 1993; PAK et al., 1994; LLOYD; FREDHOLM, 1995).

A cafeína é a droga psicoativa mais usada no mundo. Além de a cafeína ser considerada um estimulante leve, está presente em muitas bebidas como chá e café (SMITH, 2002). A ação farmacológica da cafeína ocorre por meio de (1) antagonismo não seletivo dos receptores de adenosina, (2) inibição das fosfodiesterases, (3) ativação de canais de cálcio sensíveis a rianodina encontrados nos retículos endoplasmáticos e sarcoplasmáticos e (4) antagonismo de receptores $GABA_A$ (YAMATO et al., 2002). Esses efeitos podem ser elucidados por diferentes concentrações de cafeína, uma vez que em doses baixas à moderadas, semelhante ao consumo diário de café (cerca de 10 uM), a cafeína atua principalmente como antagonista dos receptores de adenosina e, em doses maiores, inibe a atividade das enzimas fosfodiesterases (CARRILLO; BENITEZ, 2000).

Além da cafeína, outros compostos são capazes de alterar as concentrações de adenosina na circulação. Entre esses, pode-se citar o dipiridamol, que é um composto vasodilatador que aumenta a concentração extracelular de adenosina pela inibição do seu metabolismo através da inibição da ADA, e por impedir a captação da adenosina para dentro dos eritrócitos e células endoteliais vasculares (CHALELA et al., 1993, RAMAKERS et al., 2011). O dipiridamol é um agente anti-trombótico eficaz na prevenção secundária da doença cardiovascular (DIENER et al., 1996; JONES et al., 2004) e atua por vários mecanismos, incluindo efeitos anti-plaquetários, como um vasodilatador potencializando a biodisponibilidade do óxido nítrico, ou, possivelmente, através de suas propriedades antioxidantes via eliminação de radicais peroxila (PEDULLI et al., 1999; VENKATESH et al., 2010).

1.3.4 Dipeptidil peptidase IV

A Dipeptidil peptidase IV (adenosine deaminase binding protein; ADAbp; EC 3.4.14.5, DPP-IV, CD26) é uma glicoproteína multifuncional de membrana, expressa em quase todos os órgãos e tecidos, com maior concentração no túbulo proximal dos rins e sobre a membrana luminal das células epiteliais do intestino delgado

(MENTLEIN, 1999; KOS et al., 2009), além de também estar localizada nas células endoteliais dos vasos sanguíneos, sendo então encontrada como uma enzima solúvel no plasma. Entre as células do sistema imune, a DPP-IV é expressa em linfócitos T-helper ativados e em subconjuntos de macrófagos (MENTLEIN et al., 1984).

É uma protease que cliva os dipeptídeos x-Pro e x-Ala a partir da porção N-terminal de peptídeos e proteínas e é a principal inativadora de hormônios incretinas como GLP-1 e GIP. As ações desses hormônios são fundamentais para a homeostase da glicose pós-prandial e seus efeitos combinados constituem a atividade funcional do eixo enteroinsulinar (DUFFY et al., 2007). Os hormônios incretinas GIP e GLP-1 são peptídios gastrointestinais que provocam a liberação de insulina e estão envolvidos na regulação da homeostase pós-prandial de nutrientes. Estes peptídeos aumentam a liberação de insulina, de uma forma dependente de glicose, minimizando os riscos de hipoglicemia. GLP-1 ainda inibe a secreção de glucagon, estimula a biossíntese de insulina e amplia a magnitude das células beta-pancreáticas (GREEN et al., 2006).

A DPP-IV também pode potencialmente modular as respostas imunes diretamente pela regulação dos linfócitos uma vez que exibe uma função co-estimulatória em linfócitos T (MORIMOTO; SCHLOSSMAN, 1998; REINHOLD et al., 2002), além de desempenhar um papel importante no sistema imune através da sua capacidade de se ligar a ADA (Figura 5) (DONG et al.,1997).

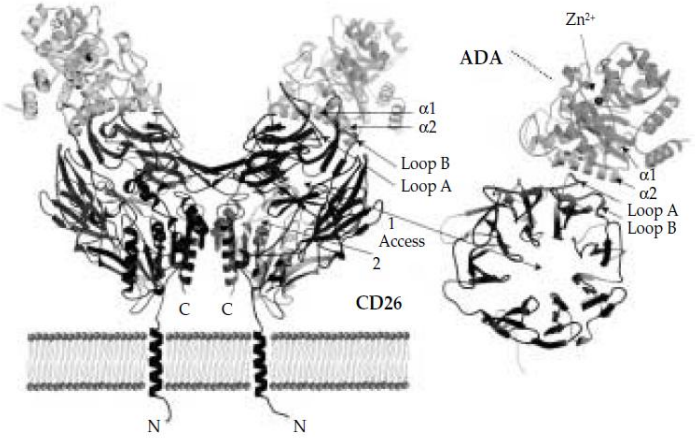

Figura 5: Interação entre ADA e CD26
Fonte: Adapatado de WEIHOFEN et al., 2004.

1.4 Sistema Colinérgico

Os principais componentes do sistema colinérgico são: a acetilcolina (ACh), colina-acetiltransferase (ChAT), o transportador de colina (CHT), o transportador de acetilcolina vesicular (vAChT), os receptores muscarínicos (mAChR) e nicotínicos (nAChR) e a acetilcolinesterase (AChE) (SARTER; PARIKH, 2005).

A ACh, primeira substância encontrada e definida como neurotransmissor, tem se mostrado presente na circulação humana, sendo produzida por linfócitos T e endotélio vascular (WESSLER et al., 2007; WESSLER; KIRKPATRICK, 2008). A acetilcolina atua na proliferação celular, organização cortical do movimento (MESULAM et al., 2002; MORETTO et al., 2004), provoca vasodilatação em artérias coronárias normais, a partir da liberação de óxido nítrico, com consequente aumento do fluxo sanguíneo (COX; VITA; TREASURE, 1989), além de facilitar a liberação de insulina em um modo dependente de glicose, através da ativação de receptores muscarínicos localizados nas células β pancreáticas (GILON; HENQUIN, 2001). Tem sido demonstrado também que a ACh tem papel importante em atenuar o processo inflamatório através da modulação na liberação de citocinas pró-inflamatórias como o fator de necrose tumoral e interleucina 1 (RAO; GUMPENY; DAS, 2007). Essa inibição acontece devido à ligação da ACh em receptores nicotínicos α7, presentes na superfície de macrófagos (ULLOA, 2005).

Assim, devido ao importante papel da ACh na supressão do processo inflamatório, investigar as colinesterases que regulam a sua concentração podem sugerir, mesmo que de forma indireta, os níveis de ACh e, consequentemente, ambas as colinesterases podem servir como marcadores de processos inflamatórios sistêmicos (DAS, 2007).

As colinesterases (ChEs) são classificadas em dois tipos de acordo com as suas propriedades catalíticas e especificidade a substratos, sensibilidade a inibidores e distribuição tecidual: a acetilcolinesterase (AChE, E.C. 3.1.1.7) também chamada de colinesterase verdadeira ou específica e a butirilcolinesterase ou pseudocolinesterase (BuChE, E.C.3.1.8) (MASSOULIÉ et al., 1993) (Figura 6).

Dada sua complexa estrutura e seu amplo modo de ação, as colinesterases parecem desempenhar importante papel no desenvolvimento de diversas patologias,

como resistência à insulina, obesidade, DM2, hipertensão, hiperlipidemias, SMet e doença de Alzheimer (DAS, 2007).

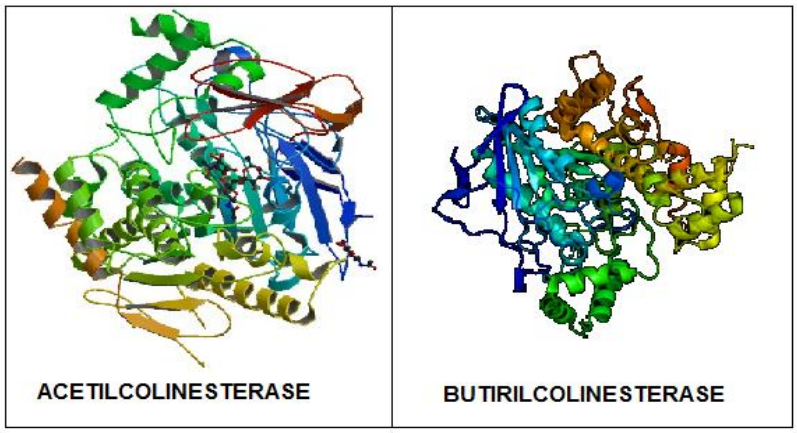

Figura 6 - Estrutura das enzimas AChE e BuChE .
Fonte: Disponível em: http://pt.wikipedia.org/wiki/Colinesterase

1.4.1 Acetilcolinesterase

A AChE é uma importante enzima regulatória que hidrolisa rapidamente o neurotransmissor ACh nas sinapses colinérgicas, bem como nas junções neuromusculares (GRISARU et al., 1999).A hidrólise da ACh ocorre a partir de sua ligação ao resíduo de serina no sítio ativo da enzima, formação do intermediário acetil-AChE e liberação de colina. Em sequência, há a hidrólise desse intermediário liberando acetato, e permitindo o "*turnover*" da enzima (SOREQ; SEIDMAN, 2001).A AChE é uma enzima heterogênea, predominantemente encontrada no cérebro, junção neuromuscular, linfócitos e eritrócitos (COKUGRAS, 2003).

A AChE mostra algumas características não encontradas em algumas outras enzimas, tais como, a organização do sítio ativo e o mecanismo catalítico. O sítio ativo da AChE é encontrado no interior de uma garganta estreita (*gorge*), e consiste de dois subsítios de ligação, um carregado negativamente ou sítio aniônico, e um sítio esterásico (SOREQ; SEIDMAN, 2001), também chamado de tríade catalítica (SHAFFERMAN et al., 1992; TALESA, 2001). Além do sítio catalítico, a AChE apresenta um sítio aniônico periférico (PAS), que está localizado na entrada do sítio

ativo, sendo este o sítio de ligação para inibidores e ativadores alostéricos (TAYLOR; LAPPI, 1975).

Além de seu papel clássico na transmissão colinérgica a AChE tem sido implicada em várias ações não colinérgicas como crescimento dos neuritos (DAY; GREENFIEL, 2002), atividade hematopoiética em células progenitoras do sangue (SOREQ; SEIDMAN, 2001), além de ter sido localizada e identificada em linfócitos onde provavelmente apresenta um papel importante na regulação das funções imunes (KAWASHIMA; FUJII, 2000).

Diversos estudos têm sido desenvolvidos com o objetivo de determinar a atividade da AChE, sendo que alterações na atividade dessa enzima têm sido encontradas em humanos e em modelos experimentais de diabetes (SANCHEZ-CHAVEZ ; SALCEDA, 2000; KUHAD; SHARMA; CHOPRA, 2008; SCHMATZ et al., 2009), em pacientes com SMet (DE BONA et al., 2012) e na leucemia linfoblástica aguda (BATTISTI et al., 2009), entre outros.

1.4.2 Butirilcolinesterase

A BuChE, também conhecida como pseudocolinesterase, colinesterase plasmática ou colinesterase inespecífica (CRAIG; STITZEL, 2005), é amplamente distribuída, sendo principalmente encontrada em plasma, rins, fígado, intestino, pulmão e tem uma distribuição neuronal muito mais restrita que a AChE (MESULAM et al., 2002). A BuChE hidrolisa com maior eficiência a butirilcolina e a propionilcolina do que a acetilcolina (MAIN et al., 1974). Ainda, essa enzima catalisa a hidrólise de uma variedade de outros ésteres, entre eles, agentes utilizados em anestesia como a procaína, ametocaína e bupivacaína (DARVESH; HOPKINS; GEULA, 2003; GALENKO-YAROSHEVSKII, 2003), e outros compostos como cocaína (GATLEY, 1991; CARMONA et al., 2000), heroína (LOCKRIDGE et al., 1980) e aspirina (MASSON et al., 1998).

A BuChE contém um grande número de sítios glicosilados que parecem contribuir com a sua longa meia vida, protegendo-a contra a proteólise e aumentando seu tempo de permanência na circulação sanguínea (NACHON et al., 2002).

Por muito tempo a função fisiológica da BuChE ficou incerta, sendo, por isso, considerada por muitos autores como “não-essencial” (PRIMO-PARMO et al.,1996). Entretanto, estudos sugerem que ela apresenta uma participação nos mais diversos processos fisiológicos. A BuChE possui uma importância toxicológica e farmacológica, pois detoxifica compostos anticolinesterásicos e várias drogas, além de proteger contra o envenenamento por organofosforados (BROOMFIELD et al., 1991; SCHWARZ et al., 1995; COKUGRAS, 2003), pode ser a primeira linha de defesa do organismo contra substâncias tóxicas inaladas ou ingeridas e também parece estar envolvida no metabolismo de lipoproteínas (KUTTY & PAYNE, 1994).

Os níveis de BuChE no soro são influenciados por diversas condições clínicas e estados fisiológicos (FURTADO-ALLE et al., 2008; BOBERG et al., 2011; BENYAMIN et al., 2011). Aumento na atividade da BuChE já foram descritas em casos de disfunções hepáticas (OGUNKEYE; ROLUGA, 2006) e na obesidade (ALCÂNTARA et al., 2005). Além disso, outros estudos demonstraram correlação positiva entre a atividade da enzima BuChE e a idade, sexo, índice de massa corporal (IMC), hipertensão e diabetes, assim como nas hiperlipidemias (KUTTY; PAYNE, 1994; ALCANTARA et al., 2002). Esses resultados reforçam o fato de que a atividade da enzima BuChE está alterada em condições clínicas importantes na patogênese da SMet (ABBOTT et al., 1993; SANCHEZ-CHAVEZ; SALCEDA, 2000; SRIDHAR et al*.,* 2005) e pode servir como um marcador de inflamação sistêmica de baixo grau (DAS, 2007).

1.5 ɣ-glutamiltransferase

A ɣ-glutamiltransferase (GGT, EC 2.3.2.2) é uma ectoenzima transmembrana, com importante papel na clivagem de grupamentos ɣ- glutamil da glutationa (GSH) ou conjugados e assim, atua na transferência desses grupamentos para um aminoácido, di-peptídeo, água ou para outra molécula de GSH (LIEBERMAN et al., 1995). O ciclo ɣ- glutamil envolvendo a GGT é considerado geralmente a principal via pela qual as células utilizam GSH extracelular para a síntese de novo de GSH intracelular (GRIFFITH; BRIDGES; MEISTER, 1978).

A GGT está localizada em vários tecidos humanos, incluindo túbulos proximais renais, canalículos biliares hepáticos, células capilares endoteliais (ECs) e macrófagos teciduais (HANIGNAN; FRIERSON, 1996). O aumento da GGT tem sido demonstrado em células imunológicas ativas tais como linfócitos B e T e macrófagos (GRISK; KUSTER; ANSORGE, 1993). Além disso, estudos têm enfatizado a importância da GGT na modulação da ativação de células T e resposta imune (LAWRENCE, et al., 2000; CARLISLE; KING; KARP, 2003).

Estudos demonstram que as células T quando submetidas a processos inflamatórios e de estresse oxidativo uniformemente expressam altos níveis de GGT, o que pode proporcionar uma vantagem adaptativa ao permitir que estas células tenham uma maior resistência ao estresse oxidativo e / ou se desenvolvam de forma mais eficaz (KARP et al., 1999). Entretanto, o papel da GGT em linfócitos ainda não está totalmente esclarecido, podendo esta enzima também estar envolvida na facilitação da proliferação celular (RAJPERT- DE MEYTS, 1992).

Ainda, a GGT pode ser encontrada no soro, sendo um marcador biológico útil em casos de ingestão alcoólica e doenças hepatobilares, além de ser um forte preditor do aumento da mortalidade cardiovascular (LEE et al., 2007; MASON; STARKE; VAN KIRK, 2010). Estudos também mostram que existe associação da GGT com as diversas morbidades da SMet (BRUCKERT, et al., 2002; SUKUGAWA et al., 2004), incluindo relação da GGT com o aumento do índice de massa corporal (IMC), sugerindo que o peso corporal pode ser o fator principal da elevação dos níveis séricos desta enzima (NAKANISHI et al., 2000; LEE; HÁ; CHRISTIANI, 2001).

1.6 Viabilidade Celular

Os ensaios de viabilidade celular são de extrema importância para diversas aplicações de rotina laboratorial, sendo utilizados para a detecção de citotoxicidade após exposição a substâncias tóxicas (FOTAKIS e TIMBRELL, 2006).

Entre os testes mais utilizados *in vitro* para a avaliação da viabilidade celular, pode-se citar o teste do MTT (brometo de [3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio]). O princípio do método é a redução de um substrato amarelo, o sal de tetrazolium MTT, por enzimas mitocondriais, resultando em um produto azul/roxo

chamado formazan que pode ser quantificado por espectrofotometria. Desta forma essa reação ocorre somente em células vivas e que estejam com suas mitocôndrias ativas, configurando-se assim um ensaio versátil e quantitativo para avaliação da viabilidade celular (MOSMANN, 1983).

Outra forma de se avaliar a viabilidade celular é através do teste do Vermelho Neutro (VN), um corante vital, solúvel em água e que passa através da membrana celular, concentrando-se nos lisossomos, fixando-se por ligações eletrostáticas hidrofóbicas em sítios aniônicos na matriz lisossomal. Muitas substâncias danificam as membranas das células resultando no decréscimo de captura e ligação do VN. Alterações na membrana lisossomal resultam numa incorporação do VN, e desta forma é possível fazer a distinção entre células viáveis e não viáveis pela medida de intensidade de cor da cultura celular (BABICH; BORENFREUND, 1991; MAMACA et al., 2005).Esta metodologia é largamente utilizada com uma sensibilidade satisfatória.

A enzima lactato desidrogenase (LDH) é uma enzima da classe das oxidorredutases que cataliza a oxidação reversível do lactato a piruvato, em presença da coenzima NAD+, que atua como doador ou aceptor de hidrogênio. Esta é uma enzima citoplasmática estável presente em todas as células, é liberada devido à perda da integridade da membrana e pode ser quantificada por espectrofotometria (CHIELLINI, 2006), sendo uma técnica conhecida pela sua velocidade e facilidade de execução (FOTAKIS e TIMBRELL, 2006).

1.7 Plantas medicinais

As plantas representaram, durante séculos, a única fonte de agentes terapêuticos para o homem. No século XIX, com o desenvolvimento da química, as plantas passaram a representar a primeira fonte de substâncias para o desenvolvimento de medicamentos (HOSTETTMANN; QUEIROZ; VIEIRA, 2003; ZARONI et al., 2004; ALBUQUERQUE; HANAZAKI, 2006). No início da década de 1990, a Organização Mundial de Saúde (OMS) divulgou que 65-80% da população dos países em desenvolvimento dependiam das plantas medicinais como única forma de acesso aos cuidados básicos de saúde (AKERELE, 1993).

No Brasil, a primeira descrição sobre o uso de plantas como remédio foi feita por Gabriel Soares de Souza, autor do Tratado Descritivo do Brasil, de 1587 (VEIGA, 2002). Essas espécies utilizadas na sabedoria popular têm se tornado objeto de estudo em muitos países e tem se tornado uma fonte importante de produtos naturais biologicamente ativos, que podem resultar na descoberta de novos fármacos, para as mais diversas doenças. Embora a medicina moderna esteja bem desenvolvida, atualmente, um sentimento geral de decepção com a medicina convencional, o desejo de adotar um estilo de vida “natural”, a facilidade de obtenção e o baixo custo de produção tem levado à utilização crescente de outras formas de terapia. Dentro deste contexto, o Brasil em 2005, através do SUS, propôs a inclusão das plantas medicinais e fitoterapia como opções terapêuticas no sistema público de saúde, contanto que esses produtos a base de plantas atendam a legislação vigente (BRASIL, 2006).

Em especial, muitas espécies de plantas têm sido usadas etnofarmacologicamente ou experimentalmente para tratar dos sintomas do DM em muitos países (LAMBA et al., 2000; NOVAES et al., 2001; MCCUNE; JONHNS, 2002; SAID et al., 2002; VOLPATO et al., 2002; SYEM et al., 2002; ELDER, 2004; SAXENA; VIKRAM, 2004). Esse fato tem causado aumento no número de pesquisas direcionadas à validação das propriedades antidiabéticas, as quais são empiricamente atribuídas a estas plantas, bem como um maior interesse nos estudos dos mecanismos de ação das substâncias responsáveis pela atividade hipoglicemiante (RAMOS et al., 1995).

1.7.1 *Syzygium cumini*

O *Syzygium cumini (L.)* Skeels (Sc) (sin. *Eugenia jambolana* Lam. *Syzygium jambolanum* DC; Figura 7) é uma planta originária da Índia, pertencente a família *Mirtaceae* e conhecida popularmente como Jambolão, que pode ser encontrado em diversos estados do Brasil, incluindo Minas Gerais, Rio de Janeiro, Rio Grande do Sul e São Paulo (BRAGANÇA, 1996).

Figura 7: *Syzygium cumini* (jambolão)
Fonte: EMBRAPA, 2008

O *Syzygium cumini* possui em seus constituintes os flavonoides, as saponinas, os ácidos graxos, o tanino, o eugenol, a antimielina e os triterpenos. Todas as partes da planta são utilizadas para fins medicinais e têm grande tradição na medicina alternativa (SIANI et al*.,* 2000) (Tabela 2). A casca, o fruto, a semente e a folha dessa planta, são frequentemente utilizadas no tratamento do diabetes e administrados na forma de diferentes preparados como o extrato aquoso ou decocção, extrato etanólico ou o suco da planta crua (PEPATO et al., 2001). O *Syzygium cumini* apresenta propriedades hipoglicêmicas, antiinflamatórias, antipiréticas, hipolipidêmicas e antioxidantes, além de ação antiviral e anticarcinogência e há uma variedade de relatos indicando a atividade antidiabética do *Syzygium cumini* com diferentes modelos experimentais (ONG; KHOO, 2000; STANLEY; KAMALAKKANNAN; MENON, 2003; SHARMA et al., 2006; BRAGA et al., 2007; LIMA et al., 2007).

O mecanismo de ação hipoglicemiante dos componentes do *Syzygium cumini*, bem como suas possíveis interações ainda não estão bem esclarecidos. O efeito de redução da glicose sanguínea pelo *Syzygium cumini* pode ser devido a um aumento da secreção de insulina a partir do pâncreas ou pela inibição da degradação de insulina (AYBAR et al., 2001). O *Syzygium cumini* também apresenta efeito na redução de lípidos através da redução dos níveis de colesterol total, triglicérides e ácidos graxos livres (SAGRAWAT; MANN; KHARYA, 2006). Este efeito tem sido relacionado à presença de flavonoides, saponinas e glicosídeos no

extrato, que podem levar a uma redução na atividade da enzima 3-HMG Co-A redutase no fígado (RAVI; RAMACHANDRAN; SUBRAMANIAN, 2004).

Entretanto, estudos mostraram controvérsias com relação aos efeitos hipoglicemiantes do *Syzygium cumini.*. Segundo TEIXEIRA e colaboradores (2000), o tratamento com extrato cru preparado das folhas em ratos com DM induzido por estreptozotocina não apresentou ação antihiperglicemiante, mostrando ainda haver controvérsias em relação às propriedades medicinais do *Syzygium cumini*. Estudos realizados em nosso laboratório demonstraram efeito do extrato aquoso das folhas de *Syzygium cumini* (ASc) sobre a atividade da enzima ADA, reduzindo-a em eritrócitos e soros de pacientes hiperglicêmicos e em membranas de eritrócitos (BOPP et al., 2009; DE BONA et al, 2011), bem como sobre a atividade das enzimas ADA e 5'NT em plaquetas de pacientes com DM tipo 2 (DE BONA et al., 2010).

Parte da planta	Uso popular
Folhas	diabetes, ação hipotensiva, diuréticas, adstringência, obstipação, leucorréia, estômago, cataplasma em doenças de pele, acalmar prurido, antiinflamatória.
Casca	atividade antidiarréica, ação inibitória contra atividade do HIV - 1 protease, hipoglicemiante, adstringência e obstipação, e é utilizada no tratamento de hemorragias e leucorréia, diabetes e úlcera venérea, indigestão e purificação do sangue, disenteria, dispepsia, anti-séptico, adstringente em ulcerações bucais, gengivas esponjosas e estomatite, inflamações locais, queimaduras, cardiotônico e estimulante do SNC, antipirético.
Sementes	anticonvulsivante, hipoglicemiante, adstringente e obstipação, atividade eupéptica, anti-hemorrágica, para alterações no estômago, antiinflamatória, bactericida, diarréia, diabetes, disenteria e hipertensão.
Frutos	hipoglicemia, adstringência e obstipação, diuréticos e estomáticos, tratamento gastrintestinal, adstringente e oralmente para úlcera de estômago, redução de acidez e diabetes, carminativo, antiescorbútico e diurético, diarréia aguda e crônica, retenção urinária, gargarejo para irritações da garganta, loção descamação do couro cabeludo, antiinflamatório, antipirético, adstringente, no tratamento de disenteria, diarréia e diabetes.
Flores	atividades antibióticas.
Raiz	antiemético, aumentar a lactação em lactantes.

Tabela 2: Uso popular do *Syzygium cumini*
Fonte: Adaptado de MIGLIATO et al., 2006.

Assim, pelo fato da hiperglicemia ser um dos componentes da SMet e uma das principais características dos pacientes com DM, e como estudos anteriores em

nosso laboratório já demonstraram efeitos protetores provocados pelo *Syzygium cumini*, *in vitro*, em pacientes diabéticos (DE BONA et al., 2010 e 2011), o mecanismo de ação do extrato aquoso de *Syzygium cumini* (ASc) através da exposição de eritrócitos à hiperglicemia, bem como a avaliação dos seus efeitos citoprotetores em condições de estresse oxidativo são objetivos deste estudo. Isto porque, em função das propriedades apresentadas pelo *Syzygium cumini*, é necessário estimular a realização de estudos científicos que comprovem o conhecimento popular existente sobre esta planta e a eficácia no tratamento das doenças, evitando assim, os malefícios decorrentes do uso indevido e proporcionando aumento dos benefícios na utilização das plantas medicinais.

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar parâmetros bioquímicos e inflamatórios em pacientes com SMet e verificar o efeito do extrato aquoso das folhas de *Syzygium cumini* sobre marcadores bioquímicos, de estresse oxidativo e viabilidade celular em eritrócitos e linfócitos expostos a condições hiperglicêmicas e oxidativas, respectivamente, *in vitro*.

2.2 Objetivos Específicos

2.2.1 Em pacientes com SMet e Saudáveis:

Em linfócitos, avaliar:

- A atividade das enzimas adenosina desaminase (ADA), dipeptidil peptidase IV (DPPIV), acetilcolinesterase (AChE),) e Gama Glutamiltransferase (GGT), bem como os níveis de espécies reativas ao ácido tiobarbitúrico (TBARS);

Em soro/plasma, avaliar:

- A atividade das enzimas ADA, DPPIV e butirilcolinesterase (BuChE) e parâmetros de estresse oxidativo, tais como os níveis de TBARS, Vitamina C e grupamentos sulfidrílicos proteicos;

- Os níveis de Proteína C reativa (CRP) e óxido nítrico (NO), além de parâmetros bioquímicos rotineiros;

Em eritrócitos, avaliar:

- Os níveis de grupamentos sulfidrílicos não protéicos;

2.2.2 Análises *in vitro:*

2.2.2.1 Em eritrócitos de controles saudáveis expostos à condições hiperglicêmicas, avaliar:

- O efeito do extrato aquoso de *Syzygium cumini* e de compostos isolados, sobre a atividade da enzima ADA;

- O mecanismo de ação pelo qual o extrato aquoso de *Syzygium cumini* age sobre a ADA em condições hiperglicêmicas;

2.2.2.2 Em linfócitos de controles saudáveis, através de um modelo de indução de estresse oxidativo *in vitro*, avaliar:

- a viabilidade celular, atividades enzimáticas e parâmetros de estresse oxidativo, bem como o efeito do extrato aquoso de *Syzygium cumini* e do ácido gálico sobre estes parâmetros

3.ARTIGOS

3.1 Lymphocytic enzymes and lipid peroxidation in patients with metabolic syndrome.

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Lymphocytic enzymes and lipid peroxidation in patients with metabolic syndrome

Karine S. De Bona [a], Gabriela Bonfanti [a], Lariane O. Cargnelutti [b], Paula E.R. Bitencourt [b], Priscila S. da Silva [b], Ronise Ceolin [b], Victor C. Pimentel [c], Maria Beatriz Moretto [a,b,*]

[a] Postgraduate Program in Pharmacology, Department of Clinical and Toxicology Analysis, Center of Healthy Sciences, Federal University of Santa Maria (UFSM), 97105-900, Santa Maria, RS, Brazil
[b] Postgraduate Program in Pharmaceutical Sciences, UFSM, Santa Maria, Brazil
[c] Department of Chemistry, UFSM, Santa Maria, Brazil

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ABSTRACT

Objectives: Metabolic syndrome (MetS) is considered a state of chronic inflammation. This study aimed to ascertain selected parameters of purinergic and cholinergic systems related to glucose metabolism and inflammation, as well as $_{\gamma}$-glutamyltransferase (GGT) and *N*-acetyl-b-glucosaminidase (NAG) activities and lipoperoxidation in lymphocytes of patients with MetS.

Design and methods: The adenosine deaminase (ADA), dipeptidyl peptidase IV (DPP-IV), acetylcholinesterase (AChE), GGT and NAG activities, as well as thiobarbituric acid reactive substances (TBARS) levels were investigated in lymphocytes of patients with MetS ($n=38$) and healthy volunteers ($n=41$). We also evaluated the insulin levels, anthropometric measurements and routine biochemical analyses.

Results: ADA ($p<0.05$), DPP-IV and AChE ($p<0.0001$) activities were higher in patients with MetS when compared to the control group. Furthermore, we observed correlations between ADA and DPP-IV activities ($p=0.0002$; $r=0.5945$), TBARS levels and ADA ($p=0.0021$; $r=0.5172$) and DPP-IV activities ($p=0.0022$; $r=0.5010$).

Conclusions: Our findings showed that MetS might cause tissue distress that disturbed lymphocytic ADA, DPP-IV and AChE activities in response to inflammatory stimuli. These alterations evidence clinical abnormalities, since these enzymatic systems are able to regulate several aspects of adipose tissue function and inflammatory state of MetS and could be used successfully both for preventing and for halting the progression of MetS.

Introduction

Metabolic syndrome (MetS) describes the clustering of abdominal obesity, lipid abnormalities, hypertension, and hyperglycemia. It is considered a strong, independent contributor to the onset of coronary heart disease and type 2 diabetes mellitus, being a major cause of morbidity and mortality in diabetic and obese patients [1,2]. The prevalence of MetS has been rapidly increasing worldwide not only in industrialized countries but also in developing countries associated with an increase in food intake [3].

The mechanisms underlying the pathogenesis of MetS are complex because they are influenced by different biological pathways including inflammation, metabolism and oxidative stress that can predict cardiovascular events [4,5]. In addition, MetS is associated with defects in the microvascular mechanisms of coronary blood flow control. During systemic inflammation, the extracellular concentration of the endogenous nucleoside adenosine increases rapidly [6]. In particular, this nucleoside can interfere with the biosynthesis of proinflammatory cytokines, it also down-regulates neutrophil functions and plays an important role in the maintenance of coronary blood flow, particularly during episodes of cardiac ischemia [7,8].

Metabolically, adenosine deaminase (ADA; EC 3.5.4.4) is involved in the purine catabolism, catalyzing the deamination of adenosine into inosine and its main physiological activity is related to T lymphocytic proliferation and maturation [9]. ADA is widely distributed in human tissues especially in lymphoid tissues [10]. Extracellularly, ADA interacts with membrane proteins, such as dipeptidyl peptidase IV (CD26/DPP-IV; EC 3.4.14.5) [11], which plays the key role in modification, processing, and/or inactivation of peptides, such as peptide hormones, various chemokines, neuropeptides, and growth factors, and in association with ADA, may be directly involved in T-cell activation [12,13].

* Correspondent author at: Department of Clinical and Toxicology Analysis, Center of Healthy Sciences, Federal University of Santa Maria (UFSM), 97105–900 - Santa Maria, RS, Brazil. Fax: +55 3220 8018.
E-mail addresses: ksbona@yahoo.com.br (K.S. De Bona), gabriela_bonfanti@yahoo.com.br (G. Bonfanti), lari.o.c@hotmail.com (L.O. Cargnelutti), paulinha.farma.bitencourt@hotmail.com (P.E.R. Bitencourt), pry_sabino@hotmail.com (P.S. da Silva), roniceolin@yahoo.com.br (R. Ceolin), victor.capi@yahoo.com.br (V.C. Pimentel), Beatriz@smail.ufsm.br, beatriz.moretto@yahoo.com.br (M.B. Moretto).

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Acetylcholinesterase (AChE, EC 3.1.1.7), another important enzyme expressed both in T and B lymphocytes [14], promotes the hydrolysis of the neurotransmissor acetylcholine and appears to be involved in the regulation of several biological functions, such as proliferation, differentiation, organization of the cytoskeleton, cell–cell contact or immune functions [15,16], besides serving as a marker of low-grade systemic inflammation. Furthermore, cell surface expression of the enzyme γ-glutamyltransferase (GGT; EC 2.3.2.2) increases markedly with T-cell activation, and this enzyme is a clinical marker of MetS [17]. Similary, *N*-acetyl-b-glucosaminidase (NAG; EC 3.2.1.30) has been proposed to play a role in the pathogenesis of chronic inflammation [18].

Lymphocytes are important immunological cells present in blood in large quantity. The changes occurring in these cells have been associated with alterations in the intracellular signaling pathways [19] as well as the pathological processes that contribute to MetS. Taking into account that MetS is considered a state of chronic inflammation associated to high mortality and possible changes in adenosinergic and cholinergic systems in MetS have not been evaluated up to this moment, the aim of this study was to evaluate the ADA, DPP-IV, and AChE activities in lymphocytes of patients with MetS. Moreover, lymphocytic GGT and NAG activities and lipoperoxidation were also examined.

Materials and methods

Study population

The sample consisted of 38 patients diagnosed with MetS (9 males and 29 females, aged 47.32 ± 11.51 years) (Table 1) before inclusion in a physical activity program of Department of Physical Education of Federal University of Santa Maria (UFSM). The controls consisted of 41 healthy volunteers (8 males and 33 females) with ages similar to those of the patients (aged 45.68 ± 8.54 years) (Table 1). The condition-specific cutoff points for MetS were applied based on the criteria of the National Cholesterol Education Program's Adult Treatment Panel, with some modifications [20] and is based on simple clinical and biochemical parameters. The subjects were classified as having MetS if they had three or more risk factors, which included obesity (waist circumference larger than 40 in. [or 102 cm] for men or 35 in. [or 88 cm] for women), hypertension (over 135 systolic or over 85 diastolic pressure), fasting hypertriglyceridemia (over 1.69 mmol/L), low high-density lipoprotein (HDL) cholesterol (lower than 1.03 mmol/L for men or 1.29 mmol/L for women) and fasting hyperglycemia (currently, glucose over 5.55 mmol/L).

Characteristics such as smoking and alcohol habits, and medication, were investigated by individual interviews, using a structured questionnaire, and all volunteers (MetS patients and healthy subjects) were informed about the nature of the study and their consent to participate of the study was obtained. The medication used included antihypertensives (31.58%), hypoglycemics (21.05%) or hypolipidemics agents (10.52%), to an adequate control of the conditions of MetS patients. Purinergic and cholinergic systems can be influenced by smoking, physical activity and other clinical or environmental risk factors. For this reason, alcoholic and smoking subjects, pregnant women, and people with cancer were excluded, and patients with diabetes mellitus were included in this study.

The study was in accordance with the guidelines of the Ethics Committee of the Federal University of Santa Maria, which approved the experimental protocol (0049.0.243.000-08), and with the Declaration of Helsinki (2000) of the World Medical Association.

Table 1
General and biochemical characteristics of the experimental groups.

	Control	MetS	*p*
n	41	38	–
♀/♂	33/8	29/9	–
Age (years)	45.68 ± 8.54	47.32 ± 11.51	ns
Weight (kg)	70.35 ± 17.07	91.28 ± 16.37	$p<0.0001$
Height (m)	1.64 ± 0.08	1.64 ± 0.09	ns
BMI (kg/m^2)	26.08 ± 4.93	33.85 ± 5.53	$p<0.0001$
WC (cm)	91.2 ± 13.01	111.2 ± 12.21	$p<0.0001$
FBG (mmol/L)	4.90 ± 0.82	6.00 ± 1.44	$p<0.0001$
TC (mmol/L)	4.67 ± 0.97	4.45 ± 0.75	ns
HDL-C (mmol/L)	1.33 ± 0.34	1.25 ± 0.42	ns
LDL-C (mmol/L)	2.6 ± 0.67	2.37 ± 0.76	ns
TG (mmol/L)	1.06 ± 0.44	1.99 ± 1.05	$p<0.0001$
Uric acid (mmol/L)	0.23 ± 0.07	0.22 ± 0.08	ns
Urea (mmol/L)	5.70 ± 1.63	5.94 ± 1.22	ns
Creatinine (μmol/L)	79.91 ± 18.56	82.21 ± 15.91	ns
Serum protein (g/L)	5.37 ± 0.39	5.87 ± 0.58	$p<0.0001$
Insulin (μUI/mL)	8.54 ± 9.33	12.54 ± 7.03	$p<0.05$
HOMA-IR index	1.95 ± 0.40	3.18 ± 0.34	$p<0.05$

Data are presented as mean ± SD. Statistically significant differences from controls, as determined by Student's *t* test; BMI = body mass index; WC = waist circumference; FBG = fasting blood glucose; TG = triglycerides; TC = total cholesterol; HDL-C = HDL cholesterol; LDL-C = LDL cholesterol; HOMA-IR = homeostasis model assessment.

Anthropometric measurements and biochemical parameters

In addition, height, weight, and body mass index (BMI) (calculated as the weight in kg divided by the square of the height in meters, kg/m^2) were estimated. Waist circumference (WC) was measured with a paper tape horizontally at the umbilicus in the standing position after normal expiration. The biochemical parameters Total cholesterol (TC), triglycerides (TG), HDL- cholesterol (HDL-C), blood glucose (FBG), creatinine, urea, uric acid, and serum protein were measured during fasting by standard methods with commercial kits. LDL-cholesterol (LDL-C) was estimated indirectly using the Friedewald's formula , following the criteria established [21]. The triglyceride concentrations were <4.5 mmol/L in all samples analyzed. Fasting plasma insulin was determined by immunoluminometric assay. The homeostasis model assessment (HOMA-IR) was used to estimate insulin resistance, which indicates proneness to developing MetS, using the formula: fasting plasma insulin (μUI/mL) × glucose (mmol/L)/22.5 [22].

Isolation of the cells

Lymphocytes were isolated from human blood collected in Vacutainer tubes (BD Vacutainer, Franklin Lakes, NJ, USA) containing lithium-heparin and separated on Ficoll-Histopaque density gradients, as described by Böyum [23]. Cell number and viability were determined by trypan blue exclusion. More than 95% of the cells were found to be viable. Final cell suspension was performed in phosphate buffered saline (PBS, pH 7.4) and 3×10^6 cells/mL were used for each analysis.

ADA activity assay

ADA activity was measured spectrophotometrically using the method of Giusti and Gakis [24], which is based on the direct measurement of the formation of ammonia produced, when ADA acts in excess of adenosine. Results were expressed in U/L.

DPP-IV activity assay

DPP-IV activity was determined as described by Schön et al. [25]. Lymphocytes suspension was incubated with 0.6 mM of Gly-Pro p-nitroanilide p-toluenesulfonate for 60 min at 37 °C. The reaction was stopped by adding 1 M acetate buffer (pH 4.5) and the absorbance was measured at 390 nm. The values were expressed in U/L

AChE activity assay

After isolation of the lymphocytes, AChE activity was determined according to the method described by Ellman et al. [26] modified by Fitzgerald and Costa [27]. 0.2 mL of intact cells were added to a solution containing 1.0 mM acetylthiocholine, 0.1 mM 5,5′-dithiobis (2-nitrobenzoic acid), and 0.1 M phosphate buffer (pH 8.0). Immediately before and after incubation for 30 min at 27 °C the absorbance was read on a spectrophotometer at 412 nm. The results are expressed as μmol AcSCh/h/mL.

NAG activity assay

The NAG activity was determined as described by Xavier et al. [28], with some modifications. Samples were incubated with p-nitrophenyl-*N*-acetyl-beta-d-glucosaminide (pH 4.5) for 10 min at 37 °C, and the reaction was quenched by the addition of glycine buffer (pH 10.6). The extent of hydrolysis of the substrate was determined by measuring the absorption at 400 nm. The results were expressed in μmol/L.

GGT activity assay

Lymphocyte GGT activity was measured using commercial kits and the results were expressed in U/L.

Thiobarbituric acid reactive substances

Lipid peroxidation was estimated in lymphocytes by measurement of thiobarbituric acid reactive substances (TBARS) according to the method of Lapenna et al. [29]. The reaction product was measured spectrophotometrically at 532 nm and the results were expressed in nmol MDA/mL.

Statistical analysis

Since data had normal distribution, according to Kolmogorov–Smirnov (KS) test, Student's *t* test was used to check difference between the groups. All the data are expressed as mean ± standard deviation (SD). The correlations were assessed by Pearson rank correlation coefficient. Differences were considered significant when the probability was $p \leq 0.05$. All the statistical analyses were conducted using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

General characteristics of the study population

We observed differences between patients with MetS and controls in some anthropometric and biochemical characteristics as weight, BMI, WC, FBG, TG, serum protein, insulin levels and HOMA-IR index, as demonstrated in Table 1.

Enzymatic assays

Both ADA and DPP-IV activities were markedly higher in lymphocytes of patients with MetS (ADA = 8.44 ± 3.79 U/L, $p<0.05$; DPP-IV = 74.26 ± 37.84 U/L, $p<0.0001$) when compared to the control group (ADA = 6.86 ± 2.52 U/L; DPP-IV = 46.02 ± 20.93 U/L) (Table 2). Experimental data also demonstrated that patients with MetS showed a significant increase on AChE activity when compared to the values of healthy subjects (MetS = 0.16 ± 0.052 vs. C = 0.11 ± 0.028 μmol AcSCh/h/mL; $p<0.0001$) (Table 2). However, no significant difference was found in NAG (MetS = 37.46 ± 18.75 vs. C = 49.06 ± 30.74 μmol/L) and GGT (MetS = 5.56 ± 3.09 vs. C = 5.22 ± 2.99 U/L) activities between the groups studied (Table 2).

Table 2
Enzymatic activities and lipid peroxidation of controls and patients with MetS.

	Control	MetS	*p*
ADA activity (U/L)	6.86 ± 2.52	8.44 ± 3.79	$p<0.05$
DPP-IV activity (U/L)	46.02 ± 20.93	74.26 ± 37.84	$p<0.0001$
AChE activity (μmol AcSCh/h/mL)	0.11 ± 0.028	0.16 ± 0.052	$p<0.0001$
NAG activity (μmol/L)	49.06 ± 30.74	37.46 ± 18.75	ns
GGT activity (U/L)	5.22 ± 2.99	5.56 ± 3.09	ns
TBARS levels (nmol MDA/mL)	5.32 ± 2.385	4.67 ± 1.384	ns

Data are presented as mean ± SD. Statistically significant differences from controls, as determined by Student's *t* test; ADA: adenosine deaminase; DPP-IV: dipeptidyl peptidase IV; AChE: acetylcholinesterase; NAG: *N*-acetyl-b-glucosaminidase; GGT: γ-glutamyltransferase; TBARS: thiobarbituric acid reactive substances.

Lipoperoxidation measure

No significant difference was found between the groups in lymphocytes TBARS levels (MetS = 4.67 ± 1.384 *vs* C = 5.32 ± 2.385 nmoL MDA/mL) (Table 2).

Correlations between analyzed parameters

Experimental data demonstrated some correlations between the parameters analyzed in patients with MetS. Interestingly, we observed a positive correlation between ADA and DPP-IV activities ($p = 0.0002$; $r = 0.5945$; Fig. 1); TBARS levels and ADA ($p = 0.0021$; $r = 0.5172$; Fig. 2A) and DPP-IV ($p = 0.0022$; $r = 0.5010$; Fig. 2B) activities.

Discussion

The results of the present investigation indicated for the first time that ADA, DPP-IV, and AChE activities are increased in lymphocytes of patients with MetS (Table 2). Stressful conditions such as inflammation can markedly increase adenosine levels [6], since it directly leads not only to the active release of adenine nucleosides, such as adenosine triphosphate (ATP), but also to the passive release due to endothelial cell damage; ATP is then quickly converted into adenosine [30]. In this regard, the strong ADA activity in response to MetS (Table 2) may modulate the adenosine regulatory mechanisms in lymphocytes. Also, we can think that the increased ADA activity in lymphocytes may evidence clinical abnormalities since adenosine can regulate several aspects of adipose tissue function including

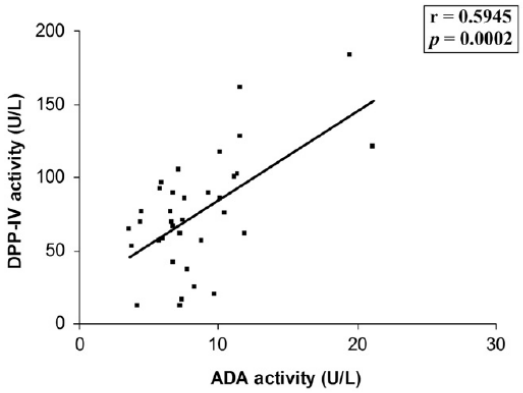

Fig. 1. Significant correlation between ADA and DPP-IV activities ($p = 0.0002$; $r = 0.5945$).

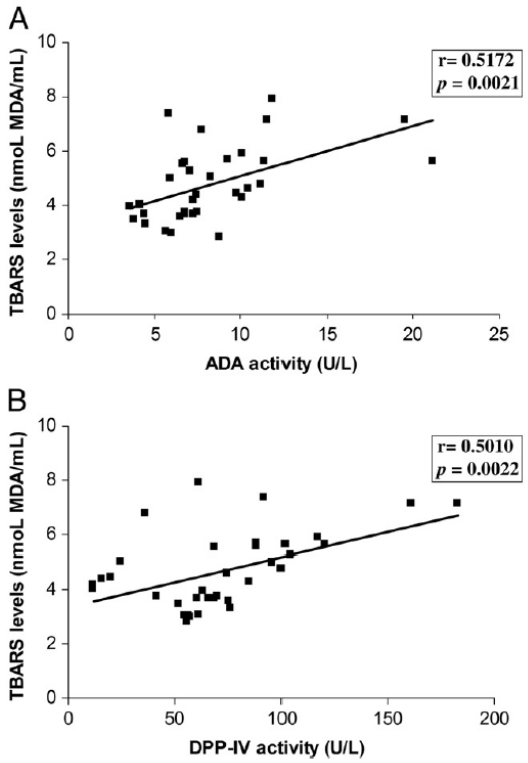

Fig. 2. Significant correlations between (2A) ADA activity and TBARS levels ($p = 0.0021$; $r = 0.5172$), (2B) DPP-IV activity and TBARS levels ($p = 0.0022$; $r = 0.5010$).

lipolysis [31,32] presents in the MetS patients. In addition , the ADA estimation may be used as a biochemical test for rapid preliminary evaluation of the severity of disease and immune performance in MetS.

In the same manner, the increase of DPP IV- activity observed in the MetS (Table 2) may be related to the interaction of ADA and DPP-IV at the T cells resulting in co-stimulatory signs responsible for the activation of the T-cell receptor [33]. In this context, DPP-IV activity may be required for T-cell activation cascade together with ADA, which has an essential role in the development of immune responses by reducing local concentration of adenosine [34,11]. Corroborating with this possibility, a positive correlation between ADA and DPP-IV activities was obtained (Fig. 1). Although previous findings in our laboratory have demonstrated an increase in the ADA activity without changes in DPP-IV activity in lymphocytes of patients with type 2 diabetes mellitus [35], in this study the increased activity of DPP-IV may be linked with several components of MetS including hyperinsulinemic obesity [36], adiposity (BMI), dyslipidemia (high triglyceride levels) and systemic inflammation.

Patients with MetS showed a significant increase in the AChE activity (Table 2), which may be explained as an inflammatory response caused by the insulin resistance state present by the MetS. When the AChE activity is increased, it occurs consequently a rapid degradation of acetylcholine, which is considered a protein with anti-inflammatory action, and it is probably involved in regulating immune functions in lymphocytes [14]. Also, some recent experimental evidence indicates that a central acting AChE inhibitor attenuates the inflammatory state, reduces body weight, and alleviates hyperglycemia, hypercholesterolemia, and insulin resistance in mice with high-fat diet–induced obesity [37]. Although the role of lymphocyte cholinergic system is still unclear, the demonstration of elevated AChE activity supports the view that a cholinergic systems may contribute to the regulation of immune function.

Contrary to our expectations, no differences were observed in TBARS levels between the studied groups (Table 2), although the positive correlation between ADA activity and TBARS levels in patients with MetS was verified (Fig. 2A). Since we observed an increase in ADA activity in patients with MetS, levels of adenosine and its antioxidant capacity were consequently altered which are therefore related to an increase in TBARS levels.

Interestingly, we also observed a positive correlation between DPP-IV activity and TBARS levels in lymphocytes of patients with MetS (Fig. 2B). Studies suggest that chronic hyperglycaemia could lead to the stimulation of DPP-IV activity [38] and is the key promoter, of reactive oxygen species and advanced glycation end products production [39]. Thus, considering that the redox potential is an important determinant of immune cell activity, the DPP-IV activity could be correlated with lipoperoxidation in these cells.

Men and women differ substantially in regard to degrees of insulin resistance, body composition and lifestyle. These differences may contribute to explain the number of women and men involved in our study (Table 1). The prevalence of women in MetS group corroborates with other studies [40] and has been proposed to result from genetic as well as environmental factors that include physical activity and nutrition, that can contribute to development of MetS.

Conclusion

In conclusion, our study has shown that ADA, DPP-IV and AChE activities are increased in lymphocytes of patients with MetS demonstrating that MetS might cause tissue distress to the point of disturbing the enzymatic activities investigated. The significant ADA, DPP-IV and AChE activities supports the view that this enzymes may evidence clinical abnormalities since its can regulate several aspects of adipose tissue function and the inflammation condition of the MetS state. We must make clear that the limitation of our study was the number of samples selected to maintain the criteria used in the design experimental. Even in light of these results, specific trials are needed for a final answer. However, we do believe that the results of this study may contribute not only to give a possible marker to assess the significance of immune function in MetS, but also to raise the alert for a need of estimations of assessments in the clinical practice to prevent MetS complications.

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3.2 Butyrylcholinesterase and γ-glutamyltransferase activities and oxidative stress markers are altered in Metabolic Syndrome, but are not affected by Body Mass Index.

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Butyrylcholinesterase and γ-Glutamyltransferase Activities and Oxidative Stress Markers Are Altered in Metabolic Syndrome, But Are Not Affected by Body Mass Index

Karine S. De Bona,[1] Gabriela Bonfanti,[1] Paula E. R. Bitencourt,[2] Lariane O. Cargnelutti,[2] Priscila S. da Silva,[2] Leidiane De Lucca,[2] Victor C. Pimentel,[3] Etiane Tatsch,[2] Thissiane L. Gonçalves,[2] Melissa Premaor,[1] Rafael N. Moresco,[1,2] and Maria Beatriz Moretto[1,2,4]

Abstract—Metabolic syndrome (MetS) leads to changes in enzymatic activities, oxidative and inflammatory parameters. Adenosine deaminase (ADA), dipeptidyl peptidase IV (DPP-IV), butyrylcholinesterase (BuChE) and γ-glutamyltransferase (γ-GT) activities, C-reactive protein (hsCRP) and nitric oxide levels (NOx), as well as oxidative stress markers were analyzed in 39 subjects with MetS and 48 controls. Also, the influence of body mass index (BMI) and anthropometric measurements were evaluated. Disturbances in antioxidant defenses and higher γ-GT and BuChE activities, NOx and hsCRP levels were observed in subjects with MetS. These findings remained associated with MetS after adjustment for BMI, except for hsCRP. ADA was correlated with age, insulin levels and HOMA-IR index in MetS. DPP-IV and total cholesterol (TC), BuChE activity and TC, and VIT C and hsCRP levels also were correlated. The analyzed parameters may reflect the inflammatory state of the MetS, and could contribute to prevention and control of various aspects of this syndrome.

KEY WORDS: body mass index; butyrylcholinesterase; metabolic syndrome; nitric oxide; γ-glutamyltransferase.

INTRODUCTION

The metabolic syndrome (MetS) is a multi-component disorder characterized by abdominal obesity, dyslipidemia, hypertension, and impaired insulin sensitivity, which has received increased attention as an important epidemiological tool for predicting cardiovascular disease and type 2 diabetes [1]. The etiology of MetS results of a complex interaction between genetic, metabolic, and environmental factors, including dietary habits and the quality of dietary fat [2]. Globally, the overall prevalence of MetS may vary according to the population, gender, age, geographic location, other correlated variables, and the diagnostic criteria used [3], and, in studies conducted in Brazil, MetS prevalence ranged from 19 to 25 % in urban populations [4].

Chronic subclinical inflammation is an intrinsic feature of MetS and during the systemic inflammation, the extracellular concentration of the endogenous nucleoside adenosine increases rapidly [5]. Adenosine deaminase (ADA; EC 3.5.4.4) is important in the degradation of endogenous adenosine and in the acute and protracted inflammatory responses [6]. Furthermore, it is known that ADA activity is elevated in diabetes and in hyperglycemic subjects and correlates positively with glucose levels [7, 8], besides being an important enzyme for modulating the bioactivity of insulin [9].

In addition, the enzyme dipeptidyl peptidase IV (DPP-IV, CD26, EC 3.4.14.5) plays an important role in the immune system *via* its ability to bind ADA. Taken together, the co-localization and interaction of the two molecules may result in regulation of extracellular concentration of adenosine [10]. Serum DPP-IV activity has been measured in

[1] Postgraduate Program in Pharmacology, Department of Clinical and Toxicology Analysis, Center of Healthy Sciences, Federal University of Santa Maria (UFSM), 97105-900 Santa Maria, RS, Brazil
[2] Postgraduate Program in Pharmaceutical Sciences, UFSM, Santa Maria, Brazil
[3] Department of Chemistry, UFSM, Santa Maria, Brazil
[4] To whom correspondence should be addressed at Postgraduate Program in Pharmacology, Department of Clinical and Toxicology Analysis, Center of Healthy Sciences, Federal University of Santa Maria (UFSM), 97105-900 Santa Maria, RS, Brazil. E-mail: beatriz@smail.ufsm.br

several immune disorders [11] and studies reported that the degree of DPP-IV activity was associated with obesity and diabetes mellitus [12, 13]. Although recent studies in our laboratory demonstrated that ADA and DPP-IV activities increased in lymphocytes of subjects with MetS [14], the precise role of these enzymes in various clinical and metabolic disorders including MetS is not well defined.

Other inflammation-related biochemical analytic is known to change with obesity in some mammalian species. For instance, studies demonstrated that Butyrylcholinesterase (E.C. 3.1.1.8, BuChE) activity that hydrolyzes acetylcholine, a molecule involved in the modulation of the immune cell activity is increased in human obesity [15]. Although its biological role has not been clearly established, this marker has been associated with inflammation, lipid metabolism, hypertension, and type 1 and type 2 diabetes mellitus suggesting the relationship of BuChE to MetS [16, 17].

MetS, besides being an inflammatory condition, is reported to be associated with vascular endothelial dysfunction and oxidative status [18]. Endothelial dysfunction has been considered to be a hallmark in the pathophysiology of MetS and has been associated with impaired metabolism and function of endothelial nitric oxide (NO); however, studies on serum NO levels in these subjects have been contradictory [19, 20]. An inappropriate release of this mediator may contribute to the development of clinically significant atherosclerosis and increase the tendency for thrombus formation in this syndrome [21]. Moreover, excessive free radical production and oxidative damage appear to explain, at least in part, the perpetuation of insulin resistance, altered energy production, and endothelial dysfunction in MetS [22].

The wide spectrum of changes that characterize the MetS, the impact in quality of life, and the importance of detection of markers could contribute to prevention and control of this syndrome and increase the interest in investigating these issues. Thus, the main aim of this study was to assess the ADA, DPP-IV, γ-glutamyltransferase (γ-GT) and BuChE activities, high sensitivity C-reactive protein (hsCRP) and NO levels, as well as oxidative stress markers in samples of subjects with MetS. In addition, we verified the influence of body mass index (BMI) on these parameters in MetS subjects studied.

MATERIALS AND METHODS

Chemicals

Adenosine, acetylthiocoline iodide and 5,5′dithiobis-2-nitrobenzoic acid (DTNB) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade and were obtained from standard commercial suppliers.

Study Population

The study was in accordance with the guidelines of the Ethics Committee of the Federal University of Santa Maria which approved the experimental protocol (23081.0040681/2008-76). The clinical trial registration number is 0049.0.243.000-08. All volunteers were informed about the nature of the study and their consent to participate in the study was obtained.

Altogether, 87 volunteers were enrolled in the study (Fig. 1). Blood samples were obtained from 48 healthy subjects (9 males and 39 females, 42.96±8.81 years old) selected in Center of Healthy Sciences of Federal University of Santa Maria (UFSM). In the control group, we selected those people who did not have characteristics related to MetS. This information was obtained through a questionnaire applied to people and was based on the results of the biochemical and anthropometric parameters of the same. Also, the volunteers selected as the control group did not practice physical activity regularly.

The MetS group included 39 subjects (9 males and 30 females, aged 47.55±11.44 years old) selected before the onset of their physical activities in a physical activity program of the Department of Physical Education of the UFSM. The subjects did not perform any physical activity until the time of sample collection.

MetS cases were selected according to the National Cholesterol Education Program's Adult Treatment Panel, with some modifications [1] based on the presence of three or more of the following five factors:

(a) waist circumference (WC) of more than 40 in. [or 102 cm] for men or 35 in. [or 88 cm] for women;

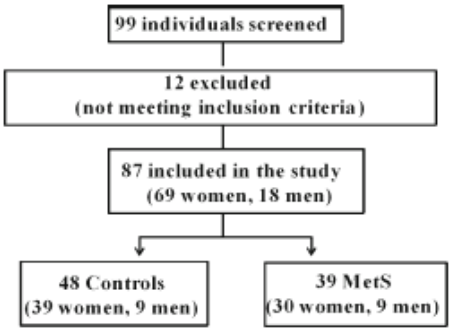

Fig. 1. Clinical design of participants study.

(b) hypertension, defined as clinical history of documented elevated blood pressure or persistent systolic blood pressure >130 mmHg and diastolic blood pressure >85 mmHg;
(c) hypertriglyceridemia, defined as fasting triglyceride levels >150 mg/dL;
(d) low HDL-cholesterol (HDL-C) values, defined as <40 mg/dL (1.04 mmol/L) for men and <50 mg/dL (1.3 mmol/L) for women;
(e) carbohydrate metabolism disorder, defined as previously documented impaired fasting glucose levels or a history of TDM2 or fasting glucose levels >100 mg/dL (5.6 mmol/L).

WC was determined using an inelastic tape to measure the circumference (in centimeter) of the abdomen midway between the lower ribs and iliac crests at the end of expiration and in a standing position. Body weight and height were measured while individuals wore light clothing and no shoes, using calibrated electronic scales (0.1-kg precision; Filizola ID 500, Brazil) and a fixed stadiometer (0.1-cm precision), respectively, and BMI was calculated as the weight in kilogram divided by the square of the height in meters (kg/m^2).

Moreover, the participants completed a questionnaire to provide general personal information such as age, medication use, presence of malignancy or other status (recent infection, chronic inflammation, coronary disease, hypertension, diabetes mellitus, smoking habits, hypothyroidism, alcohol consumption, and physical activity). The medication used included antihypertensives, hypoglycemics or hypolipidemics agents, to an adequate control of the conditions of MetS patients. In addition to responding to questionnaires, the population studied was evaluated by a physician before blood collection. Exclusion criteria for this study were pregnant women, smokers, people with cancer and alcoholic subjects, i.e., those patients who had regular and continuous alcoholic beverage (more than two drinks per week).

The samples for all biochemical measures of both groups (controls and MetS) were obtained immediately after blood collection, kept refrigerated at 4 °C, and processed within 2 h.

Biochemical Measurements

Blood samples were obtained after a 12-h fast, centrifuged to obtain the serum and plasma and analyses of the biochemical parameters such as total cholesterol (TC), triglycerides (TG), HDL-C, blood glucose (FBG), creatinine, urea, uric acid, γ-GT activity and serum protein were performed by standard methods. LDL-cholesterol (LDL-C) was calculated by using the Friedewald's formula [23] whenever triglyceride concentrations were <400 mg/dL, and all subjects involved on the study were in accordance with this rule. Fasting plasma insulin was determined by immunoluminometric assay. The homeostasis model assessment (HOMA-IR) was used to estimate insulin resistance, which indicates proneness to developing MetS, using the formula: fasting plasma insulin (μU/mL)× glucose (mmol/L)/22.5 [24]. Serum hsCRP was measured by immunoturbidimetric method on Cobas MIRA® (Roche Diagnostics, Basel, Switzerland) automated analyzer.

Enzymatic Assays

ADA and DPP-IV activities were measured spectrophotometrically in serum by the method of Giusti and Gakis [25] and Jarnolowska *et al.* [26], respectively, and were expressed in unit per liter. BuChE activity was determined by the method of Ellman *et al.* [27] modified. The reaction of BuChE was initiated by adding 0.8 mM butyrylthiocholine iodide (BuSCh), and was expressed as micromole of BuSCh per hour per milligram of protein.

Oxidative Stress Measures

Serum samples was used to assess the levels of NOx, measured on Cobas MIRA® by a method previously described and validated by Tatsch *et al.* [28].

Whole blood was centrifuged for 15 min at 2.500 rpm, the plasma was removed and the erythrocytes were washed twice in 0.9 % NaCl and recovered by centrifugation, finally, erythrocytes (RBCs) and plasma obtained were used to measure non protein thiol groups (NP-SH) and protein thiol groups (P-SH), respectively, by the method of Boyne and Ellman [29], modified by Jacques-Silva *et al.* [30], which consisted in reduction of DTNB measured at 37 °C and at 412 nm. The results were expressed as nanomole of P-SH per milliliter for plasma and nanomole of NP-SH per milliliter erythrocytes for RBCs.

Plasma ascorbic acid (VIT C) was estimated as described by Galley *et al.* [31] with some modifications by Jacques-Silva *et al.* [30]. The samples for determination of VIT C were kept refrigerated up to 1 week after collection and protected from light until the time of analysis and the content of VIT C was expressed as microgram per milliliter of plasma.

Lipid peroxidation was estimated in plasma by measurement of thiobarbituric acid reactive substances

(TBARS) according to the method of Lapenna *et al.* [32], using 1 % phosphoric acid and 0.6 % thiobarbituric acid (TBA). The reaction product was measured spectrophotometrically at 532 nm and the results were expressed as nanomole of TBARS per milliliter of plasma.

Statistical Analysis

Since variables showed a normal distribution according to Kolmogorov–Smirnov (KS) test, data were analyzed using Student's *t* test. The results are expressed as the mean±standard deviation (SD). The correlations were assessed by Pearson rank correlation coefficient. To estimate the strength of the association, odds ratios (OR) with 95 % confidence intervals (CI) was measured for each of the parameters studied by a logistic regression model with respective 95 % CI. All *p* values were two-sided and differences were considered significant when the probability was $p \leq 0.05$. Data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) and the SPSS software (ver. 9.0).

RESULTS

Main Characteristics of the Study Participants

The biochemical and anthropometrical characteristics of the study participants are illustrated in Table 1. As expected, WC, BMI as well as the weight were higher in the MetS group than in the control group ($p<0.001$). Serum FBG, TG levels, total protein ($p<0.001$), plasma insulin levels, and HOMA-IR index ($p<0.05$) were also increased in the group of subjects with MetS than in the healthy group. The other parameters analyzed did not differ significantly between the groups. Among subjects with MetS, only 13 % were diagnosed with diabetes mellitus.

ADA, DPP-IV, and BuChE Activities and Oxidative Stress Parameters

As showed in Table 2, the BuChE activity and hsCRP levels were significantly higher in the MetS group when compared to the control group ($p<0.001$; $p<0.05$, respectively). However, contrary to our expectations, no differences were obtained between the groups in ADA and DPP-IV activities.

The levels of NOx and γ-GT activity were also significantly higher in the group with MetS ($p<0.0001$). On the other hand, the levels of P-SH ($p<0.01$) and NP-SH ($p<0.001$) as well as plasma VIT C ($p<0.01$) were significantly reduced in subjects with MetS when compared with the control group. The levels of TBARS were not statistically different between the groups (Table 2).

In Table 3, we observed that the parameters such as P-SH ($p=0.035$), NP-SH ($p=0.001$), VIT C ($p=0.007$),

Table 1. General and Biochemical Characteristics of the Experimental Groups

	Control	MetS
N	48	39
♀/♂	39/9	30/9
Age (years)	42.96±8.81	47.55±11.44
BMI (kg/m^2)	25.83±4.68	33.85 ±5.53***
WC (cm)	91.13±12.37	111.3±12.21***
FBG (mmol/L)	4.88±0.78	6.03±1.42***
TC (mmol/L)	4.69±1.05	4.46±0.75
HDL- C (mmol/L)	1.35 ±0.33	1.28±0.44
LDL- C (mmol/L)	2.65±0.83	2.45±0.87
TG (mmol/L)	1.05±0.44	1.97±1.05***
Uric acid (mmol/L)	0.24±0.079	0.22±0.027
Urea (mmol/L)	5.62±1.55	5.97±1.22
Creatinine (μmol/L)	75.14±16.79	82.21±15.02
Serum protein (g/L)	5.32±0.4	5.85±0.58***
Insulin (μUI/mL)	8.60±1.51	12.7±1.20*
HOMA-IR index	1.2±0.40	3.18±0.34*

Data are presented as mean±SD. Statistically significant differences from controls, as determined by Student's *t* test
BMI body mass index, *WC* waist circumference, *FBG* fasting blood glucose, *TG* triglycerides, *TC* total cholesterol, *HDL-C* HDL cholesterol, *LDL-C* LDL cholesterol, *HOMA-IR* homeostasis model assessment
*$p<0.05$; ***$p<0.001$

Table 2. NOx Levels, ADA, DPP-IV, BuChE and γ-GT Activities and Parameters of Inflammation and Oxidative Stress of Controls and Subjects with MetS

	Control	MetS
ADA (U/L)	17.26±7.51	17.18±7.97
DPP-IV (U/L)	58.52±21.20	55.38±12.37
BuChE (BuSCh/h/mg of protein)	4.24±1.30	5.72±1.40***
TBARS (nmoL TBARS/mL)	5.74±1.79	5.42±1.68
P-SH (nmoL P-SH/mL)	116.2±26.04	99.29±20.45**
NP-SH (nmoL NP-SH/mL)	1074±244.4	744.20±125.4***
VIT C (μg/mL)	26.22±8.11	20.21±7.2**
NOx (μmol/L)	67.89±41.49	190.9±163.70***
hsCRP (mg/L)	5.26±3.96	7.52±4.83*
γ-GT (μKat/L)	0.22±0.15	0.58±0.43***

Data are presented as mean±SD. Statistically significant differences from controls, as determined by Student's *t* test
ADA adenosine deaminase, *DPP-IV* dipeptidyl peptidase IV, *BuChE* butyrylcholinesterase, *TBARS* thiobarbituric acid reactive substances, *P-SH* protein thiol groups, *NP-SH* non-protein thiol groups, *VIT C* ascorbic acid, *NOx* nitric oxide, *hsCRP* high sensitivity C-reactive protein, *γ-GT* γ-glutamyltransferase
*$p<0.05$; **$p<0.01$; ***$p<0.001$

Table 3. Odds Ratio of MetS Adjusted by BMI Values

	OR	95 % CI
TBARS	0.965	0.696–1.337
P-SH	0.972	0.946–0.998
VIT C	0.852	0.758–0.957
NP-SH	0.993	0.989–0.996
ADA	1.007	0.936–1.084
DPP-IV	0.979	0.945–1.014
hsCRP	1.065	0.926–1.226
NOx	1.017	1.006–1.028
BuChE	2.173	1.327–3.557
γ-GT	1.180	1.073–1.297

OR odds ratio, *CI* confidence interval, *BMI* body mass index, *TBARS* thiobarbituric acid reactive substances, *P-SH* protein thiol groups, *VIT C* ascorbic acid, *NP-SH* non-protein thiol groups, *ADA* adenosine deaminase, *DPP-IV* dipeptidyl peptidase IV, *hsCRP* high sensitivity C-reactive protein, *NOx* nitric oxide, *BuChE* butyrylcholinesterase, *γ-GT* γ-glutamyltransferase

NOx (p=0.003) levels, BuChE (p=0.002), and γ-GT (p=0.001) activities remained associated with MetS, even after adjustment for BMI values, thereby discarding the possibility of the influence of this anthropometric index on analyzed parameters. Also, a logistic regression model was done in order to evaluate the influence of BMI, sex, and age in the parameters analyzed in this study and no significant results were observed, i.e., these variables together did not affect the parameters analyzed (data not shown).

Associations Between Clinical, Oxidative, and Inflammatory Markers in MetS Group

We observed positive correlations between ADA activity and insulin levels (p=0.0177; Fig. 2b), HOMA-IR (p=0.0367; Fig. 2c) and age (p=0.0223; Fig. 2d) in subjects with MetS. Besides, DPP-IV activity was correlated positively with TC (p=0.0376; Fig. 3a). Also, BuChE activity showed the same relationship with TC (p=0.0389; Fig. 2a). On the other hand, VIT C levels were negatively associated with hsCRP levels (p=0.0140; Fig. 3b). The same correlations were made to the control group and no significant results were found.

DISCUSSION

Epidemiological studies of the general population indicated the close association of inflammation with MetS and its component conditions [33, 34], such as insulin resistance and obesity, which are closely and reciprocally interrelated. As can be observed in Table 1, WC, BMI, as well as the TG levels were higher in the MetS group than in the control group. Inflammation can produce a wide variety of changes in the plasma concentrations of lipids and lipoproteins [35], and is now widely accepted that fat can produce biologically active substances, all of which can contribute to strengthen the link between overweight/adiposity and inflammatory process seen in MetS [36].

The enzyme BuChE has been associated with different parameters of adiposity in humans and studies suggested that increased activity of BuChE seen in various clinical conditions could serve as marker with a high clinical informative power of low-grade systemic inflammation [37]. In this study, we demonstrated that the BuChE activity was higher in subjects with MetS when compared to the control group (Table 2), suggesting it like an interesting tool to evaluated the pathological processes mediating the MetS. Also, other studies have suggested that the BuChE activity may be considered as a secondary marker for MetS in obese individuals [38] and do not have a direct pathophysiological role in the development of this syndrome. In addition, we observed a positive correlation between the BuChE activity and TC levels (Fig. 2a). High serum lipid concentrations may induce stereoscopic alteration in an enzymatic configuration that modifies BuChE activity or altered expression of enzyme-encoding gene that determines BuChE concentration and activity [39].

Recent studies in our laboratory have observed increased ADA and DPP-IV activities in hyperglycemic and MetS subjects [8, 14], but, in this study we did not observe these differences between the groups studied probably because the glucose levels of MetS subjects were not enough to affect the enzymatic activities in serum (Table 2). However, we found a positive correlation between the ADA activity and insulin levels and HOMA-IR index (Fig. 2b, c), raising the possibility that decreased adenosine concentrations may be related to the development of insulin resistance. Also, a positive correlation was observed between the ADA activity and age of the subjects with MetS (Fig. 2d), demonstrating that there are changes in the adenosinergic system, adenosine formation and degradation that occur with advancing age [40]. We can also suppose that the possible relationship between ADA and insulin and HOMA-IR could display clinical significance, since it may reflect a poor glycemic control which generally is worsened with age.

Interestingly, DPP-IV activity was positively correlated with TC levels (Fig. 3a), suggesting that DPP-IV, essential in the development of normal immune responses,

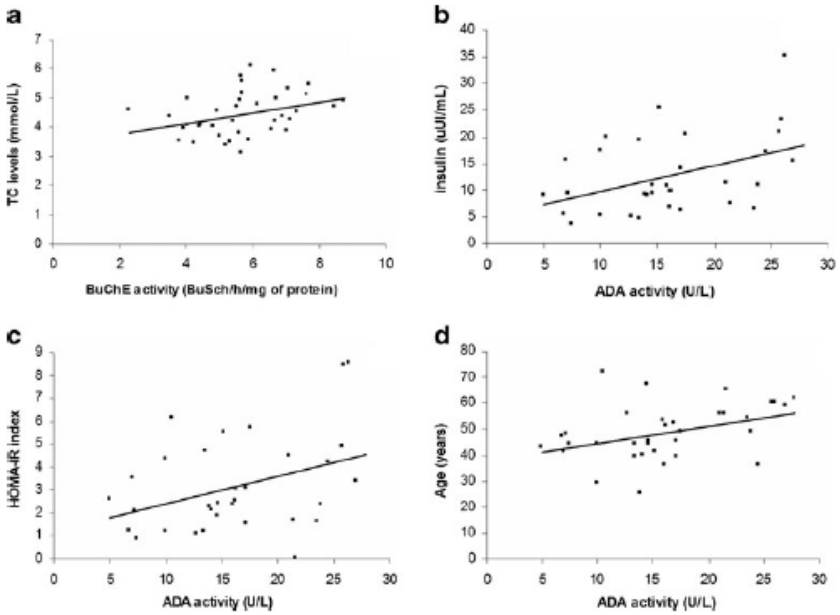

Fig. 2. Significant correlations between **a** BuChE activity and TC levels (r=0.3365, p=0.0389), **b** ADA activity and insulin levels (r=0.4230, p=0.0177), **c** ADA activity and HOMA-IR index (r=0.3707, p=0.0367), **d** ADA activity and age (r=0.3907, p=0.0223).

may be affected by TC levels in subjects with MetS. Also, studies demonstrated that DPP-IV inhibitors have been reported to reduce TC levels, so have an interesting profile of action on cardiovascular risk [41].

Serum γ-GT is associated with some atherosclerotic risk factors and several possible mechanisms for this association have been proposed, as oxidative stress and subclinical inflammation [42]. Furthermore, the γ-GT activity was higher in subjects with MetS and it has also been closely associated with a potentially prooxidant profile of circulating thiol compounds [43] as demonstrated by reduced levels of P-SH and NP-SH (Table 2). In fact, several sources of oxidative stress are related to hyperglycemia, hyperinsulinemia and hypertriglyceridemia, as well as inadequate antioxidant defenses, therefore the uncontrolled free radical production may be one of the mechanisms underlying the development of comorbidities in MetS subjects [44].

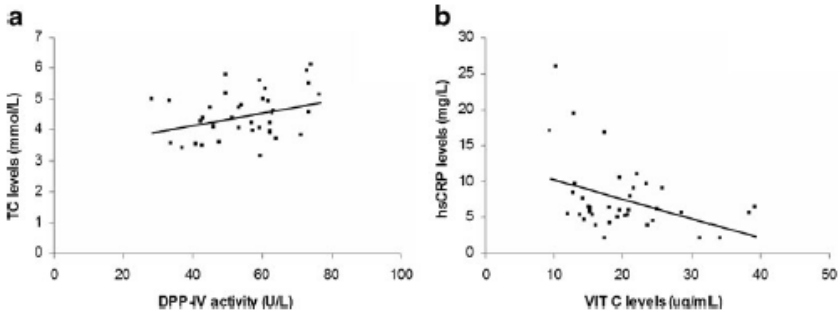

Fig. 3. Significant correlations between **a** DPP-IV activity and TC levels (r=0.3385, p=0.0376) and **b** VIT C and hsCRP levels (r=−0.4006, p=0.0140).

As a measure of the inflammatory component of MetS, hsCRP is reported as an independent risk factor of diabetes mellitus [45] and cardiovascular disease [34]. hsCRP is induced by cytokines produced by accumulated adipocytes and because of this, it increases in subjects with MetS [46] corroborating with our results (Table 2). Also, we demonstrated that hsCRP was correlated negatively with VIT C levels (Fig. 2b) in subjects with MetS, demonstrating that the inflammatory conditions lead to a reduction of the antioxidant protective shield of the body.

Since a strong correlation between endogenous NO production and serum NOx (nitrite/nitrate) levels has been established, determination of these inorganic NO metabolites in the circulation has been considered an index of generalized NO production. In this context, another finding relevant in this study was that the subjects with MetS had higher levels of NOx when compared to the control group (Table 2). It has been shown that insulin stimulates NO production, which could explain the increases in serum NOx concentrations observed in this study [20]. This marked production of NO may lead to pathological changes in various physiological systems aggravating the insulin resistance.

In Table 3, we presented the results of a logistic regression model that was used to exclude the possibility of interference of a parameter; in this case, the obesity represented by BMI values, on the other determinations analyzed in this study. The results showed that P-SH, NP-SH, VIT C, NOx levels, BuChE, and γ-GT activities were not affected by BMI that besides being measure body fat, it is a anthropometric indices associated with MetS risk factors. These results indicate that the changes observed in set of parameters evaluated may reflect the inflammatory state of the MetS subjects, once that MetS is usually accompanied by "low-grade systemic inflammation" and augmented oxidative stress [47], both of which are believed to have pivotal roles in development of MetS itself and in its further morbidities [48].

As our study is based on a selected population, it exhibits some limitations. Indeed, as described in study population section the sample size is small, but all subjects were criteriously selected to be part of the group with MetS based in the questionnaire applied to the participants.

CONCLUSION

Taking into consideration that the analyzed parameters were not affected by the BMI, except for levels of hsCRP, we conclude that the increase of BuChE and γ-GT activities together with NO levels observed in MetS subjects and, especially the correlations obtained could be involved in the development of state in MetS. Despite serum ADA and DPP-IV activities were not changed in MetS subjects, the associations demonstrated may play a role in the cascade of abnormalities present in the MetS. Although it is not possible to conclude that the effects observed in this group can be generalized to subjects with more severe impairments of MetS conditions, we should emphasize that our results could contribute to the related metabolic/inflammatory/disease-prone status of MetS subjects and play a role in the prevention and control of various aspects of this syndrome. This study provides new and interesting insights to MetS, however, future studies are required in order to determine if physical activity was able to affect the studied parameters in subjects with MetS.

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Conflict of Interest. No competing financial interests exist.

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3.3 *Syzygium cumini* is more effective in preventing the increase of erythrocytic ADA activity than phenolic compounds under hyperglycemic conditions *in vitro*

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Dear Beatriz,

We are pleased to inform you that your manuscript, "Syzygium cumini is more effective in preventing the increase of erythrocytic ADA activity than phenolic compounds under hyperglycemic conditions in vitro", has been accepted for publication in Journal of Physiology and Biochemistry.

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With kind regards,

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Editor-in-Chief
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Syzygium cumini is more effective in preventing the increase of erythrocytic ADA activity than phenolic compounds under hyperglycemic conditions *in vitro*

Karine S. De Bona[1], Gabriela Bonfanti[1], Paula E. R. Bitencourt[2], Lariane O. Cargnelutti[2], Priscila S. da Silva[2], Thainan P. da Silva[2], Régis A. Zanette[1], Aline S. Pigatto[3], Maria B. Moretto[1,2*]

[1]Postgraduate Program in Pharmacology
Department of Clinical and Toxicology Analysis
Center of Health Sciences
Federal University of Santa Maria (UFSM)
97105-900 - Santa Maria, RS, Brazil
[2]Postgraduate Program in Pharmaceutical Sciences- UFSM-Santa Maria- Brazil
[3]Franciscan University Center– UNIFRA - Santa Maria –RS – Brazil

Corresponding author:
Maria Beatriz Moretto
beatriz@smail.ufsm.br
Fax: +55 - 3220 8749

Abstract

Syzygium cumini (Sc) is a plant known for its antidiabetic properties. The aim of this study was to evaluate the effect of Sc aqueous leaf extract (ASc) on adenosine deaminase (ADA) activity in erythrocytes (RBCs) exposed to high glucose concentrations (30mM) *in vitro*. We also investigated the effects of the main phenolic compounds found in ASc (gallic acid, rutin and chlorogenic acid) and the effects of insulin, caffeine and dipyridamole, which are substances involved in the adenosine metabolism, on ADA activity *in vitro*. Blood samples were obtained from healthy volunteers and a suspension of RBCs was used for the determination of ADA activity. The results showed that: (i) the effect of ASc on ADA activity was more significant than the combination of phenolic compounds; (ii) insulin, caffeine or dipyridamole prevented high-glucose increase of ADA activity at doses as low as 50 µU/mL, 25 µM and 1 µM, respectively ; (iii) the inhibitory effect caused by ASc on erythrocyte ADA activity remained practically the same after the combination of the extract with insulin or caffeine; (iv) when RBCs were exposed to ASc plus dipyridamole, this chemical attenuated the effect of ASc on ADA activity, suggesting an antagonism or a competition with ASc by the same site of action. Therefore, ASc was more effective in preventing the increase in ADA activity than phenolic compounds, suggesting that ASc may collaborate to improve endothelial dysfunction, antioxidant, anti-inflammatory and antithrombotic properties of adenosine by affecting its metabolism. The results of this study help to provide evidence of the empirically supported benefits of the use of Sc in diabetes.

Keywords*:* Adenosine deaminase. Diabetes *mellitus.* Erythrocytes. Glucotoxicity. *Syzygium cumini* extract

Introduction

Diabetes mellitus (DM) is a clinical term denoting a group of metabolic disorders characterized mainly by hyperglycemia, which is the factor responsible by membrane damage and death of erythrocytes (RBCs). The development of diabetes complications is complex, and not yet fully understood, but involves the direct toxic effects of high glucose levels, along with the impact of elevated blood pressure, abnormal lipid levels, oxidative stress and chronic inflammatory condition [44, 34].

During conditions associated with metabolic stress, such as inflammation, the purine nucleoside adenosine can be formed and released into the extracellular space as a result of a rapid degradation of intracellular adenosine triphosphate (ATP) [37, 33, 15]. Adenosine diffuses to the surface of surrounding cells, where it binds specific membrane receptors [46]. Evidence indicates that adenosine helps to maintain tissue integrity by reducing energy demand, increasing nutrient availability, modulating the immune system in tissues subjected to injurious stimuli, including ischemia and inflammation, and also plays an important role in the modulation of insulin action on glucose metabolism in different tissues [49, 55, 27]. Adenosine deaminase (ADA: EC 3.5.4.4) is a key enzyme in purine metabolism, catalyzing the irreversible deamination of adenosine and deoxyadenosine, to inosine and deoxyinosine, respectively, closely regulating extracellular adenosine concentrations [19].

Several studies have investigated the components that contribute to determining extracellular adenosine concentrations, once that adenosine levels are driven by a complex interplay of enzymes and transporters [42, 36]. In line with this, dipyridamole is known to increase extracellular levels of adenosine, by inhibition of cellular reuptake of the latter and by inhibition of ADA activity into multiple cells, including RBCs, platelets and endothelial cells [63, 21]. Caffeine is a methylxanthine derivative and a multifunctional compound that inhibits phosphodiesterase activity. However, at concentrations associated with typical consumption, the predominant effect of caffeine is direct antagonism of adenosine at the receptor level [20].

In spite of great therapeutic innovations in type 2 DM, the existing pharmacological approaches such as oral hypoglycemics have limited use because of undesirable side effects and high rates of secondary failure [48]. In milieu of these observations, many plants have been observed to be useful [39, 7]. Plants not only offer better protection and a lesser side effect profile but also ameliorate and prevent

β-cell failure [41]. According to ethnobotanical information, more than 800 plants are used as traditional remedies in one or other form for the treatment of diabetes [3].

In this context, *Syzygium cumini* (L.) Skeels (Sc, family: Myrtaceae), popularly known as Jamun, has been the major constituent of herbal formulations, and many bioactive compounds such as flavonoids, glycosides, tannins, anthocyanins and ascorbic acid, from its different parts (e.g., fruits, leaves, stem and roots), have been isolated and advocated for the management of diabetes over many centuries [24, 28, 53, 32]. Various mechanisms have been proposed for the antidiabetic actions of *Syzygium cumini*. These include stimulation of pancreatic insulin secretion [56, 54] restoration of beta architecture [52], amelioration of dyslipidemia [25], upregulation of the glucose transporter GLUT-4 [4], rise in cathepsin-B activity and increasing glycogen content in liver and muscle [1, 52]. *Syzygium cumini* also causes an increase in the activity of key enzymes of glycolysis and a decrease in the activity of important enzymes involved in gluconeogenesis [54].

Syzygium cumini is a powerful antioxidant and presents anti-inflammatory, hypoglycemic, antibacterial and antidiarrheal effects [40, 43, 60, 10, 38, 5]. Recent studies have demonstrated that *Syzygium cumini* leaves extract (ASc) inhibited ADA activity in serum and cells of diabetic and hyperglycemic patients *in vitro* [9, 13, 14]. However, the cellular and molecular mechanisms of action are not yet completely elucidated.

The biochemical organization, as well dynamic properties of RBCs membranes are considerably altered in the hyperglycemic state, once that uncontrolled glucose regulation causes tissue damage by mechanisms involving repeated acute changes in cellular metabolism [61]. Nevertheless, it is not well established how the elevated glucose, at short incubation times, affects erythrocyte enzymes and very little is known about the ASc effects at cellular level. Thus, the present study was carried out to evaluate the effect of ASc on ADA activity in RBCs exposed to high glucose concentrations, under *in vitro* conditions. Moreover, in order to clarify the mechanism by which ASc acts on erythrocyte ADA activity, the effects of insulin, caffeine and dipyridamole, substances which are known to affect adenosine metabolism, and the effects of biologically and pharmacologically active compounds present in ASc were analyzed in hyperglycemic conditions, *in vitro*.

Materials and methods

Chemicals

Adenosine, rutin, gallic acid, chlorogenic acid, insulin and dipyridamole were obtained from Sigma Chemical Co (St. Louis, MO, USA). All other chemicals were of analytical grade and were obtained from standard commercial suppliers.

Plant material and *Syzygium cumini* aqueous leaves extract (ASc) preparation

Leaves of *Syzygium cumini* were freshly locally collected, cleaned, dried, and powdered. They were identified by the Laboratory of Botanic and Pharmacognosy of the Franciscan University Center – UNIFRA, Santa Maria, RS, Brazil. The leaves were dried in a greenhouse with air circulation at 40°C for approximately 48 h. Then, they were ground in a knife mill. The products were submitted to extraction in a Soxhlet apparatus until exhaustion. After extraction, the solvent was evaporated in a rotavapor, to give the crude extract. The stock solution was made dissolving 1 g of the crude extract in 100 mL NaCl 0.9% [16]. A voucher specimen (SMDB 14.001) was identified and deposited at the Herbarium of the Federal University of Santa Maria (UFSM).

High-performance liquid chromatography (HPLC) characterization of the extract

The chemical characterization of the extract was determined in recent study where a high-performance liquid chromatography (HPLC) analysis, described below, revealed that the phenolic compounds such as gallic acid, rutin and chlorogenic acid are the major components of the extract [13].

Chromatographic analyses were carried out in isocratic conditions using RP-C18 column (4.6 mm x 250 mm) packed with 5 μm diameter particles and the mobile phase was methanolacetonitrile- water (40:15:45, v/v/v) containing 1.0% acetic acid. The solutions of standards used for the chromatographic analyses were rutin, kaempferol, chlorogenic acid and gallic acid. The chromatographic peaks were confirmed by comparing its retention time with those of reference standards and

quantification was performed by peak integration using the external standard method. All chromatographic operations were performed at room temperature [64].

Preparation of samples

Blood samples were obtained from healthy volunteers in heparinized tubes and the plasma was removed by centrifugation at 3400 rpm for 10 min. The remaining RBCs were washed three times with a saline solution (NaCl 0.9%). After washing, the RBCs were resuspended in phosphate buffer saline and a 10% hematocrit suspension was immediately used for ADA activity determination. All experiments were performed at least three times using RBCs obtained at different occasions and a total of 12 samples were used for each incubation performed.

The study was in accordance with the guidelines of the Ethics Committee of the Federal University of Santa Maria, which approved the experimental protocol (0049.0.243.000-08).

In vitro experimental protocol

Initially, RBCs at 10% hematocrit were incubated for 2 h at 37°C with glucose (5, 10 and 30 mM, which correspond to 90mg/dL, 180 mg/dL and 540mg/dL, respectively) in order to determine the glucose concentration able to alter the ADA activity *in vitro*.

Subsequently, RBCs were pre-incubated with various concentrations of ASc (100, 200 and 500 µg/ml), rutin (40, 80 and 120 µM), gallic acid (50, 100 and 200 µM) and chlorogenic acid (20, 80 and 120 µM) at 37°C for 30 min, followed by incubation in hyperglycemic conditions (glucose 30 mM) at 37°C for 2 h. Also, in order to evaluate the possible interactions among rutin, gallic acid and chlorogenic acid, a combination of the three compounds, in the highest concentrations, was studied under the same conditions mentioned above. The concentrations of the compounds used in the incubations were selected on the basis of earlier experiments and adapted for this study [35, 63, 50]. A control containing only RBCs was also used.

RBCs were incubated with different concentrations of caffeine (25-500 µM), dipyridamole (1-100 µM) and insulin (5-250 µU/ml) in the presence of glucose (30

mM). The highest concentrations of these substances were used for incubation with the ASc (500 µg/mL), using the above mentioned protocol.

ADA activity

Firstly, the RBCs were exposed to various concentrations of the compounds mentioned above at 37°C for 30 min, followed by incubation in hyperglycemic conditions for 2 h (37°C). After incubations, ADA activity was measured spectrophotometrically in RBCs by the method of Giusti and Gakis [22]. The reaction was started by addition of the substrate (adenosine) to a final concentration of 21 mmol/L and incubations were carried out for 1 h at 37°C. The reaction was stopped by adding phenol–nitroprusside and alkaline-hypochlorite. Ammonium sulfate was used as ammonium standard. Also, one blank of sample was used in order to eliminate a possible interference of the color of the extract that could alter the measure of enzyme activity. Readings for samples and standard were corrected by subtracting readings for sample blank and reagent blank, respectively, yielding ΔA sample and ΔA standard, and ADA activity (U/L) was calculated according to the following formula: ADA (U/L) = ΔA sample/ ΔA standard x 50 U/L. The results were expressed as U/L.

Statistical analysis

Statistical differences of experiments were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's post hoc test using Statistica software (version 6.0). The results were expressed as mean ± standard error of the mean (SEM). Differences were considered statistically significant when $p \leq 0.05$.

Results

In vitro effect of glucose on ADA activity

The results obtained for ADA activity in RBCs in the presence of different concentrations of glucose, *in vitro*, are depicted in Fig. 1. High glucose levels were used to mimic the hyperglycemia observed in types 1 and 2 diabetic patients. A

significant increase (p<0.05) in the ADA activity was observed when RBCs were exposed to the highest glucose concentration (30 mM) after 2 h of incubation. Glucose levels lower than 30 mM showed only a slight stimulatory effect. Thus, the remaining incubations were performed in the presence of 30 mM of glucose, which was the only concentration capable of significantly alter ADA activity.

In vitro effect of ASc, rutin, gallic acid and chlorogenic acid on ADA activity in hyperglycemic conditions

The experimental data demonstrated that ASc prevented the increase in ADA activity caused by the exposure of RBCs to 30 mM of glucose in all concentrations tested (Fig. 2A). We also observed a similar effect of the phenolic compounds, rutin, gallic acid and chlorogenic acid, on the ADA activity when RBCs were exposed to the same conditions (Fig. 2B).

The effect provoked by the combination of the highest concentrations of rutin, gallic acid and chlorogenic acid was compared to the effect of ASc on the ADA activity (Fig. 3). The results showed that the decrease in the ADA activity was more pronounced in the presence of ASc (p<0.0001) than in comparison to the compounds in combination (p<0.01). Reductions of 48.2% and 35.4% in the enzyme activity were observed for ASc and for the compounds in combination, respectively, when compared to the values obtained with glucose alone. It is important to emphasize that the concentrations of the phenolic compounds used *in vitro* are in similar proportions to those found in the extract, since 0.0304% of rutin, 0.729% of gallic acid and 0.0093% of chlorogenic acid were found in the crude extracts after HPLC analysis.

In vitro effect of insulin, caffeine and dipyridamole on ADA activity

The results showed that caffeine, insulin or dipyridamole attenuated the increase in the ADA activity caused by glucose independently of the concentration used (Fig. 4). When RBCs were incubated with all concentrations tested for each substance alone (caffeine, insulin or dipyridamole without glucose), the ADA activity was maintained at a level similar to that observed in the control samples (data not shown) (n=12).

As can be observed in Fig. 5, ASc, insulin, caffeine and dipyridamole, in the highest concentrations tested, caused a reduction of 37.2%, 16.1%, 15.2% and 16.4%, respectively, in the ADA activity of RBCs subjected to hyperglycemic conditions. However, when RBCs were exposed to the combination of ASc plus insulin, was observed a reduction of ADA activity of 37.5% (p<0.0001 compared with G 30 mM; p<0.01 compared with Ins 250 µU/mL). Also, after incubation with ASc plus caffeine, a reduction of 39.1% in the ADA activity was observed (p<0.0001 compared with G 30 mM; p<0.01 compared with Caf 500µM) and with ASc plus dipyridamole, a decrease of 26.7% in the enzymatic activity was noted (p<0.01 compared with G 30 mM).

In order to clarify the results regarding the interaction between dipyridamole and ASc on the ADA activity, we tested the incubations of dipyridamole at 100µM plus the ASc in graduated concentrations (500, 750 and 1000 µg/mL). The effect of dipyridamole 100µM plus ASc 1000 µg/mL (reduction of 62.8 % in the ADA activity) was similar to the effect of ASc at 1000 µg/mL (reduction of 61.0%); however, the effect of dipyridamole plus ASc at the concentrations of 500 µg/mL and 750 µg/mL (reduction of 41.7% and 44.29%, respectively) was less efficient than ASc alone (reduction of 49.0% to ASc 500 µg/mL and 48.8% to ASc 750 µg/mL) (data not shown).

Discussion

The *in vitro* exposure of cells to high glucose concentrations is often used as a model to investigate the deleterious effects of hyperglycemia in diabetes. RBCs have been the subject of many studies because of their readily accessible and relatively simple metabolism [30, 29, 11].

In our study, a significant increase in the ADA activity was observed when RBCs were exposed to the highest glucose concentration (Fig. 1). It has been shown that augmented ADA activity reflects accelerated purine turnover and high salvage pathway activity which may lead to reduced adenosine levels [31, 26]. Moreover, it might be assumed that disturbed homeostasis of adenosine greatly contributes to the impaired function of immune cells in diabetes and may play a role in the development of insulin resistance [51].

Interestingly, ASc, at all concentrations tested, prevented the increase in the ADA activity in hyperglycemic conditions (Fig. 2A). Hence, this result is beneficial in situations of hyperglycemia, since the reduction of ADA activity results in increased levels of adenosine, which affect various physiological and pathological processes in cells. Moreover, *Syzygium cumini* has been used in the traditional system of medicine and it has presented promising therapeutic value because of its various phytoconstituents [62]. Therefore, the investigation of the effect of the active principles individually and the elucidation of the extract mechanism of action are fundamentally important.

We have performed a detailed evaluation of the main compounds present in ASc, and as expected, a similar effect on ADA activity was observed with rutin, gallic acid and chlorogenic acid when incubated separately (Fig. 2B), corroborating with previous reports that showed the inhibition of a variety of enzymes by secondary metabolites [45, 12, 2]. In addition, Fig. 3 shows that ASc was more effective in preventing the increase in the ADA activity than the combination of phenolic compounds. The analyses of the compounds combinations let us understand how interactions among compounds are important for the biological activity of crude extracts. Although we have used only the main compounds that were detected in the extract, we cannot exclude the possibility of having other substances in the extract that could act separately or synergistically to cause the effects of this plant [59] on the enzyme activity. Moreover, the curves of the compounds rutin, gallic acid and chlorogenic presented in this study demonstrated that the effect of these compounds on the activity of ADA was not dependent of the dose, *in vitro*. Therefore, we standardized for the incubation of the compounds together, the higher dose of each compound for comparison with the highest dose of the extract.

Considering that human RBCs contain specific insulin receptors, which have binding characteristics similar to those found in other classical target cells [57], we studied the effects of insulin on the ADA activity of RBCs exposed to hyperglycemic conditions, *in vitro,* and a reduction on the ADA activity was observed (Fig. 4). Insulin may modulate its own action on glucose metabolism by changing ADA activity and hence local concentration of adenosine [49], since it was proposed that the increased effectiveness of insulin resulted from an increased accumulation of adenosine [58].

Likewise, we observed that caffeine reduced the ADA activity independently of the concentration in hyperglycemic conditions (Fig. 4). The complex effects produced

by caffeine probably cannot be accounted for a single mechanism, once at low and moderate doses, similar to the daily consumption of coffee (approximately 10 µM), caffeine acts mainly as adenosine receptor antagonists and, at higher doses, caffeine additionally inhibits the activity of phosphodiesterases [20]. Therefore, we can suggest that caffeine caused an inhibition of phosphodiesterase, blocking the hydrolysis of cyclic adenosine-3', 5'- monophosphate (cAMP) and decreasing the levels of AMP and, consequently, of adenosine. Therefore, this likely reduction in the levels of adenosine may indirectly affect the ADA activity, reducing it.

To further explore the possible mechanism by which ASc decreased the ADA activity, we studied the effects of dipyridamole in RBCs under hyperglycemic conditions. RBCs contain nitrobenzylthioinosine-sensitive equilibrative nucleoside transporter (ENT), which plays an important role in nucleoside and nucleobase uptake for salvage pathways of nucleotide synthesis, facilitating the transmembranous diffusion of adenosine [6]. In line with this, our findings showed that dipyridamole significantly reduced the ADA activity once that this chemical increases the plasma concentration of adenosine by inhibiting adenosine uptake [23] and attenuating adenosine catabolism [18] (Fig. 4). Another possible mechanism of action of dipyridamole on the ADA activity is related to its role as an inhibitor of phosphodiesterase increasing cAMP levels. Thus, dipyridamole may act as a promising therapeutic agent, once in whole tissues this inhibitory effect can lead to increased extracellular concentrations of adenosine [8, 47].

Fig. 5 shows the effect of ASc in combination with insulin, dipyridamole or caffeine in hyperglycemic conditions. The decrease in the ADA activity caused by ASc plus caffeine was more significant than the effect of caffeine alone and similar to the effect caused by ASc alone, demonstrating that when the two compounds are incubated together the effect of ASc is highlighted. Likewise, we observed that the inhibitory effect caused by the extract remained practically the same after the combination with insulin and was statistically different from insulin *per se* in hyperglycemic conditions. Thus, we can suppose that ASc was more effective than insulin in reducing the enzyme activity in cells exposed to high glucose concentrations. In the presence of insulin or caffeine, the effect of ASc on the ADA activity was maintained and the remaining activity is higher than expected when mixing two inhibitors, leading us to suggest that the *in vitro* action of the extract is

similar to the compounds; however, this effect was not additive, prevailing the effect caused by ASc.

In addition, the effect of dipyridamole plus ASc on the ADA activity was less evident than the ASc alone, demonstrating that the presence of dipyridamole attenuated the effect of the extract by antagonizing its effect or by competition with ASc by the same site of action. Based on the obtained results, we can suggest that one mechanism of action of ASc may be through of the inhibition of phosphodiesterase activity. However, this is not the only mechanism of action by which the ASc inhibits the enzyme activity and other mechanisms may contribute to the effect of the extract. The inhibition of ADA activity by plant phenolic compounds derivates and the subsequent accumulation of endogenous adenosine would explain some of the pharmacological effects of these natural compounds. Also, ASc may be acting as an antioxidant compound, since studies have shown that ADA can be used as a marker of oxidative stress [17].

To the best of our knowledge, this is the first study that evaluated the possible mechanism of action of ASc on the adenosinergic system involved in hyperglycemic state*, in vitro,* in RBCs. We believe that our study is clinically relevant for several reasons. Firstly, as this plant is one of the most popular medicinal plants used worldwide for diabetes treatment, the knowledge of its bioactivity and possible mechanisms of action are critical for informed public health decisions. Secondly, it is especially important the role that ASc exerts to avoid the increase in ADA activity, once it collaborates for the maintenance of the adenosine levels in circulation. Thirdly, the discovery of antidiabetic drugs that simulate the normal physiological mechanisms of tissues which are involved in glucose homeostasis, possibly provides a new paradigm for the treatment and management of type 2 diabetes.

Conclusions

From the abovementioned observations, we can conclude that ASc alters ADA activity, but not only because of the content of secondary metabolites present in the extract. The present findings demonstrate evidences that one of the possible mechanisms of action of the ASc on the ADA activity in RBCs may be through of the inhibition of phosphodiesterase activity. The inhibition of ADA activity by ASc leads to an increase in the adenosine levels, collaborating to improved endothelial

dysfunction, and the antioxidant, anti-inflammatory and antithrombotic properties of this nucleoside. We consider that these findings have helped to better understand the effect of potential properties of *Syzygium cumini* on the adenosinergic system in hyperglycemic RBCs which is under current investigation in our group.

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Conflict of interest

The authors declare that there are no conflicts of interest**.**

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FIGURA 1

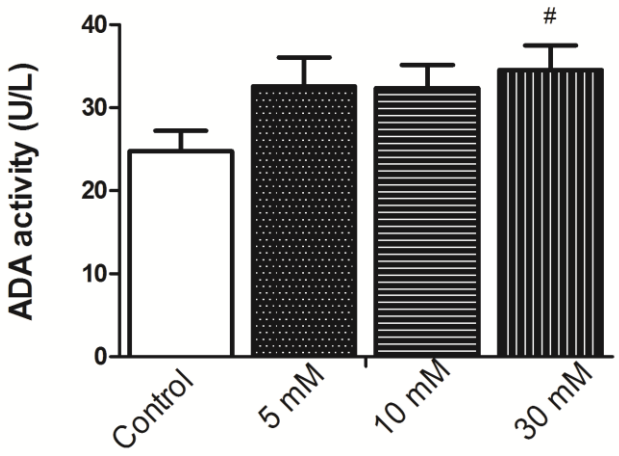

Fig. 1: Effect of glucose levels on ADA activity in RBCs after 2 hours of incubation, *in vitro.* Data are reported as mean ± SEM and expressed as U/L. Statistically significant differences as determined by one-way ANOVA, followed by Duncan multiple range test ($^{\#}p<0.05$ compared with control) (n=12).

FIGURA 2

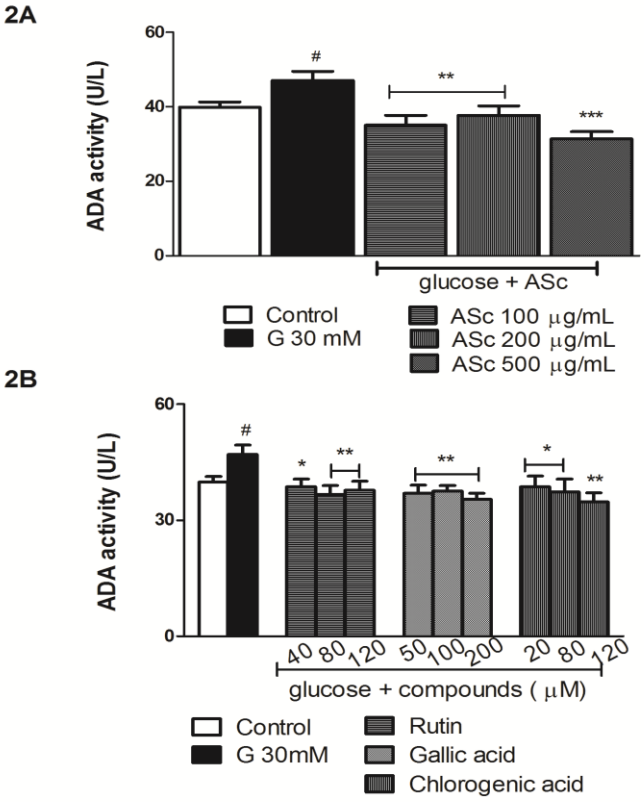

Fig. 2: Effect of different concentrations of (A) ASc, (B) rutin, gallic acid and chlorogenic acid on ADA activity in RBCs exposed to hyperglycemic conditions after 2 hours of incubation, *in vitro*. Data are reported as mean ± SEM and expressed as U/L. Statistically significant differences as determined by one-way ANOVA, followed by Duncan multiple range test ($^{\#}p<0.05$ compared with control; *p<0.05

compared with G 30mM; ** p<0.01 compared with G 30mM; ***p<0.001 compared with G 30 mM) (n=12).

FIGURA 3

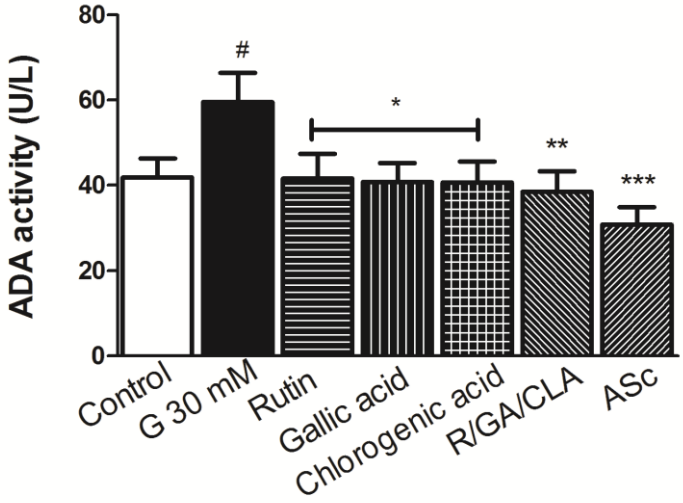

Fig. 3: Effect of rutin (120 µM), gallic acid (200 µM), chlorogenic acid (120 µM), ASc (500 µg/mL) and compounds incubated together (R/GA/CLA) on ADA activity in RBCs exposed to hyperglycemic conditions after 2 hours of incubation, *in vitro*. Data are reported as mean ± SEM and expressed as U/L. Statistically significant differences as determined by one-way ANOVA, followed by Duncan multiple range test (#p<0.05 compared with control; *p<0.05 compared with G 30 mM; ** p<0.01 compared with G 30 mM; ***p<0.001 compared with G 30 mM;) (n=12).

FIGURA 4

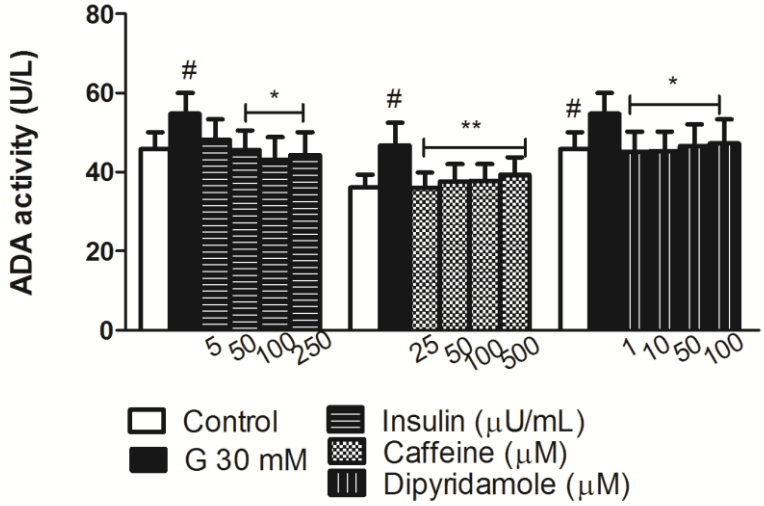

Fig 4: Effect of different concentrations of insulin, caffeine or dipyridamole on ADA activity, *in vitro*, in hyperglycemic conditions after 2 hours of incubation. Data are reported as mean ± SEM and expressed as U/L. Statistically significant differences as determined by one-way ANOVA, followed by Duncan multiple range test (#p<0.05 compared with control; *p<0.05 compared with G 30mM; ** p<0.01 compared with G 30mM) (n=12).

FIGURA 5

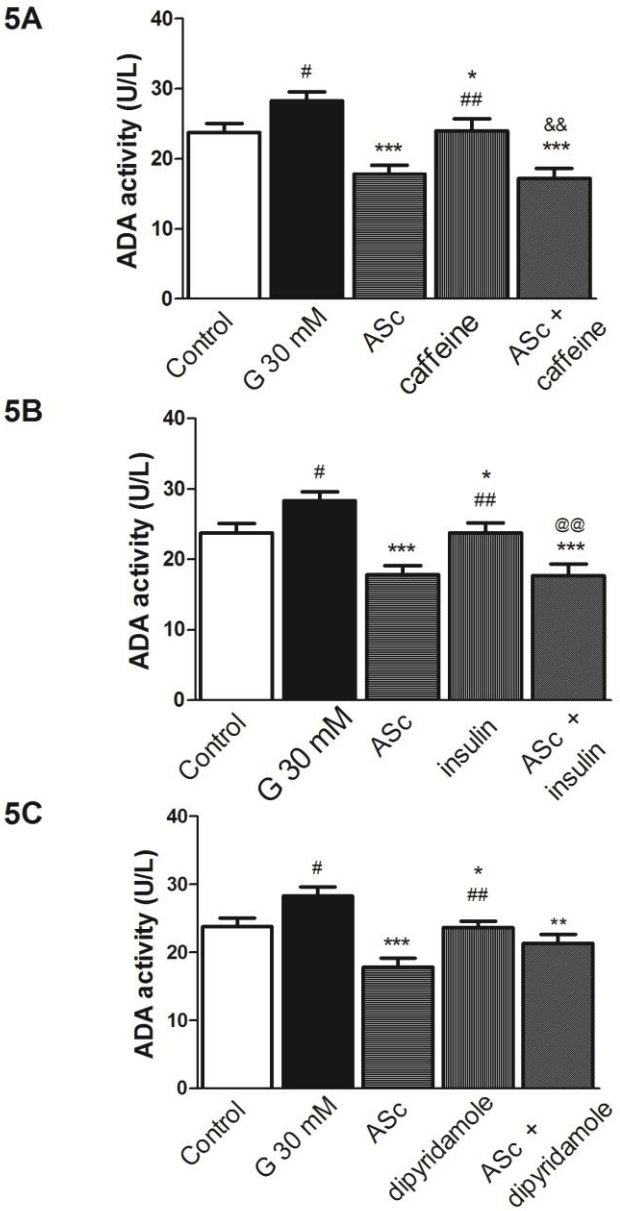

Fig. 5: Effect of (A) ASc plus caffeine, (B) ASc plus insulin and (C) ASc plus dipyridamole on ADA activity, *in vitro*, in hyperglycemic conditions after 2 hours of incubation. Data are reported as mean ± SEM and expressed as U/L. Statistically significant differences as determined by one-way ANOVA, followed by Duncan multiple range test ($^{\#}p<0.05$ compared with control; * $p<0.05$ compared with G 30mM; ** $p<0.01$ compared with G 30Mm; ***$p<0.001$ compared with G 30 mM;; $^{\#\#}$ $p<0.01$ compared with ASc 500 µg/mL; $^{\&\&}$ $p<0.01$ compared with Caf 500µM; $^{@@}$ $p<0.01$ compared with Ins 250 µU/mL) (n=12).

4. MANUSCRITO

4.1 Protective effect of gallic acid and *Syzygium cumini* extract against oxidative stress-induced cellular injury in human lymphocytes

Manuscrito submetido ao periódico **Cell Biochemistry and Function**

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Protective effect of gallic acid and *Syzygium cumini* extract against oxidative stress-induced cellular injury in human lymphocytes

Karine Santos De Bona[1], Gabriela Bonfanti[1], Paula E. R. Bitencourt[2], Thainan P. da Silva[2], Raphaela Maleski Borges[2], Aline Boligon[2], Aline S. Pigatto[3], Margareth Lynde Athayde[2], Maria B. Moretto[1,2*]

[1]Postgraduate Program in Pharmacology
Department of Clinical and Toxicology Analysis
Center of Health Sciences
Federal University of Santa Maria (UFSM)
97105-900 - Santa Maria, RS, Brazil
[2]Postgraduate Program in Pharmaceutical Sciences, UFSM, Santa Maria, Brazil
[3]Franciscan University Center, UNIFRA, Santa Maria, RS, Brazil

Corresponding author:
Maria Beatriz Moretto
beatriz@smail.ufsm.br
Fax: +55 55 3220 8749

Abstract

Plants constitute an important source of active natural products and the focus on plant research has increased all over the world as a potential therapeutic approach of a number of diseases. Adenosine deaminase (ADA) and dipeptidyl peptidase IV (DPP-IV) play a role in the immune system by having a co-stimulatory function in human lymphocytes. Moreover, the viability of these cells is limited in the presence of excessive free radical production. Thus, the aim of this study was to evaluate the effect of gallic acid and *Syzygium cumini* leaf extract (ASc) on ADA and DPP-IV activities, cell viability and oxidative stress parameters in lymphocytes exposed to 2,2'-azobis-2-amidinopropane dihydrochloride (AAPH), *in vitro*. A suspension of lymphocytes obtained from healthy volunteers was used for the determination of ADA, DPP-IV, and lactate dehydrogenase (LDH) activities, lipoperoxidation, protein thiol (P-SH) group levels and cellular viability. The results showed that: (i) HPLC analysis identified and quantified the presence of phenolic acids and flavonoids as the main compounds of ASc; (ii) ASc reduced the AAPH-induced increase in ADA activity, but no effect was observed on DPP-IV activity; (iii) ASc was not able to reduce the AAPH-induced lipid peroxidation, although the extract increased P-SH groups and cellular viability and decreased LDH activity; (iv) gallic acid showed less protective effects than ASc. In conclusion, ASc affects the purinergic system and may modulate adenosine levels, indicating that this plant exhibits immunomodulatory properties. ASc also may potentially prevent the cellular injury induced by oxidative stress, highlighting its cytoprotective effects.

Keywords*:* Adenosine deaminase; cellular viability; dipeptidyl peptidase IV; lymphocytes; oxidative stress. *Syzygium cumini* extract

Introduction

Plants constitute an important source of active natural products which differ widely in terms of structure and biological properties. They have a remarkable role in the traditional medicine in different countries, and, in the recent years, the focus on plant research has increased all over the world [1]. The consumption of plant products is associated with a lowering risk of a number of chronic diseases [2], and natural compounds have been proposed as potentially therapeutic and immunomodulatory agents in the treatment of several diseases [3-5].

Syzygium cumini (*S. cumini*; Syn. *Eugenia jambolana*; Family: Myrtaceae), commonly known as 'Jamun', is a medicinal plant native to India. Scientific studies have shown that extracts of different parts of *S. cumini* possess a range of pharmacological properties such as antidiabetic, anti-inflammatory, anti-ulcerogenic, antimicrobial, and antioxidant activities, as well as cardioprotective and hepatoprotective effects [6-12]. Previous studies have also demonstrated that *S. cumini* leaf extract (ASc) exhibits protective properties in serum and cells of diabetic and hyperglycemic patients *in vitro* [13-15]. Furthermore, the polyphenolic compounds found in the *S. cumini* extract, including flavonoids and phenolic acids, are known to have various physiological activities [16]. One of these compounds is gallic acid (3,4,5-trihydroxybenoic acid), which is especially abundant in processed beverages such as red wine and green tea and has antioxidant, anti-inflammatory, antimicrobial, and anticancer activities [17].

The normal function of the immune system is essential for health, and dysfunction of the same leads to several diseases. The most relevant cells involved in the immune response are the lymphocytes [18], and the majority of immune diseases are linked to a loss of T-cell homeostasis. The proliferation, activation, and cytokine secretion by T-cells are regulated by the formation of intracellular reactive oxygen species (ROS), suggesting that intracellular ROS could play a role in peripheral T-cell homeostasis [19]. Nevertheless, excessive ROS production can attack cellular components of lymphocytes leading to cell damage or improper lymphocyte function and, thus, limit their viability [20].

In this context, an important enzyme altered in various conditions associated with oxidative stress [21] is adenosine deaminase (ADA), which has been also shown to be increased in diseases characterized by T lymphocyte proliferation [22, 23] and

is considered as a nonspecific marker of T-cell activation. Metabolically, ADA is an enzyme of the purine metabolism which catalyzes the conversion of adenosine to inosine, thus downregulating the biologic effects of adenosine *in situ* and playing a key role in normal immune function [24]. ADA is released into the extracellular medium by immune cells where the enzyme can bind to certain membrane proteins such as CD26 or dipeptidyl peptidase IV (DPP-IV, EC 3.4.14.5) [25]. DPP-IV may also potentially modulate immune responses by directly regulating lymphocytes [26] and, via its ability to bind ADA, exhibits a co-stimulatory function on human T cells [25].

The discovery of the biological ambivalence of oxidative stress, as well as its numerous metabolic, structural, and functional effects, has generated a large number of experimental and clinical investigations concerning the association between the generation of free radicals and development of many diseases. Oxidative stress is implicated as playing a significant role in the development of many chronic diseases [27], ranging from cancer to cardiovascular and neurodegenerative disorders [28-30].

In vitro models, particularly cell-based methods/assays, have been widely used in various areas of life sciences and could help to define mechanisms and impacts of tested compounds prior to *in vivo* studies. Notwithstanding, there is paucity of data regarding the effect of natural compounds on lymphocyte metabolism under oxidative stress conditions, *in vitro*. The present investigation has therefore been designed to study the effect of *S. cumini* leaf extract (ASc) and gallic acid on ADA and DPP-IV activities and to assess their effect on cellular viability and oxidative parameters in lymphocytes exposed to 2,2’-azobis-2-amidinopropane dihydrochloride (AAPH), a water soluble free radical generator used to stimulate oxidative stress in *in vitro* cellular models.

Materials and methods

Chemical apparatus and general procedures

Acetonitrile and phosphoric, chlorogenic, caffeic, and ellagic acids were purchased from Merck (Darmstadt, Germany). Catechin, epicatechin, quercetin, isoquercitrin, quercitrin, kaempferol, rutin, and gallic acid, adenosine, 2,2’-azobis-2-amidinopropane dihydrochloride (AAPH), 5,5’dithiobis-2-nitrobenzoic acid (DTNB),

Ficoll–Histopaque, 3-[4,5-dimethylthiazol- 2-yl]-2,5-diphenyl tetrazolium bromide (MTT), thiobarbituric acid, and gly-pro-p-nitroanilide p-toluenesulfonate were obtained from Sigma Chemical Co (St. Louis, MO, USA). High performance liquid chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software. All other chemicals were of analytical grade and were obtained from standard commercial suppliers.

Plant material and ASc preparation

Fresh locally collected *S. cumini* leaves were cleaned, dried, and powdered. They were identified by the Laboratory of Botanic and Pharmacognosy of the Franciscan University Center, Santa Maria, Brazil. The leaves were dried in a greenhouse with air circulation at 40 °C for approximately 48 h. Then, they were ground in a knife mill. The products were submitted to extraction in a Soxhlet apparatus until exhaustion. After extraction, the solvent was evaporated in a rotavapor to give the crude extract. The stock solution was made dissolving 1 g of the crude extract in 100 mL NaCl 0.9% [31]. A voucher specimen (SMDB 14.001) was identified and deposited at the herbarium of the Federal University of Santa Maria.

Quantification of compounds by HPLC-DAD

Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} columns (4.6 mm x 150 mm) packed with 5 µm diameter particles. The mobile phase was: (A) acetonitrile: water (95:5, v/v) and (B) water: phosphoric acid (98:2, v/v), and the composition gradient was: 5% of A until 10 min and changed to obtain 20%, 40%, 50%, 60%, 70%, and 100% A at 20, 30, 40, 50, 60, and 80 min, respectively, following the method described by Kamdem et al. [32] with slight modifications. *S. cumini* leaf extract was analyzed at a concentration of 1 mg/mL. The presence of eleven antioxidant compounds was investigated, namely gallic, chlorogenic, caffeic, and ellagic acid, catechin, epicatechin, quercetin, quercitrin, isoquercitrin, kaempferol, and rutin. Identification of these compounds was

performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. The flow rate was 0.6 mL/min, injection volume was 40 µl and wavelength was 254 nm for gallic acid, 280 nm for catechin and epicatechin, 325 nm for chlorogenic, ellagic, and caffeic acids, and 366 nm for rutin, isoquercitrin, quercitrin, kaempferol, and quercetin. The samples and mobile phase were filtered through 0.45 µm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.025 - 0.300 mg/ml for quercetin, quercitrin, isoquercitrin, kaempferol, and rutin, 0.040 - 0.250 mg/ml for gallic, chlorogenic, caffeic, and ellagic acids, and 0.03 - 350 mg/ml for catechin and epicatechin. The chromatography peaks were confirmed by comparing their retention time with those of reference standards and by DAD spectra (200 to 600 nm). Calibration curve for gallic acid: Y = 12674x + 1185.3 (r = 0.9995); catechin: Y = 12840x + 1178.2 (r = 0.9999); epicatechin: Y = 13042x + 1363.7 (r = 0.9998); chlorogenic acid: Y = 11969x + 1241.8 (r = 0.9994); caffeic acid: Y = 13158x + 1196.4 (r = 0.9997); ellagic acid: Y = 12547x + 1243.9 (r = 0.9996); rutin: Y = 12873x + 1265.4 (r = 0.9993); isoquercitrin: Y= 11987x + 1273.2 (r = 0.9996); quercitrin: Y = 11970x + 1283.7 (r = 0.9995); kaempferol: Y = 12650x + 1359.5 (r = 0.9999) and quercetin: Y = 12693x + 1365.2 (r = 0.9997). All chromatographic operations were carried out at ambient temperature and in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves, as defined by Boligon et al. [33]. LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

Isolation of lymphocytes from human blood

Lymphocytes were isolated from blood samples obtained from healthy volunteers, collected in Vacutainer tubes (BD Vacutainer, Franklin Lakes, NJ, USA) containing lithium-heparin, and separated on Ficoll-Histopaque density gradients, as described by Böyum [34]. Cell number and viability were determined by trypan blue exclusion. More than 95% of the cells were found to be viable. Final cell suspension was performed in phosphate buffered saline (PBS, pH 7.4) and 3 x 10^6 cells/ml were

used for each analysis. All experiments were performed at least three times using lymphocytes obtained at different occasions and a total of eight samples were used for each incubation performed. The study was in accordance with the guidelines of the Ethics Committee of the Federal University of Santa Maria, which approved the experimental protocol (0049.0.243.000-08).

In vitro experimental protocol

Initially, lymphocytes were incubated with two concentrations of ASc (100 and 500 µg/ml) and gallic acid (50 and 200 µM) at 37 °C for 30 min, followed by incubation with AAPH (1 mM) at 37 °C for 2 h. The 2 h preincubation time with AAPH was chosen because at 37 °C and pH 7 the half-life of AAPH is about 175 h; consequently, the free-radical generation rate is virtually constant for the first few hours [35]. The effects of ASc and gallic acid *per se* were also observed in all experiments. The concentrations of the extract and gallic acid used in the incubations were selected on the basis of earlier experiments and adapted for this study [15, 36]. A control containing only lymphocytes was also used. After the incubation time, the lymphocyte suspensions were used for determinations of enzymatic activities, oxidative stress parameters, and viability assays as described below.

Enzymatic assays

ADA activity in lymphocyte suspensions was measured spectrophotometrically using the method of Giusti and Gakis [37], which is based on the direct measurement of the formation of ammonia, produced when ADA acts in excess of adenosine. The reaction was started by adding the substrate (adenosine) to a final concentration of 21 mmol/L and incubations were carried out for 1 h at 37 °C. The reaction was stopped by adding phenol-nitroprusside and alkaline-hypochlorite. Ammonium sulfate solution (75 mM ammonia) was used as the standard. Also, a blank of each sample was used in order to eliminate a possible interference of the color of the extract that could alter the measure of enzyme activity. Readings for samples and the standard were corrected by subtracting readings for sample blank and reagent blank, respectively, yielding ΔA sample and ΔA standard, and ADA activity (U/L) was

calculated according to the following formula: ADA (U/L) = ΔA sample/ ΔA standard x 50 U/L. The values were expressed as U/mg protein.

DPP-IV activity was determined as described by Schön et al. [38]. The lymphocyte suspensions were incubated with 0.6 mM of Gly-Pro q-nitroanilide q-toluenesulfonate for 120 min at 37 ºC. The reaction was stopped by adding 1 M acetate buffer (pH 4.5) and the absorbance was measured at 390 nm. The results were expressed as U/mg protein.

Parameters of oxidative stress

Thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation was estimated in lymphocytes by measurement of TBARS according to the method of Okawa et al [39]. Lymphocytes were mixed with 8% SDS solution and incubated for 5 min at room temperature. Glacial acetic acid (20%) was added to the reaction mixture and incubated for 2 - 5 min at room temperature. Finally, 0.8% TBA solution was added to the reaction mixture followed by incubation for 1 h in a boiling water bath. The reaction mixture was cooled, centrifuged, and the absorbance of the supernatant was measured spectrophotometrically at 532 nm. The results were expressed in nmol MDA/mg protein.

Protein thiol (P-SH) groups

P-SH groups in lymphocyte suspensions were determined by the method of Boyne and Ellman [40], modified by Jacques-Silva et al [41], which consisted in the reduction of DTNB measured at 37 °C and at 412 nm. The results were expressed as nmol P-SH/mg protein.

Cell viability

Tetrazolium salt method (MTT assay)

Viability assay was performed by the colorimetric MTT method. After the incubation time, 1.2 mM of MTT was added to the cell suspension following by incubation at 25 °C for 60 min. The formazan product generated during the incubation was solubilized in dimethyl sulfoxide and quantified spectrophotometrically at 560 nm. Only viable cells are able to reduce MTT, thus, each value obtained indicates the percentage of cell viability in relation to that of control cells, considered as 100% of viability.

Lactate dehydrogenase (LDH) activity assay

Following exposure with ASc and gallic acid with or without AAPH, the cells were centrifuged at 3000 rpm for 5 min in order to obtain a cell free supernatant. The activity of LDH was immediately measured using a commercial kit (Bioclin) and the results were expressed as U/L.

Neutral red assay

The neutral red assay is based on protocol described by Borenfreund and Puerner [42] and determines the accumulation of the neutral red dye in the lysosomes of viable, uninjured cells. Following incubation time, the cells were incubated for 3 h with neutral red dye (50 µg/ml). Cells were then washed with PBS, and 1 ml of the developing solution (EtOH/AcCOOH/H_20) was added followed by gentle shaking for 10 min for complete dissolution. The absorbance was determined at 540 nm and the results were presented as percentage of control values.

Protein quantification

Protein concentration of lymphocytes was measured by the method of Lowry et al. [43] using bovine serum albumin as the standard

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan’s post hoc test using Statistica software (version 6.0). The results were

expressed as mean ± standard error of the mean (SEM). Differences were considered statistically significant when $p \leq 0.05$.

Results

HPLC analysis

HPLC fingerprinting of ASc revealed the presence of gallic acid (t_R = 10.39 min; peak 1), catechin (t_R = 16.54 min, peak 2), chlorogenic acid (t_R = 19.98 min; peak 3), caffeic acid (t_R = 24.13 min; peak 4), ellagic acid (t_R = 28.75 min; peak 5), epicatechin (t_R = 32.05 min; peak 6), rutin (t_R = 38.01 min; peak 7), quercitrin (t_R = 43.81 min; peak 8), isoquercitrin (t_R = 46.57 min; peak 9), quercetin (t_R = 50.23 min; peak 10), and kaempferol (t_R = 55.42 min; peak 11) (Fig. 1).

In vitro effect of ASc and gallic acid on enzymatic activities in AAPH-induced oxidative stress conditions

Initially, we observed a significant increase in ADA activity when lymphocytes were exposed to AAPH 1 mM ($p<0.001$) when compared to the control. However, no change was observed in DPP-IV activity after the exposure of lymphocytes to oxidative stress conditions. ASc, *per se*, was capable to reduce ADA activity at the concentration of 500 µg/mL. On the other hand, gallic acid, *per se*, at the concentration of 200 µM caused an increase in DPP-IV activity (Fig. 2a and 2b). Moreover, ASc was able to protect the cells from the AAPH-induced increase in ADA activity at the concentrations of 100 µg/mL ($p<0.05$) and 500 µg/mL ($p<0.001$), whereas gallic acid was effective only at the concentration of 200 µM ($p<0.001$) (Fig. 2a and 2b).

In vitro effect of ASc and gallic acid on parameters of oxidative stress induced by AAPH

The results shown in Table 1 revealed that the incubation of lymphocyte suspensions in the presence of AAPH caused a significant increase ($p<0.05$) in lipid

peroxidation when compared with the control. However, neither ASc nor gallic acid were able to reduce TBARS levels at all concentrations tested.

A decrease in P-SH groups was observed when the lymphocytes were exposed to AAPH. ASc at 500 µg/mL was able to protect the cells by increasing P-SH group levels, whereas gallic acid did not cause any change on P-SH groups, neither in the presence nor in the absence of AAPH (Table 1).

In vitro effect of ASc and gallic acid on cell viability in AAPH-induced oxidative stress conditions

The MTT assay showed that 1 mM AAPH caused a decrease of 39.3% in lymphocyte viability after 2 h of incubation when compared to control ($p<0.05$; Fig. 3a). ASc at 500 µg/mL caused an increase in MTT levels *per se* and in the presence of AAPH; notwithstanding, this parameter was not altered in the presence of gallic acid.

AAPH reduced the levels of neutral red by 34.1% in lymphocytes ($p<0.05$). However, distinctive effects were observed when the cells were co-incubated with ASc and gallic acid. Increased neutral red levels were observed for ASc at all concentrations tested ($p<0.001$ to ASc 100 µg/mL and $p<0.01$ to ASc 500 µg/mL), whereas gallic acid did not show this protective effect (Fig. 3b).

LDH activity in the cells exposed to AAPH was 62.4% higher than that of the control ($p<0.05$; Fig. 3c). ASc protected the cells by reducing the enzyme activity at the concentrations of 100 and 500 µg/mL ($p<0.001$). A similar effect was observed for gallic acid, which also reduced LDH activity at all the concentrations tested ($p<0.01$).

Discussion

The present study reports the differential protective effects of ASc and gallic acid, a natural compound present in this extract, in lymphocytes subjected to oxidative stress conditions using AAPH as free radical generator. AAPH readily penetrates the cellular membrane and accumulates within lymphocytes, generating peroxyl radicals that contribute to adverse changes in biomembrane composition, potentially leading to an interference with cell-signaling pathways and ultimately to loss of cell function and viability [44]. A significant finding in this study was that ADA

activity increased in lymphocytes exposed to AAPH (Fig. 2a), while the generation of free radicals, *in vitro*, did not affect DPP-IV activity (Fig. 2b). The increase in ADA activity may affect adenosine levels available for stimulation of adenosine receptors expressed on the T-cell surface, contributing to impaired immune regulation [45, 46]. Moreover, the reduction of adenosine levels collaborates to further tissue damage by interfering with the role of this nucleoside in the regulation of inflammation [47]. Despite increasing amounts of knowledge regarding the physiological role of DPP-IV activity, there is currently no conclusive information on this enzymatic function under disease conditions, particularly under oxidative stress conditions.

Interestingly, ASc, at all concentrations tested, prevented the increase in ADA activity under oxidative stress conditions (Fig. 2a) and may represent an important mechanism to preserve adenosine levels in the circulation. Consequently, ADA inhibition by ASc may serve as a therapeutic strategy in conditions where ADA activity is enhanced such as rheumatoid arthritis, systemic lupus erythematosus, and tuberculosis [22, 23]. Furthermore, inhibition of ADA activity by ASc reduces the formation of hypoxanthine and xanthine (substrates for xanthine oxidase that have been shown to be the central mechanism in the generation of oxygen free radicals) and blocks free radical generation [48]. Gallic acid (200 µM), in turn, was able to reduce ADA activity and to increase DPP-IV activity. These different effects of gallic acid on the enzymes activities may be attributed to the fact that the efficiency of phenolic compounds is variable and related to many factors, such as the number, site of bounding, and mutual positions of hydroxyls on the aromatic ring [49].

It has been well documented that AAPH enhances lipid peroxidation in cellular systems and also causes the complete and rapid depletion of intracellular reduced glutathione [50], which corroborates with our results (Table 1). Although in previous studies we have demonstrated that ASc reduced lipid peroxidation in erythrocyte membranes and platelets of diabetic patients *in vitro* [14, 15], here we show that the pretreatment with ASc and gallic acid was not able to reverse the lipid peroxidation damage induced by AAPH in lymphocyte suspension. Therefore, we can suppose that ASc and gallic acid, under the conditions of this study, were not able to prevent the damage caused by AAPH, since that varying biological effects could be obtained in an *in vitro* system over a broad range of concentration levels of polyphenols, but these effects depend on the specific assay or model system used [51]. Moreover, ASc (500 µg/mL) was able to increase the levels of P-SH groups, demonstrating that

natural compounds are important in the protection of normal cell structure and adequate function of lymphocytes [52], since sulphydryl groups protect cells against free radicals, peroxides, and other toxic compounds [53].

Corroborating with other studies [54, 55], we have clearly demonstrated that AAPH impaired the viability of lymphocytes (Fig. 3), and, interestingly, only ASc improved cell viability, as determined by MTT assay and neutral red test, whereas gallic acid did not change these parameters. Moreover, pretreatment with the plant extract decreased LDH activity, indicating its potential application in the treatment of ROS-mediated cell membrane damage and confirming the cytoprotective effects rendered by *S. cumini* [56]. Thus, the suppression of the LDH leakage as well as the improvement of cell viability by the extract could be due to the presence of bioactive principles and the possible synergistic effect of its different constituents. Likewise, gallic acid significantly attenuated the AAPH-induced increase in LDH activity, which might be due to the ability of this compound to protect membrane integrity, thereby decreasing enzyme leakage [57].

Gallic acid, although found in higher amounts in the extract, showed little protective effect in this *in vitro* cell model. Therefore, we cannot exclude the hypothesis that other substances present in the extract are acting individually or synergistically to cause the effects of this plant on the enzymatic activities, cellular viability, and antioxidant defenses.

In conclusion, for the first time, the present study demonstrated the protective properties of the crude extract of *S. cumini* on blood lymphocytes subjected to oxidative stress using AAPH as free radical generator, *in vitro*. The results demonstrate that ASc protects against cell death caused by AAPH and contributes to membrane integrity, indicating that this plant extract is a significant source of natural antioxidants and may potentially be used as a food supplement in patients with certain diseases in which the oxidative stress-induced cellular injury is pathologically involved. ASc was also able to reverse the increase in ADA activity, although it did not affect the activity of DPP-IV, indicating that this plant affects the purinergic system and may modulate adenosine levels present in the circulation. From a molecular point of view, these results represent important findings suggesting that ASc exhibits immunomodulatory, antioxidant, and cytoprotective properties. Therefore, this study evokes a special interest in the protective properties of *S.*

cumini, but further studies are required to determine the exact biological role of the analyzed parameters, particularly in terms of lymphocyte homeostasis.

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Conflict of interest

The authors declare that there are no conflicts of interest

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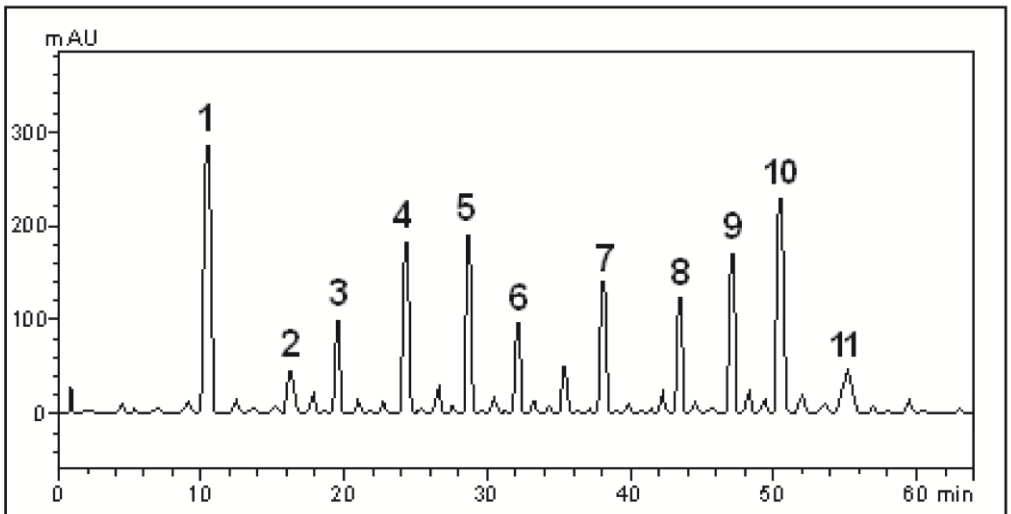
FIGURA 1



Compounds	*Syzygium cumini*		LOD	LOQ
	mg/g	%	µg/m	µg/m
Gallic acid	27.41 ± 0.02	2.74	0.016	0.054
Catechin	3.98 ± 0.01	0.39	0.035	0.118
Chlorogenic	7.85 ± 0.09	0.78	0.007	0.023
Caffeic acid	15.36 ± 0.03	1.53	0.023	0.072
Ellagic acid	15.81 ± 0.02	1.58	0.018	0.059
Epicatechin	8.27 ± 0.05	0.82	0.020	0.067
Rutin	11.46 ± 0.01	1.14	0.009	0.030
Quercitrin	10.09 ± 0.03	1.07	0.043	0.141
Isoquercitrin	14.95 ± 0.07	1.49	0.025	0.082
Quercetin	19.38 ± 0.01	1.93	0.010	0.034
Kaempferol	4.27 ± 0.05	0.42	0.014	0.045

Fig. 1 Representative high performance liquid chromatography profile of *Syzygium cumini* leaf extract. Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), ellagic acid (peak 5), epicatechin (peak 6), rutin (peak 7), quercitrin (peak 8), isoquercitrin (peak 9), quercetin (peak 10) and kaempferol (peak 11). Results are expressed as mean ± SEM of three determinations.

FIGURA 2

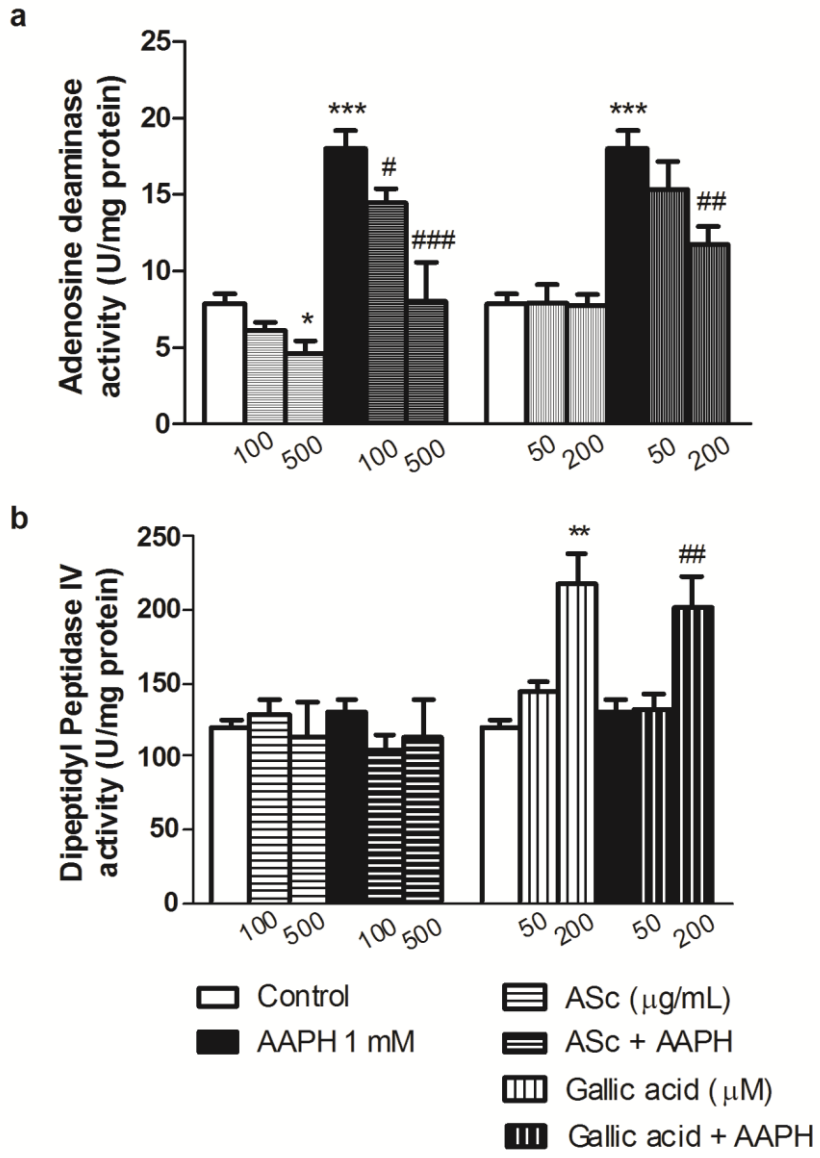


Fig. 2 Effect of ASc and gallic acid on ADA (a) and DPP-IV (b) activities in lymphocytes after 2 h of incubation with AAPH, *in vitro*. Data are reported as mean ± SEM (n=8) and expressed as U/mg protein. Statistically significant differences were determined by one-way ANOVA followed by Duncan's multiple range test (**p*<0.05, ***p*<0.01, and ****p*<0.001 compared with control; $^{\#}p$<0.05, $^{\#\#}p$<0.01, and $^{\#\#\#}p$<0.001 compared with AAPH 1mM).

FIGURA 3

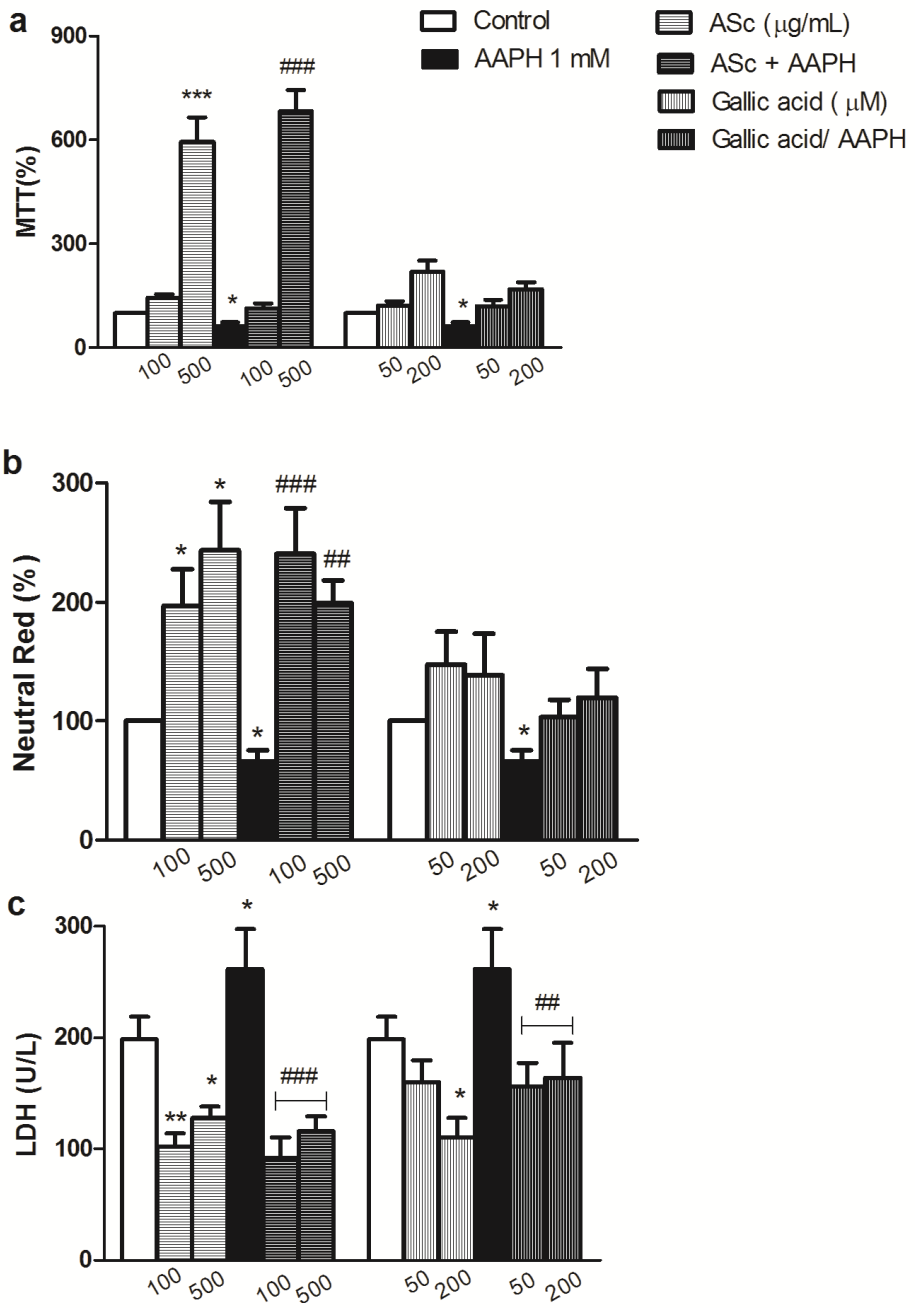


Fig. 3 Effect of ASc and gallic acid on MTT (a) and neutral red (b) assays and on LDH activity (c) in lymphocytes after 2 h of incubation with AAPH, *in vitro*. Data are reported as mean ± SEM (n=8). Statistically significant differences were determined by one-way ANOVA followed by Duncan's multiple range test (**p*<0.05, ***p*<0.01, and ****p*<0.001 compared with control; $^{\#\#}p$<0.01 and $^{\#\#\#}p$<0.001 compared with AAPH 1mM).

TABELA 1

Table 1 *In vitro* effect of ASc and gallic acid on TBARS and P-SH levels in the presence or absence of AAPH**.**

	TBARS (mmol/mg protein)	P-SH (nmol/mg protein)
Control	4.62 ± 1.43	184.6 ± 23.8
ASc 100 µg/mL	6.36 ± 2.06	172.9 ± 25.4
ASc 500 µg/mL	5.52 ± 1.06	371.7 ± 42.6**
Gallic acid 50 µM	4.37 ± 1.91	146.1 ± 35.4
Gallic acid 200 µM	5.13 ± 1.6	188.5 ± 31.6
AAPH 1 mM	10.2 ± 1.32*	75.4 ± 19.5*
AAPH + ASc 100 µg/mL	9.76 ± 1.62	161.4 ± 25.3
AAPH + ASc 500 µg/mL	9.83 ± 2.08	368.6 ± 37.6$^{###}$
AAPH + Gallic acid 50 µM	8.42 ± 1.77	175.3 ± 39.6
AAPH + Gallic acid 200 µM	9 ± 1.25	141.2 ± 31.1

Data are reported as mean ± SEM (n=8). Statistically significant differences were determined by one-way ANOVA followed by Duncan's multiple range test (*p<0.05 and **p<0.01 compared with control; $^{###}p$<0.001 compared with AAPH 1mM).

5. DISCUSSÃO

No presente estudo avaliamos, inicialmente, parâmetros bioquímicos e inflamatórios em pacientes com SMet. Um aumento na atividade das enzimas ADA e DPP-IV foi observado em linfócitos de pacientes com SMet, sendo que a ativação dessas duas enzimas apresenta um papel essencial na resposta imune, em especial a ADA que está diretamente envolvida na regulação das concentrações de adenosina na circulação. Sendo assim, essa alteração nos níveis de adenosina pode estar associada à diversos componentes da SMet, incluindo a obesidade, adiposidade corporal (IMC) e inflamação sistêmica, fatores esses que auxiliam no desenvolvimento do processo dessa síndrome (KIRINO et al., 2011).

Os pacientes com SMet também apresentaram aumento da atividade das enzimas AChE em linfócitos e BuChE no soro. O aumento na AChE pode estar associada a uma resposta inflamatória provocada pelo estado de resistência à insulina, uma vez que a rápida degradação da acetilcolina leva a uma redução nas ações anti-inflamatórias exercidas pela mesma, bem como um prejuízo na regulação da função imune em linfócitos (TAYEBATI et al., 2002). Já o aumento encontrado na atividade da BuChE pode se dar pelo fato de que a resistência à insulina e o fluxo de ácidos graxos do tecido adiposo para o fígado poderiam estimular a síntese hepática da BuChE e o aumento da sua atividade no soro (CUCUIANU et al., 2002), e corrobora com outros estudos que demonstraram ativação dessa enzima em pacientes com diabetes, obesidade e hiperlipidemias (KUTTY e PAYNE, 1994; ALCÂNTARA et al., 2005).

Outro fator diretamente relacionado à SMet é a disfunção endotelial, uma vez que a insulina estimula a produção de NO a partir do endotélio (KIM et al., 2006; JANSSON, 2007), o que explica, em parte, o aumento dos níveis de NOx encontrados nos pacientes com SMet avaliados neste estudo. Os estados de resistência à insulina também estão associados com a produção aumentada de EROs. Embora, neste estudo, não tenha sido observado um aumento nos níveis de TBARS na SMet, houve uma diminuição nas defesas antioxidantes, o que pode levar ao dano de organelas e enzimas, bem como ao desenvolvimento de comorbidades nesses pacientes. Nesse contexto, também foi observado um aumento na atividade

da GGT no soro dos pacientes com SMet, e considerando que a enzima está envolvida no catabolismo da glutationa (GIRAL et al., 2008), salienta-se o seu papel como biomarcador de estresse oxidativo.Por fim, pode-se observar que os parâmetros analisados no soro, com exceção dos níveis de CRP, não foram afetados pelo IMC.

Com relação aos resultados encontrados em pacientes com SMet, as alterações observadas em linfócitos demonstram que a SMet pode causar dano celular com alteração de importantes marcadores enzimáticos, como a ADA, DPP-IV e AChE, enzimas essencias na regulação de vários aspectos da função do tecido adiposo e da condição de inflamação presente na SMet. Além disso, as alterações observadas nos parâmetros analisados no soro e, especialmente as correlações obtidas neste estudo podem desempenhar um papel importante na cascata de anormalidades presentes na SMet. Assim, estes parâmetros podem ser utilizados na prática clínica como possíveis marcadores para a avaliação da função imune de pacientes com SMet, além de serem úteis na prevenção e controle de vários aspectos desta síndrome.

Em relação aos estudos *in vitro* ficou demonstrado que o ASc foi capaz de prevenir o aumento na atividade da ADA em condições hiperglicêmicas, além de ter apresentado efeito mais significativo do que os compostos fenólicos testados quando incubados juntos. Pode-se sugerir a partir deste resultado que os efeitos do ASc sejam atribuídos a outros possíveis mecanismos em situações de hiperglicemia, que não somente devido aos efeitos relacionados a presença dos compostos fenólicos em sua composição. Ao explorar o mecanismo de ação do ASc, observamos que o mesmo atua de maneira semelhante a cafeína e a insulina. Por outro lado, o efeito do ASc em combinação com o dipiridamol foi menos evidente que o efeito do ASc sozinho, demonstrando que o dipiridamol atenuou o efeito do ASc sobre a atividade da ADA, por antagonizar o efeito do mesmo ou por competição com o extrato. Assim, um dos possíveis mecanismos de ação propostos neste estudo para o efeito do ASc sobre a atividade da ADA em eritrócitos expostos à condições hiperglicêmicas seria através da inibição na atividade da enzima fosfodiesterase, com consequente redução nos níveis de AMP e adenosina.

Por fim, no quarto manuscrito deste estudo o ASc reduziu a atividade da ADA e aumentou a viabilidade celular em linfócitos expostos ao AAPH, mas não alterou a lipoperoxidação causada por este composto. Ainda, os efeitos observados para o

ácido gálico foram menos eficientes que os demonstrados pelo ASc. É possível sugerir que o ASc ao reduzir a atividade da ADA, pode estar aumentando os níveis de adenosina e colaborando para a manutenção dos efeitos benéficos provocados pela mesma, como ações antioxidantes, antiinflamatórias e antitrombóticas. Além disso, podemos atribuir ao ASc um efeito citoprotetor através do aumento da viabilidade celular e por contribuir na integridade da membrana das células. Portanto, o extrato de *Syzygium cumini* apresentou efeitos significativos nos desenhos experimentais efetuados e este estudo contribui para o melhor entendimento das propriedades e mecanismos de ação do *Syzygium cumini*.

6. CONCLUSÕES

Com base nos resultados obtidos no presente estudo podemos concluir que:

- A atividade das enzimas ADA, DPP-IV, AChE e BuChE encontram-se aumentadas em pacientes com SMet, demonstrando que os sistemas purinérgico e colinérgico são influenciados por esta situação clínica e refletem o estado inflamatório desses pacientes;

- Os pacientes com SMet também apresentaram defesas antioxidantes reduzidas e aumento nos níveis de NOx e CRP, resultando em alterações em vários sistemas fisiológicos e agravando a resistência à insulina presente nesses pacientes;

- A atividade da enzima ADA encontra-se aumentada em RBCs expostos à altas concentrações de glicose, indicando que a sinalização purinérgica pode estar alterada no estado hiperglicêmico;

- Um dos possíveis mecanismo de ação do *Syzygium cumini* sobre a atividade da ADA em eritrócitos expostos à condições hiperglicêmicas é através da inibição da enzima fosfodiesterase. Este resultado é benéfico em situações de hiperglicemia, pois evita a redução nos níveis de adenosina e, assim, contribui para a manutenção das propriedades exercidas por esse nucleosídeo, tais como atividades antiinflamatórias, antioxidantes e antitrombóticas;

- O ASc preveniu o aumento da atividade da ADA em linfócitos expostos ao AAPH, assim, representando um importante mecanismo de preservação dos níveis de adenosina na circulação;

- O ASc aumentou a vibilidade celular e contribuiu para a manutenção da integridade da membrana de linfócitos, demonstrando seu efeito citoprotetor em condições de injúria celular induzidas por estresse oxidativo;

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