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ATIVIDADE DAS ECTONUCLEOTIDASES, COLINESTERASE SÉRICA E PERFIL OXIDATIVO NO DIABETES MELITO TIPO 2 E HIPERTENSÃO EM HUMANOS

TESE DE DOUTORADO

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Santa Maria, RS, Brasil

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ATIVIDADE DAS ECTONUCLEOTIDASES, COLINESTERASE SÉRICA E PERFIL OXIDATIVO NO DIABETES MELITO TIPO 2 E HIPERTENSÃO EM HUMANOS

por

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

Orientador: Profa. Dra. Maria Rosa Chitolina Schetinger

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Bioquímica Toxicológica

A Comissão Examinadora, abaixo assinada,
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DEDICATÓRIA

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas:
Bioquímica Toxicológica
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ATIVIDADE DAS ECTONUCLEOTIDASES, COLINESTERASE SÉRICA E PERFIL OXIDATIVO NO DIABETES MELITO TIPO 2 E HIPERTENSÃO EM HUMANOS

AUTOR: GILBERTO INÁCIO LUNKES
ORIENTADOR: MARIA ROSA CHITOLINA SCHETINGER
Data e Local de Defesa: Santa Maria, 5 de março de 2008

O aumento na atividade enzimática da NTPDase e 5'-nucleotidase, em pacientes com diabetes e hipertensão, desencadeou a investigação dos possíveis mecanismos envolvidos nas alterações na atividade das ectonucleotidases. O nível de estresse oxidativo e também a resposta dos sistemas antioxidantes foram avaliados em pacientes com diabetes tipo 2, hipertensão e diabetes tipo 2 com hipertensão associada. A interferência da concentração de glicose foi avaliada na atividade enzimática das ectonucleotidases, colinesterase sérica e também das enzimas antioxidantes. As curvas *in vitro* demonstraram que o aumento na atividade dos sistemas enzimáticos foi proporcional à elevação nas concentrações de glicose, demonstrando uma interferência direta da hiperglicemia. O aumento na expressão da enzima NTPDase demonstrou uma importante correlação com a hidrólise dos nucleotídeos de adenina ATP e ADP em pacientes com diabetes e hipertensão associada. O incremento nos níveis de marcadores de estresse oxidativo e dos sistemas antioxidantes, em pacientes com diabetes e hipertensão associada, parecem estar relacionados com um mecanismo compensatório para prevenir o dano oxidativo. Os baixos níveis de ácido ascórbico sérico aumentam a exposição dos pacientes, com diabetes e hipertensão associada, aos danos oxidativos resultantes do aumento na geração de espécies reativas de oxigênio. O aumento na

atividade da enzima colinesterase sérica, em pacientes com diabetes e hipertensão associada, pode estar potencialmente relacionado com os níveis de glicemia e com o metabolismo dos lipídios. Os medicamentos administrados aos pacientes não alteraram as respostas enzimáticas nos grupos analisados. Portanto, houve uma possível interferência do diabetes e da hipertensão no mecanismo catalítico da colinesterase sérica. Os dados obtidos nos estudos permitem sugerir que os elevados níveis de glicose sangüínea constituem um dos principais fatores capazes de promover alterações nas respostas enzimáticas em pacientes diabéticos e com hipertensão associada.

Palavras-chave: Ectonucleotidases, estresse oxidativo, colinesterase sérica, Diabetes melito tipo 2, hipertensão, humanos, plaquetas.

ABSTRACT

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

ACTIVITY OF THE ECTONUCLEOTIDASES, SERUM CHOLINESTERASE AND OXIDATIVE PROFILE IN TYPE 2 DIABETES MELITO AND HYPERTENSION IN HUMANS

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ADVISER: MARIA ROSA CHITOLINA SCHETINGER
Date and Place of the defense: Santa Maria, March 5th, 2008

The increase in the NTPDase and 5'-nucleotidase enzymatic activities, in patients with diabetes and hypertension, unchained the investigation of the possible mechanisms involved in the alterations in ectonucleotidases activities. The oxidative stress level and also the answer of the antioxidant systems were evaluated in patients with type 2 diabetes, hypertension and type 2 diabetes with associated hypertension. The interference of the glucose concentration was evaluated in the enzymatic activity of the ectonucleotidases, serum cholinesterase and also antioxidant enzymes. The curves *in vitro* demonstrated that the increase in enzymatic activity was proportional to the elevation in the glucose concentrations, demonstrating a direct interference of the hyperglycemia. The increase in the NTPDase expression demonstrated an important correlation with adenine nucleotide ATP and ADP hydrolyze in patients with diabetes and associated hypertension. The increment in markers of oxidative stress and antioxidant systems levels in patients with diabetes and associated hypertension seems to be related with a compensatory mechanism to prevent oxidative damages. Low serum acid ascorbic levels increase exposes to oxidative damages in patients with diabetes and associated hypertension, resultant of the increase in reactive oxygen species generation. The increment in the serum cholinesterase activity can be potentially related with glycemia levels and lipid

metabolism in patients with diabetes and associated hypertension. The medicines administered to the patients did not alter the enzymatic responses in the analyzed groups. Therefore, there were a possible interference of the diabetes and hypertension in the catalytic mechanism of the serum cholinesterase enzyme. Data obtained in the studies permit to suggest that high blood glucose levels constitute one of the principal factors capable to promote alterations in the enzymatic responses in patients with diabetes and associated hypertension.

Key words: Ectonucleotidases, oxidative profile, serum cholinesterase, type 2 Diabetes melito, hypertension, platelets.

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LISTA DE ABREVIATURAS

ATP – adenosina trifosfato

ADP – adenosina difosfato

CAT – catalase

DM – Diabetes melito

DNA – ácido desoxirribonucléico

ERO – espécies reativas de oxigênio

HAS – hipertensão arterial sistêmica

NDP – nucleotídeo difosfato

NPSH – grupos tióis não protéicos

NMP – nucleotídeo monofosfato

NTP – nucleotídeo trifosfato

E-NTPDase – ecto-nucleosídeo trifosfato difosfohidrolase

PRP – plasma rico em plaquetas

SOD – superóxido dismutase

TBARS – espécies reativas ao ácido tiobarbitúrico

UDP – uridina difosfato

UTP – uridina trifosfato

LISTA DE ANEXOS

SUMÁRIO

1 INTRODUÇÃO

A complexidade epidemiológica de doenças crônicas como diabetes e hipertensão têm atingido níveis alarmantes (GANNE et al., 2007). Estudos recentes demonstram que o Diabetes melito está se consagrando como uma das maiores catástrofes de saúde pública (MEETOO et al., 2007). Atualmente, cerca de 6% da população adulta mundial tem diabetes diagnosticada e há uma previsão de 366 milhões de pessoas com diabetes até 2030 (WILD et al., 2004). A estimativa de pacientes hipertensos no Brasil é de aproximadamente 30 milhões de pessoas e no mundo de 600 milhões (SOCIEDADE BRASILEIRA DE HIPERTENSÃO, 2006). Por constituírem doenças de extrema relevância em saúde pública requerem contínuos estudos.

A perda funcional do endotélio vascular no diabetes está intrinsecamente ligada ao desenvolvimento de doença cardiovascular e é responsável pela aceleração de processos aterotrombóticos (HAMILTON et al., 2007). Estudos têm demonstrado que cerca de 80% dos pacientes com Diabetes melito tipo 2 desenvolvem hipertensão (SAVOIA & SCHIFFRIN, 2007). O comprometimento bioquímico do paciente diabético hipertenso deve ser amplamente investigado na tentativa de buscar explicações para as alterações desenvolvidas.

O presente estudo tem como propósito avaliar os fatores envolvidos na alteração da atividade das enzimas NTPDase e 5'-nucleotidase em plaquetas de humanos com diabetes e hipertensão. Estudos anteriores, em humanos e modelo experimental, demonstraram um aumento na hidrólise de ATP e ADP pela enzima NTPDase e AMP pela 5'-nucleotidase em pacientes hipertensos, diabéticos tipo 2 e diabéticos tipo 2 hipertensos (LUNKES et al., 2003; LUNKES et al., 2004). A investigação em humanos também revelou que os medicamentos, administrados aos pacientes diabéticos e hipertensos, não foram capazes de alterar a atividade enzimática das ectonucleotidases (LUNKES et al., 2003). Então, com a finalidade de investigar os fatores capazes de interferir na atividade enzimática foram realizadas

curvas de glicose e frutose, assim como, foi avaliada a expressão da enzima NTPDase (CD39) em plaquetas de pacientes com diabetes tipo 2 e hipertensão.

O incremento na produção de radicais livres, proveniente do aumento de dano oxidativo em lipídios e proteínas, está sendo associado com complicações micro e macrovasculares em pacientes diabéticos (PENNATHUR & HEINECKE, 2007). Com o propósito de avaliar o nível de estresse oxidativo, foram investigados os sistemas antioxidantes enzimáticos e não-enzimáticos em pacientes com diabetes tipo 2 e hipertensão.

A colinesterase sérica está envolvida na detoxicação de xenobióticos circulantes e é capaz de hidrolisar acetilcolina (LOCKRIDGE, 1988). A acetilcolina além das funções cognitivas está relacionada com ações antiinflamatórias (RAO et al., 2007). O diabetes tipo 2 e a hipertensão podem apresentar baixos níveis de inflamação sistêmica. Portanto, a colinesterase sérica, que está elevada em baixos níveis de inflamação sistêmica, pode constituir um marcador para predisposição ao desenvolvimento de diabetes tipo 2 (DAS, 2007).

Com a finalidade de investigar as alterações pertinentes aos pacientes com diabetes tipo 2 e hipertensão associada foram elaborados os seguintes objetivos:

Objetivo Geral

Avaliar os fatores que possam estar promovendo aumento na atividade das enzimas que degradam nucleotídeos da adenina em plaquetas de pacientes diabéticos, hipertensos e diabéticos hipertensos, bem como a geração de estresse oxidativo e as alterações na atividade da colinesterase sérica.

Objetivos específicos

a. Verificar atividade enzimática *in vitro* da colinesterase sérica frente a diferentes concentrações de glicose em voluntários humanos.

b. Verificar a atividade enzimática *in vitro* das ectonucleotidases frente a diferentes concentrações de glicose e frutose em plaquetas de voluntários humanos.

c. Verificar se há alteração na expressão da enzima NTPDase em plaquetas de pacientes com diabetes tipo 2, hipertensão e diabéticos tipo 2 - hipertensos.

d. Avaliar os sistemas anti-oxidantes enzimáticos e não-enzimáticos em pacientes com diabetes tipo 2, hipertensão e diabéticos tipo 2 - hipertensos, bem como o efeito da glicose e de micronutrientes.

2 REVISÃO DA LITERATURA

2.1 Diabetes melito

O diabetes melito (DM) constitui uma síndrome de etiologia múltipla, caracterizada por hiperglicemia crônica e elevado risco de alterações aterotrombóticas afetando o sistema coronário, o cerebral e o arterial (MINISTÉRIO DA SAÚDE, 2006).

Esta síndrome apresenta diferentes etiologias para os distúrbios de glicemia. O DM tipo 1 apresenta uma restrição total no fornecimento de insulina, com tendência a cetoacidose e necessidade de tratamento com insulina. O DM tipo 2 resulta de graus variáveis de resistência à insulina e deficiência relativa de secreção de insulina. Neste caso, os pacientes apresentam suscetibilidade à obesidade e complicações micro e macrovasculares (MINISTÉRIO DA SAÚDE, 2006).

A hiperglicemia tem um marcado efeito na estrutura funcional da fibrina, gerando coágulos com estrutura mais densa, resistente a fibrinólise. A combinação desses fatores com alteração na reatividade plaquetária cria um risco trombótico para o desenvolvimento de doença cardiovascular (GRANT, 2007). A presença de disfunção endotelial, aumento da geração de processo trombótico e resposta inflamatória anormal são características de DM tipo 2. Os pacientes com DM tipo 2 e doença arterial coronariana têm hiperatividade de fatores trombóticos vasculares enquanto que os fatores anticoagulatórios ficam suprimidos (BONDAR et al., 2007).

A homeostasia da glicemia é obtida por meio da secreção de insulina. O tratamento com insulina tem efeito benéfico nas funções vasculares. Esse efeito resulta provavelmente do controle da glicemia, como um mecanismo secundário (GAENZER et al., 2002). O tratamento com insulina em DM tipo 2 interfere na expressão de citocinas inflamatórias. Subseqüentemente, aumenta os processos trombóticos em pacientes com aterosclerose, independentemente do tempo de duração do diabetes e da extensão da doença arterial coronariana (ANTONIADES et al., 2007).

No Brasil, o DM tipo 2 associado com a hipertensão arterial sistêmica constitui a principal causa de mortalidade, hospitalizações, amputações de membros inferiores e representa ainda 62,1% dos diagnósticos primários em pacientes com insuficiência renal crônica submetidos à diálise (MINISTÉRIO DA SAÚDE, 2006).

2.2 Hipertensão Arterial Sistêmica

A hipertensão arterial sistêmica (HAS) constitui um dos principais fatores de risco para o desenvolvimento de doenças cardiovasculares, cerebrovasculares e renais. Esta alteração da pressão arterial é responsável por aproximadamente 40% das mortes por acidente vascular cerebral, por 25% das mortes por doença arterial coronariana e, em combinação com o diabetes, por 50% dos casos de insuficiência renal terminal. A prevalência na população urbana adulta brasileira varia entre 22 e 44% (MINISTÉRIO DA SAÚDE, 2006).

No DM tipo 1, a HAS pode estar associada a nefropatia diabética. Nestes casos, o controle da pressão arterial é crucial para retardar a perda da função renal. No DM tipo 2, a hipertensão pode estar associada à síndrome de resistência à insulina e ao alto risco cardiovascular. Estudos com pacientes diabéticos hipertensos ressaltam a importância da redução da pressão arterial como um fator capaz de diminuir a morbi-mortalidade cardiovascular e as complicações microvasculares relacionadas ao diabetes (SOCIEDADE BRASILEIRA DE HIPERTENSÃO, 2006). O controle adequado da hiperglicemia previne a progressão de distúrbios microcirculatórios coronarianos. Porém, a presença concomitantemente de hipertensão retarda o efeito no sistema circulatório coronariano resultante do controle da glicemia (TAKIUCHI et al., 2002).

As doenças cardiovasculares são diretamente afetadas pela hipertensão arterial sistêmica. A hipertensão quando associada com o diabetes pode exacerbar as complicações no sistema cardiovascular. Portanto, a combinação dessas duas doenças é responsável pelo desenvolvimento mais precoce de doenças coronárias. A prevalência de hipertensão é muito maior em diabéticos que em pacientes não diabéticos (GARCÍA DONAIRE & RUILOPE, 2007).

Em normotensos, a insulina que tem propriedade vasodilatadora, pode estimular a atividade nos receptores neuronais simpaticomiméticos sem elevar a

pressão arterial sistêmica. Estudos sugerem que quadros de resistência e/ou hiperinsulinemia podem causar um aumento na pressão arterial em pacientes com diabetes (AGATA et al., 1998; MATAYOSHI et al., 2007).

2.3 Colinesterase sérica

A colinesterase sérica está envolvida na detoxicação de xenobióticos circulantes e é responsável pela hidrólise da acetilcolina. O neurotransmissor acetilcolina está envolvido com funções cognitivas e também com ações antiinflamatórias (RAO et al., 2007). Portanto, a colinesterase, pelo aumento na hidrólise de acetilcolina, pode realçar a inflamação (DAS, 2007).

O DM tipo 2 e a hipertensão são doenças que podem apresentar baixos níveis de inflamação sistêmica. A elevação da atividade enzimática da colinesterase sérica, observada em diferentes condições clínicas, poderia servir como um marcador de baixos níveis de inflamação sistêmica (DAS, 2007). Neste contexto, a colinesterase sérica pode constituir um marcador para predisposição ao desenvolvimento de diabetes tipo 2 (RAO et al., 2007).

Estudos prévios têm demonstrado uma elevação na atividade da colinesterase sérica em pacientes com diabetes, hipertensão e resistência à insulina (RUSTEMEIJER et al., 2001; DAVE & KATYARE, 2002). O aumento da colinesterase sérica pode ser proveniente do aumento de fluxo de ácidos graxos livres, que estimula a síntese hepática desta enzima em pacientes diabéticos (CUCUIANU et al., 2002). A elevação da atividade enzimática da colinesterase parece estar relacionada com a hipertensão e com os distúrbios provenientes do diabetes (ALCANTARA et al., 2002).

Pacientes com hiperlipidemia tipo IIb apresentaram um aumento na atividade da colinesterase sérica, quando comparados com pacientes hígidos para dislipidemia (KÁLMÁN et al., 2004). A colinesterase sérica tem sido relacionada com parâmetros de adiposidade e perfil de lipídios séricos (IWASAKI et al., 2007). Esta influência do metabolismo dos lipídios foi observada em pacientes com hiperlipoproteinemia tipo IIa e IIb tratados com sinvastatina, que tiveram uma diminuição na atividade da colinesterase sérica (MUACEVIC-KATANECA et al., 2005).

2.4 Plaquetas

As plaquetas estão envolvidas na hemostasia sangüínea, onde desempenham atividade mecânica e bioquímica. Dentre as funções das plaquetas destacam-se a ativação e a agregação. As plaquetas são ativadas quando entram em contato com colágeno, trombina, ADP (DANIEL et al., 1998).

No DM tipo 2 há um incremento na reatividade plaquetária e em conseqüência um risco maior de complicações cardiovasculares (ANGIOLILLO et al., 2007). Estudo prévio demonstrou que a reatividade plaquetária em pacientes diabéticos se mantém elevada, mesmo frente à terapia antiplaquetária. Este tipo de resposta demonstra que o paciente diabético esta mais exposto ao processo aterotrombótico (EVANGELISTA et al., 2007). Além disso, níveis elevados de insulina, em jejum, estão associados com uma redução da fibrinólise e com a hipercoagulabilidade em pacientes com tolerância normal à glicose. A hiperinsulinemia aumenta o risco de doenças cardiovasculares (MEIGS et al., 2000). A hiperinsulinemia e a hiperglicemia, mas particularmente a combinação de ambos proporciona um estado pró-trombótico e pode em adição, ser pró-inflamatório e pró-aterogênico (BODEN & RAO, 2007).

A presença de hiperatividade plaquetária em pacientes diabéticos com glicemia controlada e sem complicações está associada ao aumento no estresse oxidativo e com um deficiente sistema antioxidante em pacientes com DM tipo 2. A associação dessas alterações proporciona um risco maior de ocorrência de doenças vasculares em pacientes DM tipo 2 (VÉRICEL et al., 2004).

Em pacientes diabéticos o aumento da atividade de plaquetas sangüíneas contribui para as complicações vasculares. Nestes pacientes, a estimulação da plaqueta, com a trombina, promove uma liberação de nucleotídeos de adenina. A hiperglicemia crônica promove a liberação aumentada de ATP/ADP das plaquetas, que pode constituir um importante fator para hiperatividade plaquetária (MICHNO et al., 2007).

2.5 Nucleotídeos e nucleosídeos

Os nucleotídeos extracelulares constituem importantes moléculas sinalizadoras (ERB et al., 2006; INOUE et al., 2007). Os nucleotídeos modulam uma

grande variedade de funções nos tecidos onde interferem em efeitos inflamatórios, na agregação plaquetária e em reações imunes (ATKINSON et al., 2006; BOURS et al., 2006; BELDI et al., 2008).

O ATP quando secretado para o meio extracelular de plaquetas é capaz de mediar a reatividade plaquetária (BIRK et al., 2002). A hidrólise subseqüente de ATP e ADP até AMP e adenosina inibem a agregação plaquetária (MARCUS et al., 2005). O ATP atua como um inibidor das ações do ADP (STAFFORD et al., 2003).

O ADP constitui um importante agonista fisiológico para a hemostasia (MURUGAPPA & KUNAPULI, 2006). A interação do ADP com os receptores P2X e P2Y em plaquetas tem uma importante função na trombogênese, pois o ADP extracelular ativa a agregação plaquetária (CATTANEO, 2007). A hidrólise do ADP até adenosina, através das ectonucleotidases, estimula o processo de inibição da agregação plaquetária (ROBSON et al., 2005). Portanto, há uma conseqüente inibição da agregação plaquetária através dos receptores de adenosina (CRISTALLI et al., 2003; KAHNER et al., 2006). A adenosina também pode ser um agente vasodilatador (ABBINK-ZANDBERGEN et al., 1999; BIJLSTRA et al., 2004).

Os purinoceptores constituem receptores para ATP, ADP e adenosina. Normalmente, o receptor purinérgico P1 responde a adenosina, enquanto o receptor ionotrópico P2X responde ao ATP e o receptor metabotrópico P2Y pode ser ativado pelo ATP, ADP, UTP e UDP (BURNSTOCK & KNIGHT, 2004). O receptor P2X1 não é ativado pelo ADP e portanto não induz a agregação plaquetáriaa, via plasmina (ISHII-WATABE et al., 2000). A ativação do receptor P2Y(12) em presença de altas concentrações de ADP induz a um parcial agregação plaquetária (KAUFFENSTEIN et al., 2001).

2.6 NTPDase e 5'-nucleotidase

NTPDase (nucleosídeo trifosfato difosfohidrolase, CD39, EC 3.6.1.5) é o termo genérico para designar uma família de enzimas presentes na membrana plasmática de diversos tecidos. As NTPDases catalisam a hidrólise de nucleotídeos difosfatados (NDP) e trifosfatados (NTP), com diferentes graus de preferência por um tipo individual (ROBSON et al., 2006).

A família das E-NTPDases (ecto-nucleosídeo trifosfato difosfohidrolase) é composta por 8 membros. Embora possam ser divididos em grupos, de acordo com suas características topográficas, todos os membros apresentam cinco regiões conservadas de NTPDase, as quais estão envolvidas na atividade catalítica da enzima e/ou integridade estrutural das E-NTPDases (ROBSON et al., 2006).

A NTPDase 1 hidrolisa ATP e ADP de forma igualitária, a NTPDase 3 e a NTPDase 8 hidrolisam preferencialmente ATP como substrato e NTPDase 2 tem uma elevada preferência por nucleotídeo trifosfatado (ZIMERMMANN, 2001; KUKULSKI et al., 2005). As NTPDases 1, 2, 3 e 8 estão fortemente ligadas a membrana via dois domínios transmembrana, que no caso da NTPDase 1 são importantes para a manutenção da especificidade da atividade catalítica e do substrato (Figura 1). Os dois domínios também podem sofrer movimentos coordenados durante o processo de ligação e hidrólise dos nucleotídeos (GRINTHAL & GUIDOTTI, 2006).

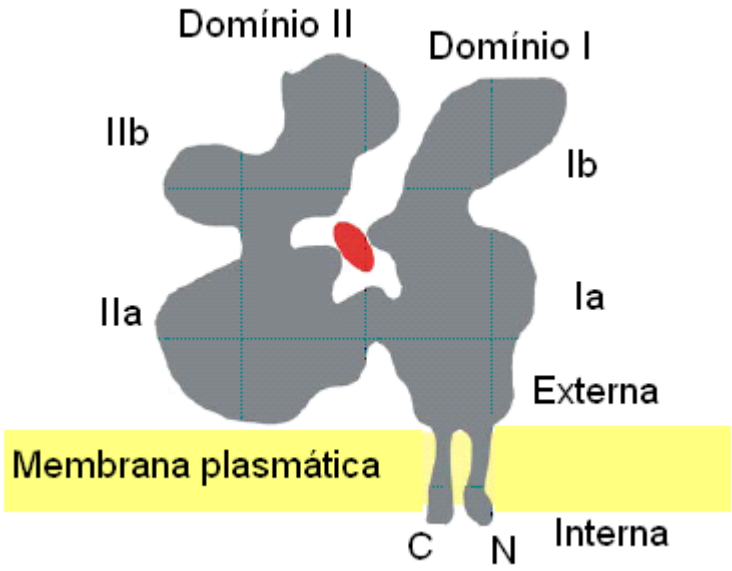

FIGURA 1: Topologia da enzima NTPDase localizada na superfície da membrana com dois domínios transmembrana. (Adaptado de ROBSON et al., 2006).

A NTPDase 4 tem duas isoformas α e β – que diferem na especificidade por nucleotídeos e na dependência de cátions divalentes. Esta ectoenzima tem localização lisossomal (WANG & GUIDOTTI, 1998; BIEDERBICK et al., 2000).

A NTPDase 5 apresenta um alto grau de especificidade para nucleotídeos difosfatos (NTPs) e possui as porções C e N-terminal não hidrofóbicas (PÁEZ et al., 2001).

A NTPDase 6 hidrolisa preferencialmente nucleosídeos 5'-difosfatos. A análise imunohistoquímica sugere que a NTPDase 6 é associada ao Complexo de Golgi e a pequenas extensões da membrana plasmática (BRAUN et al., 2000).

A NTPDase 7 possui localização subcelular e tem preferência por nucleosídeos trifosfatos (SHI et al., 2001). A NTPDase 8 possui dois domínios transmembrana, uma porção C-terminal e outra N-terminal. Essa NTPDase possui poucos sítios de N-glicosilação e dois resíduos de aminoácidos na porção C-terminal (SÉVIGNY et al., 2000; BIGONNESSE et al., 2004).

A atividade da NTPDase já foi caracterizada inicialmente em plaquetas de ratos e posteriormente em plaquetas de humanos (FRASSETTO et al., 1993; PILLA et al., 1996). As NTPDases em plaquetas intactas de humanos podem estar envolvidas com a inibição da agregação plaquetária e regulação do tônus vascular (SÉVIGNY et al., 2002). A CD39 solúvel bloqueou *in vitro* a agregação plaquetária induzida por ADP e inibiu a reatividade plaquetária induzida por colágeno, demonstrando uma importante função na tromboregulação (GAYLE III et al., 1998; ENJYOJI et al., 1999; MARCUS et al., 2005). As respostas tromboregulatórias da NTPDase podem ser observadas em estudos *in vivo* e *in vitro* que demonstraram sua participação na homeostasia através de um potente efeito anti-trombótico (MARCUS et al., 2005; COSTA et al., 2004).

A enzima 5'-nucleotidase (CD73, EC 3.1.3.5), catalisa especificamente a hidrólise de NMP a adenosina (BARMAN, 1969; SARKIS et al.,1995). A 5'-nucleotidase é uma glicoproteína intrínseca da membrana plasmática de diferentes tipos celulares como as plaquetas e também pode ser encontrada em tecidos como nervoso, renal e hepático (ZIMMERMANN et al., 1993). A ativação da enzima 5'-nucleotidase contribui para a inibição da agregação plaquetária por células endoteliais humanas (KAWASHIMA et al., 2000). Na cascata de coagulação as enzimas NTPDase e 5'-nucleotidase têm importante função na regulação da agregação plaquetária (ENJYOJI et al., 1999).

As ecto-nucleotideo pirofosfatase/fosfodiesterase (E-NPPs) são encontradas na superfície das células como proteínas transmembrana. As NPPs hidrolisam pirofosfato ou fosfodiesterase em uma variedade de compostos extracelulares

incluindo nucleotídeos (STEFAN et al., 2005). Essas NPPs foram caracterizadas com componentes de um múltiplo sistema de hidrólise de nucleotídeos em plaquetas de ratos (FÜRSTENAU et al., 2006).

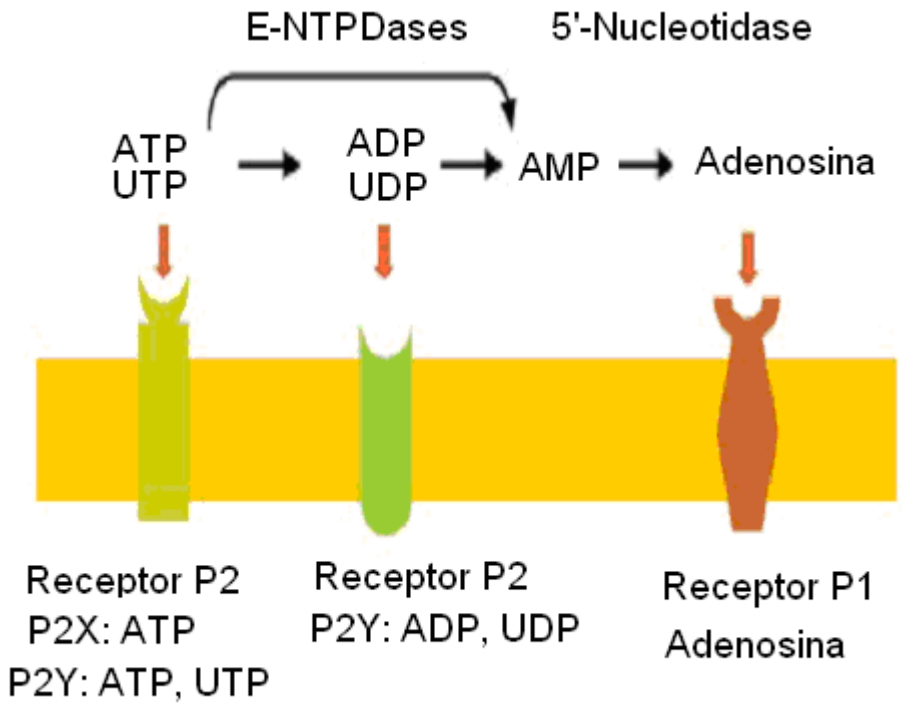

FIGURA 2: Catabolismo de nucleotídeos extracelulares e ativação dos receptores para nucleotídeos (receptores P2) e adenosina (receptores P1). (Adaptado de ROBSON et al., 2006).

2.6.1 Ectonucleotidases em patologias humanas

O grupo de estudo do Laboratório de Enzimologia Toxicológica, da Universidade Federal de Santa Maria, investiga a atividade das ectonucleotidases em diferentes patologias humanas, com a finalidade de elucidar os mecanismos envolvidos nas alterações enzimáticas.

A atividade das enzimas NTPDase e 5'-nucleotidase foram avaliadas em pacientes com diabetes e hipertensão associada e também em modelo experimental. Estes estudos sugerem a interferência dessas patologias no

mecanismo catalítico das ectonucleotidases (LUNKES et al., 2003, LUNKES et al., 2004). Foi observado que em pacientes diabéticos sobrecarga com ferro promove aumento na hidrólise de nucleotídeos de adenina (MIRON et al., 2007). A investigação em gestantes com elevado risco de trombose sugere que as ectonucleotidases estão envolvidas na tromboregulação (LEAL et al., 2007). O aumento na expressão de CD39, em pacientes com hipercolesterolemia, foi uma resposta compensatória ao processo inflamatório e pró-oxidativo associado com a hipercolesterolemia (DUARTE et al., 2007).

Compostos de pirimidina foram observados como inibidores da atividade da NTPDase em córtex cerebral de ratos (CECHIN et al., 2003). A interferência de tratamento sub crônico de $HgCl_2$ foi avaliada na atividade das enzimas NTPDase e 5´-nucleotidase em córtex cerebral de ratos tratados com este metal (MORETTO et al., 2004). A interferência da exposição crônica de alumínio na atividade das enzimas NTPDase e 5'-nucleotidase foi avaliada em plaquetas, córtex cerebral e hipocampo de modelo experimental, indicando que as plaquetas podem servir como marcadores da toxicidade do alumínio no sistema nervoso central (KAIZER et al., 2007).

A atividade da enzima NTPDase foi inicialmente caracterizada em linfócitos humanos (LEAL et al., 2005). Posteriormente, foi observado um aumento na atividade da NTPDase em pacientes com a infecção por HIV e sua correlação positiva com CD39 em linfócitos (LEAL et al., 2005). A hidrólise de nucleotídeos de adenina em pacientes com câncer de mama demonstrou que a atividade da NTPDase depende do estágio do câncer (ARAÚJO et al., 2005).

A atividade das ectonucleotidases também foi avaliada em modelos experimentais de desmielinização pelo brometo de etídio. No tratamento com interferon beta pode se observar que a hidrólise dos nucleotídeos de adenina está modificada em plaquetas de ratos desmielinizados (SPAVANELLO et al., 2007). Por outro lado, o tratamento com ebselen e vitamina E não modificou a atividade da enzima 5´-nucleotidase. Porém, a atividade da enzima NTPDase ficou diminuída em ratos desmielinizados e o ebselen e a vitamina E interfere na hidrólise dos nucleotídeos da adenina (MAZZANTI et al., 2007).

A atividade das enzimas NTPDase e 5'-nucleotidase foi avaliada em pacientes com insuficiência renal. Os dados demonstraram uma alteração na hidrólise de nucleotídeos em plaquetas de pacientes com alteração renal submetidos à

hemodiálise. Possivelmente, as mudanças na atividade das ectonucleotidases poderiam contribuir para as alterações na homeostasia de pacientes com insuficiência renal crônica (SILVA et al., 2005).

2.7 Estresse oxidativo

A presença de estresse oxidativo nas células é proveniente essencialmente da perda de equilíbrio entre os processos oxidantes e antioxidantes, com conseqüente falência no reparo do dano oxidativo (SCHAFER & BUETTNER, 2001). Com isso, as células danificadas promovem a produção de espécies reativas de oxigênio e nitrogênio, que compreendem radicais hidroxil, superóxido, peróxido de hidrogênio e peroxinitrito (VALKO et al., 2005). As espécies reativas de oxigênio são altamente reativas e constituem estruturas moleculares que reagem com diversos componentes celulares como o DNA, as proteínas, os lipídios e os produtos finais da glicação avançada. Essas reações entre os componentes celulares e as espécies reativas de oxigênio e nitrogênio promovem danos no DNA, distúrbios na formação mitocondrial, danos na membrana celular e eventualmente morte celular (LELLI et al., 1998).

O nível de estresse oxidativo tem sido relacionado com patologias crônicas, como o diabetes, o Alzheimer e o Parkinson (McGRATH, L.T. et al., 2001; JENNER, 2003). O estresse oxidativo pode ser importante no diabetes, porque a cronicidade da hiperglicemia e também a resistência à insulina podem induzir ao dano oxidativo e contribuir para a destruição de células beta pancreáticas (KING & LOEKEN, 2004).

Os sistemas antioxidantes de defesa constituem-se em estruturas moleculares capazes de capturar os radicais livres e com isso prevenir os danos oxidativos nas células. A hiperglicemia estimula o aumento na geração de espécies reativas de oxigênio e induz a um incremento na atividade das enzimas superóxido dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), nos níveis de grupos tióis não protéicos (NPSH), proteína carbonil e TBARS em pacientes diabéticos. Esses dados sugerem que sistemas antioxidantes podem ser considerados como marcadores de injúria vascular em diabéticos (AHMED et al., 2006; RAMAKRISHNA & JAILKHANI, 2007). O ácido ascórbico faz parte de um sistema antioxidante não enzimático responsável pela remoção de radicais livres. A vitamina C, em baixas

concentrações em pacientes diabéticos, tem sido relacionada com o incremento dos níveis de estresse oxidativo (SKRHA et al., 2003).

A produção de espécies reativas de oxigênio constitui um importante mecanismo de ativação e agregação plaquetária, com extrema relevância no recrutamento de plaquetas para a formação do trombo (KRÖTZ et al., 2004). O estresse oxidativo, induzido pela hiperglicemia, é responsável pela ativação da condição pró-trombose, ativação inicial de plaquetas, adesão e subseqüente formação de agregação plaquetária. Por isso, o controle metabólico da glicemia é fundamental para as funções plaquetárias em diabéticos (FERRONI et al., 2004). Também, a atividade da enzima NTPDase em plaquetas pode ser suscetível a radicais livres (FRASSETTO et al., 1997).

3 ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos e manuscritos, os quais encontram-se aqui organizados. O artigo está disposto da mesma forma que foi publicado na edição da revista científica (**Artigo 1**). Os manuscritos estão dispostos da mesma forma que foram submetidos na edição da revista científica (**Manuscrito 1 e 2**) e na fase de redação (**Manuscrito 3**).

3.1 Artigo 1

O artigo “Serum cholinestease activity in diabetes and associated pathologies” foi publicado no periódico “Diabetes Research and Clinical Practice”.

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Serum cholinesterase activity in diabetes and associated pathologies

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Abstract

Serum cholinesterase activity was measured in diabetes, hypertensive and diabetic/hypertensive patients. The sample consisted of volunteer patients and was divided in a control group ($n = 26$), type 2 diabetic group ($n = 16$), hypertensive group ($n = 12$) and type 2 diabetic/hypertensive group ($n = 26$). In addition, blood glucose, cholesterol and triglyceride levels were determined. Serum cholinesterase activity in the control group was significantly lower in relation to the other groups ($p < 0.001$). Blood glucose levels were elevated in type 2 diabetic and type 2 diabetic/hypertensive groups. In vitro studies showed increased cholinesterase activity in the presence of glucose 5–100 mM or insulin 0.5–25 UI ($p < 0.001$). Cholesterol and triglycerides were at normal levels only in the control group. Possibly, a relationship exists between the increase in serum cholinesterase and the vascular complications in the diabetic patients, potentially stimulated by the levels of glycemia and dyslipidemia. Although patients were receiving different medicines, the increase in enzyme activity was similar in all groups. This enzymatic profile suggests a possible interference of the diseases in the catalytic mechanism of the serum cholinesterase enzyme.

Keywords: Cholinesterase; Human serum; Diabetes mellitus; Hypertension

1. Introduction

Diabetes is a common chronic illness characterized by alterations in the ability to use or produce insulin, affecting a million people around the world, promoting structural and functional tissue injuries. Resistance to insulin and compensatory hyperinsulinemia are decisive factors for predisposition to the cardiovascular disease and other complications [1,2]. Type 2 diabetic patients present an overload in the cardiovascular system [3] and hypertension, which accelerates the atherosclerotic process and promotes vascular endothelial lesions and the deposition of lipids [4].

Cholinesterase is present in mammals and includes two classes: acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BuChE; EC 3.1.1.8). AChE is situated in the central nervous system, platelet and erythrocyte membranes while BuChE is more abundant in the serum that is synthesized by the liver [5]. BuChE is able to act on hydrophilic and hydrophobic choline esters [6,7] and it is well known that this enzyme hydrolyses a variety of xenobiotics [8]. BuChE has

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grown of interest as a bioscavenger of drugs and organophosphate and carbamate insecticides [9]. An increase in serum cholinesterase activity has been observed in type 1 and type 2 diabetic patients, which is possibly associated with vascular complications in diabetes [10,11]. In fact, many studies have suggested an association of altered BuChE activity with lipid metabolism, hypertension and disturbances found in diabetes mellitus [12–14].

Thus, the objective of the present analysis was to determinate the serum cholinesterase activity in diabetic, hypertensive and diabetic/hypertensive patients.

2. Patients and methods

The sample consisted of patients from the diabetic, hypertensive and diabetic/hypertensive patient assistance program associated with the Municipal Secretary's Office of Health and Environment in Cruz Alta (RS, Brazil) as well as of healthy volunteers. All subjects gave written informed consent to participate in the study. The protocol was approved by the Human Ethics Committee of the Health Science Center from the Federal University of Santa Maria. The sample was divided into four groups. The control group consisted of 26 individuals aged 49.8 ± 13.2 years, 50% males and 50% females, who did not present any disease and who had not been submitted to any pharmacological therapy during the last month. Controls were carefully selected by clinical evaluation, matched by sex, age and body mass index similar to that of the patients. All type 2 diabetic, hypertensive and type 2 diabetic/hypertensive patients were submitted to medical treatment with appropriate medicines. The type 2 diabetic group consisted of 16 patients aged 54.5 ± 4.9 years, 50% males and 50% females, with type 2 diabetes mellitus treated with chlorpropamide (250 mg/day) or glibenclamide (5 mg/day). The hypertensive group was formed by 12 patients aged 55.8 ± 7.5, 50% males and 50% females, with different hypertension levels treated with captopril (25 mg/day), furosemide (40 mg/day), acetylsalicylic acid (100 mg/day) or propranolol (40 mg/day). The type 2 diabetic/hypertensive group was formed by 26 patients aged 58.2 ± 6.2, 50% males and 50% females, with type 2 diabetes mellitus plus hypertension who received appropriate medications for the associated diseases.

Data are analyzed statistically by one-way ANOVA followed by the Tukey–Kramer's test when the F-test was significant ($p < 0.05$) to determine the differences between the study groups and the control. Data are presented as mean values ± S.D.

3. Results and discussion

The hypertensive and type 2 diabetic/hypertensive patients presented stage 2 hypertension (moderate) with systolic blood pressure ranging from 162 to 169 mmHg and diastolic blood pressure ranging from 101 to 107 mmHg. The blood pressure in the other groups was at normal range. The patients that were selected to participate in this study were hypertensive, diabetics or diabetic/hypertensive without any other sequel. None of those patients presented any clinical sign of retinopathy, neuropathy or nephropathy.

Ten milliliters of blood was obtained from each participant and serum was used for biochemical and cholinesterase activity determinations. Blood was collected, pooled and then centrifuged at 160 × g for 10 min. Protein was adjusted to 0.3–0.5 mg/mL. Cholinesterase activities were determined by the method of Ellman et al. [15], modified by Rocha et al. [16]. Hydrolysis rates were measured at acetylthiocholine concentration (0.8 mM) in 2 mL assay solutions with 100 mM phosphate buffer, pH 7.5, and 1.0 mM 5-5′-dithio-bis(2-nitrobenzoic acid) (DTNB) at 25 °C. One hundred microliters of human serum were added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by the formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s) using a Hitachi 2001 spectrophotometer. All samples were run in duplicate or triplicate. Protein was assayed by the method of Bradford [17] using bovine serum albumin as standard.

The control (78 ± 9 mg/dL) and hypertensive (88 ± 19 mg/dL) groups presented normal blood glucose levels, whereas the type 2 diabetic (178 ± 38 mg/dL) and type 2 diabetic/hypertensive (166 ± 42 mg/dL) groups presented elevated blood glucose levels. The cholesterol (186 ± 9 mg/dL) and triglycerides (107 ± 36 mg/dL) were at normal levels in the control group, whereas type 2 diabetics, hypertensive and type 2 diabetic/hypertensive patients presented higher values to cholesterol (225 ± 21, 218 ± 30 and 240 ± 27 mg/dL, respectively) and triglycerides (303 ± 63, 242 ± 58 and 310 ± 221 mg/dL, respectively). HDL-cholesterol levels were higher in the control group (58 ± 7), whereas in type 2 diabetes (35 ± 14), hypertension (39 ± 13) and type 2 diabetes/hypertension (40 ± 8) presented lower values. The type 2 diabetic and type 2 diabetic/hypertensive patients presented hemoglobin A1c values of 6.7 ± 0.2 and 5.6 ± 0.1%, respectively, and the other groups were at normal levels.

To evaluate the interference of glucose and insulin in human serum cholinesterase activity, we performed experiments with glucose concentrations ranging from 5 to 100 mM, or insulin ranging from 0.5 to 25 UI/mL. The time of preincubation used in the in vitro analysis was 30 min and serum was obtained from control subjects.

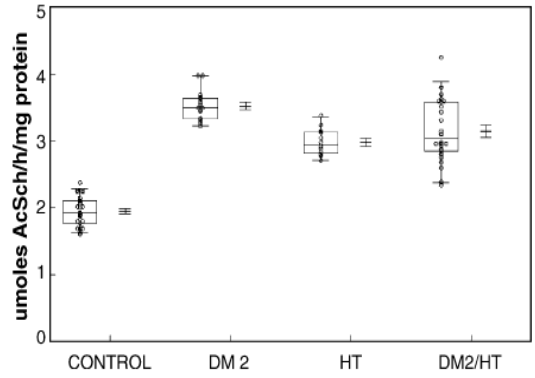

Fig. 1. Distribution of the values of serum cholinesterase activity from control ($n = 26$); type 2 diabetes (DM2; $n = 16$); hypertensive (HT, $n = 12$); diabetic and hypertensive (DM2/HT; $n = 26$) groups. Details are described in Section 2. Activity is expressed as μmol AcSCh/(h mg) protein.

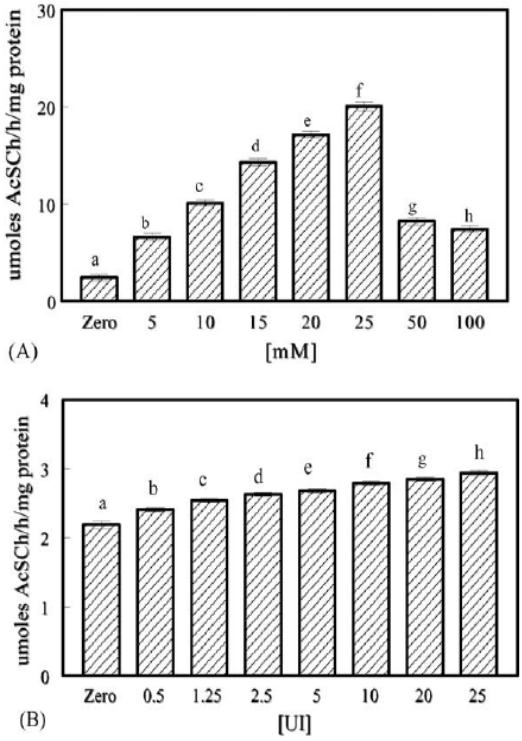

Fig. 2. In vitro effect of glucose (A) and insulin (B) in the serum cholinesterase activity from control patients ($n = 3$). Activity is expressed as μmol AcSCh/(h mg) protein. Values represent mean ± S.D. Groups not sharing the same letters, are different from each other (ANOVA, Tukey–Kramer test, $p < 0.001$).

The activity of serum cholinesterase related with diabetes is present in the literature [19–21], but the effect of the medicines and the assessment in patients with coexistent hypertension, as presented in this work, is a novelty. Serum cholinesterase activity was expressed as μmol AcSCh/(h mg) of protein. The activity was enhanced [$F(3.76) = 37.39, p < 0.001$] and post-hoc comparisons by Tukey–Kramer's test revealed that diabetic, hypertensive and diabetic/hypertensive were different from control. The individual values of cholinesterase activities are represented in the Fig. 1. The results suggest that these pathologies could affect the catalytic mechanism of the enzyme instead to only increase the synthesis or release of the liver serum cholinesterase. All medicines used by the patients were described in Section 2 and in Table 1. No differences among the medicines were observed in serum cholinesterase activity when comparing chlorpropamide, glibenclamide, captopril, furosemide, acetylsalicylic acid and propranolol. Probably, it indicates that the hepatic metabolism of these different compounds did not change, in a different manner, the release of cholinesterase from the liver. This enzymatic profile suggests a direct interference of the diseases in the catalytic mechanism of the enzyme. It appears to be as important as the potential increase in the cholinesterase synthesis or release by the liver.

Table 1
Activity of enzyme serum cholinesterase

Groups	Drugs	Serum cholinesterase (μmol AcSCh/(h mg))
Control ($n = 26$)	None ($n = 26$)	1.92 ± 0.32[a]
Type 2 diabetic ($n = 16$)	Chlorpropamide ($n = 8$)	3.45 ± 0.53
	Glibenclamide ($n = 8$)	3.48 ± 0.52
Hypertensive ($n = 12$)	Captopril, acetylsalicylic acid, furosemide ($n = 7$)	2.96 ± 0.45
	Propranolol, acetylsalicylic acid, furosemide ($n = 5$)	2.99 ± 0.49
Type 2 diabetic/hypertensive ($n = 26$)	Chlorpropamide, captopril, furosemide ($n = 14$)	3.15 ± 0.65
	Glibenclamide, captopril, furosemide ($n = 12$)	3.16 ± 0.68

Values represent mean ± S.D. from individual experiments.
[a] Different from others in the same column (ANOVA, Tukey–Kramer test, $p < 0.01$).

In fact, the in vitro curves reinforce this idea, once glucose concentration ranging from 5 to 100 mM increased serum cholinesterase activity [$F(7.16) = 22.72$, $p < 0.001$]. This effect was more pronounced between 10 and 25 mM, being all groups different from each other, as can be observed on Fig. 2A. It is important to point out that glucose levels observed in practice clinic (5–15 mM) enhanced substantially cholinesterase activity. The stimulatory effect of insulin in serum cholinesterase activity in vitro began in 0.5 UI (Fig. 2B), being all concentrations significantly different from the control (zero) [$F(7.16) = 16.5$, $p < 0.001$]. The serum cholinesterase activity in type 2 diabetes mellitus patients has a significant correlation with serum levels of insulin and free fatty acids and can be observed that insulin resistance and an increment flux of free fatty acids from adipose tissue to the liver would stimulate the hepatic synthesis of serum cholinesterase [18]. High levels of serum cholinesterase was determined in patients with obesity [22], in which sometimes is plausible to found high levels of insulin and glucose. Randell et al. [23] showed that high insulin levels might stimulate the production of BuChE in the CaCo-2 intestinal cell line. Thus, it is plausible to suppose a correlation among hepatic intermediary metabolism, glucose and insulin levels with serum cholinesterase activity. In fact, we observed enhanced cholinesterase activity, correlating positively with high triglyceride and total cholesterol levels and negatively with HDL-cholesterol levels from patients with type 2 diabetes, hypertension and type 2 diabetes/hypertension. These findings obtained from type 2 diabetes patients are in agreement with the literature [14] and reinforce the relationship between serum cholinesterase and lipid metabolism.

Additionally, the repetitive exhibition of the endothelium vascular to hyperglycemia can result in irreversible dysfunction, involving biochemical and molecular factors [24]. Gomes et al. [25] reported that impaired endothelium-dependent vasodilatation resulting from acute exposure to glucose levels, corresponding to those measured during post-prandial period in patients with type 2 diabetes, could be related with dysfunction of the L-arginine-NO signaling pathway. Another important aspect to be discussed is that the increase in cholinesterase activity may accelerate the hydrolysis of choline esters present in the circulation. Probably, could be a rapid acetylcholine hydrolysis diminishing the rate of action of this neurotransmitter in the near neighboring endothelium, affecting acetylcholine-induced relaxation.

Concluding, diabetic, hypertensive and diabetic/hypertensive patients presented elevated serum cholinesterase activities, not influenced by the medicines utilized. The consequences of the increase on the serum cholinesterase activity in these pathologies are not still clear, but it could be related with long-term complications, potentially stimulated by the levels of glucose and insulin.

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3.2 Manuscrito 1

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Effect of high glucose levels in human platelet NTPDase and 5'-nucleotidase activities

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Abstract

***Objectives*:** We attempt to evaluate the effect of glucose levels in human platelet ectonucleotidases activities in patients with diabetes or hypertension.

***Methods*:** The activities of the enzymes NTPDase (CD39) and 5'-nucleotidase (CD73), and CD39 expression were analyzed in human blood platelets of type 2 *Diabetes* mellitus (DM-2), hypertension (HT) and type 2 *Diabetes* mellitus/hypertension (DM-2/HT) goups. The interference of glucose and fructose on the NTPDase and 5'-nucleotidase in platelets from control patients was also verified.

***Results*:** NTPDase and 5'-nucleotidase activities increased with increasing glucose and fructose concentrations ($p < 0.001$) and the different times of pre-incubation did not interfere in ectonucleotidase activities ($p > 0.5$). NTPDase and 5'-nucleotidase activities demonstrated a positive correlation between serum glucose levels and ATP and ADP hydrolysis in DM-2 and DM-2/HT patients. CD39 expression demonstrated that DM-2, HT and DM-2/HT groups presented a significant increase ($p < 0.004$) when compared to the control group.

***Conclusion*:** The hydrolysis of adenine nucleotides is enhanced in platelets of patients with diabetes and hypertension. We observed that an increasing glucose concentration had a direct effect on ectonucleotidases activities. Furthermore, CD39 expression was enhanced in all patients groups. These results suggest that hyperglycemia interferes in platelet homeostasis and hydrolysis nucleotides are important facto to thromboregulation.

Keywords: Hyperglycemia; NTPDase; 5'-nucleotidase; Diabetes; Hypertension; Platelet

1. Introduction

Platelets play an important role in hemostasis and thrombosis. These anuclear cells act via adhesion and aggregation which allow thrombus formation at the site of vascular injury [1,2,3]. Increased platelet aggregation may result in thromboembolic events and contribute to acute coronary syndromes [4,5].

Adenine nucleotides are released from dense granules during platelet activation [6,7]. Adenosine triphosphate (ATP) has been suggested to have a role in the regulation of platelet aggregation [8,9]. Adenosine diphosphate (ADP) plays a very important role in thrombogenesis being the main promoter of platelet aggregation [10,11]. Adenosine is an endogenous inhibitor of platelet aggregation and interferes in vascular tone [12]. These adenine nucleotides and nucleosides act via purinoreceptors [6-12].

The family of ectonucleotidases includes enzymes that degrade extracellular nucleotides. The enzymes NTPDase (nucleoside triphosphate diphosphohydrolase CD39) and 5'-nucleotidase (CD73) are located in the platelet membrane and complete the hydrolysis of ATP to adenosine [13,14]. Both enzymes CD39 and CD73 play an important role in hemostasis and platelet aggregation mainly by regulating ADP catabolism and adenosine production [15-19]. Recently, studies observing alterations in NTPDase and 5'-nucleotidase activities in blood platelets suggested that these ectonucleotidases are involved in the thromboregulation process in several physiological and pathological conditions [20-22].

Diabetes mellitus is an important risk factor for vascular complications and thrombus formation [23]. Chronic hyperglycemia promotes platelet activation and can contribute to vascular events [24,25]. Platelet hyperactivation contributes to

increased risk of atherothrombosis in type 2 diabetes [26]. High glucose concentrations, when chronic, promote alteration in ATP/ADP levels and may be an important factor involved in platelet hyperactivity in the course of diabetes [27]. The ectonucleotidases, CD39 and CD73, in platelets, are altered in type 2 diabetic and hypertensive patients and probably such modifications are compensatory physiological responses related with the thromboregulation process [21,28].

Previous studies in our laboratory demonstrated an increase in ectonucleotidase activities in patients with diabetes and associated pathologies [21]. However, the mechanism by which it occurred was not completely understood. Thus, in the present study we attempt to evaluate the effect glucose levels in human blood platelets ectonucleotidases activities in these groups of patients.

2. Material and Methods

2.1 Chemicals

Nucleotides, sodium azide, HEPES, and Trizma base were purchased from Sigma (St. Louis, MO). Antibodies for flow cytometry analysis [R-phycoerythrin-conjugated mouse monoclonal antibody against human CD39, and fluorescein isothiocyanate-conjugated mouse monoclonal antibody against human CD61 were purchased from Serotec Ltd. (Kidlington, Oxford, UK) and BD PharMingen Technical Data Sheet (San Jose, CA, USA), respectively. The glucose, cholesterol, HDL-cholesterol, triglycerides and lactate dehydrogenase (LDH) commercial kits were obtained from Labtest (Lagoa Santa, MG, Brazil). All other reagents used in the experiments were of analytical grade and of the highest purity.

2.2 Patients

The sample consisted of patients from the Assistance Program for diabetic and hypertensive patients associated with the Municipal Secretary's Office of Public Health in Cruz Alta (RS, Brazil) as well as of healthy volunteers. All subjects gave written informed consent to participate in the study. The protocol was approved by the Human Ethics Committee of the Health Science Center from the Federal University of Santa Maria (Protocol number: 013/2004).

The sample was divided into four groups consisting of 50% males and 50% females. The control group (n=9) consisted of individuals with ages ranging from 28 to 52 years, who did not present any disease and who had not been submitted to any pharmacological therapy during the last month. Controls were carefully selected by clinical evaluation and presented sex, age and body mass indices similar to those of the patients. The type 2 diabetic (DM-2, n=8) group consisted of patients with ages ranging from 56 to 68 years. The patients of the DM-2 group had type 2 diabetes mellitus and were treated with glibenclamide (10 mg/day) or mettformin (850 mg/day). The hypertensive (HT, n=9) group was made up of patients with ages ranging from 30 to 70 years. The patients of the HT group had different hypertension levels and were treated with captopril (25 mg/day), furosemide (40 mg/day), acetylsalicylic acid (100 mg/day) or propranolol (40 mg/day). The type 2 diabetic/hypertensive (DM-2/HT, n=9) group consisted of patients with ages ranging from 51 to 69 years. All patients of the DM-2/HT had type 2 diabetes mellitus plus hypertension and received appropriate medication for the associated diseases. Ten milliliters of blood was obtained from each participant and used for platelet-rich plasma preparations, biochemical determinations and hematological determinations.

2.3 Hematologic determinations

Quantitative determinations of platelets obtained by venipuncture were performed using a Coulter-STKS analyzer (Miami, USA).

2.4 Biochemical determinations

Serum glucose, cholesterol, triglycerides and lactate dehydrogenase (LDH) were determined by spectrophotometry, using commercial kits.

2.5 Platelet-rich plasma (PRP) preparation

PRP was prepared from human donors by methods previously published [13]. Briefly, blood was collected into 0.129 mol/L citrate and centrifuged at 160 g for 10 min. The PRP was centrifuged at 1400 g for 15 min and washed twice with 3.5 mmol/L Hepes isosmolar buffer containing 142 mmol/L NaCl, 2.5 mmol/L KCl, and 5.5 mmol/L glucose. The washed platelets were resuspended in Hepes isosmolar buffer, and protein was adjusted to 0.3–0.5 mg/mL.

2.6 NTPDase and 5'-nucleotidase assays

Platelet NTPDase was incubated as previously described [13], with 5.0 mmol/L $CaCl_2$, 100 mmol/L NaCl, 4.0 mmol/L KCl, 5.0 mmol/L glucose, and 50 mmol/L Tris–HCl, pH 7.4, at a final volume of 200 μL. The total quantity of 20 μL of the enzyme preparation (10–15 μg of protein) was added to the reaction mixture and pre-incubated for 10 min at 37°C. The reaction was started by the addition of 1.0 mmol/L of ATP or ADP. The activity of 5'-nucleotidase was assayed using the same conditions except that 5.0 mmol/L of $MgCl_2$ and 2.0 mmol/L of AMP were used. The ectonucleotidases reactions were stopped after one hour of incubation with

trichloroacetic acid (TCA 10%), at a final concentration of 5%. The Pi released was measured by the method of Chan et al. (1984) [29] using Malachite Green as coloring reagent. The enzymatic activities were described in nmol Pi/min/mg of protein. All samples were run in triplicate.

2.7 Glucose and fructose curve

To evaluate the glucose and fructose levels in NTPDase and 5'-nucleotidase activities, we performed experiments with glucose/fructose concentrations ranging from 5 to 100 mM in platelet-rich plasma (PRP) from control subjects. Pre-incubation times of 10, 120 minutes and 24 hours were used.

2.8 Flow cytometry analysis

Peripheral blood cells were incubated with anti-CD39 and anti-CD61 (20 μL per 10^6 cells) for 25 min, lysed with fluorescence activated cell sorter (FACS) reagent, and incubated again for 15 min in the dark. Cells were washed twice in NaCl/Pi buffer (pH 7.4) containing 0.02% (w/v) sodium azide and 0.2% (w/v) BSA. The cells were then resuspended in NaCl/Pi buffer (pH 7.4) and immediately analyzed with a FACScalibur flow cytometer using cellquest software (Becton Dickinson, San Jose, CA, USA), without fixation.

2.9 Protein determination

Proteins were determined by the Coomassie Blue method [30], using bovine serum albumin (BSA) as standard.

2.10 Statistical analysis

Data were analyzed statistically by two-way and one-way ANOVA, followed by Duncan's multiple range test. Differences between groups were considered to be significant when $p < 0.05$. All data were expressed as mean ± S.D. Correlation was evaluated with the Pearson test. Linear correlation between variables was also carried out.

3. Results

The patient's characteristics are shown in Table 1. Glucose levels were normal (3.8–6.1 mmol/L) in control and HT groups, and higher in DM-2 (141.5%) and DM-2/HT (136.6%) groups ($p<0.05$). The lipid profile of the pathological groups were different from the control group ($p<0.05$). Total cholesterol levels (<5.1 mmol/L) presented an increase in DM-2 (30.1%), HT (33.3%) and DM-2/HT (47.6%). Triglyceride levels (<2.2 mmol/L) presented an increase of 135% in DM-2, HT and DM-2/HT groups. Quantitative analysis demonstrated that platelet counts obtained from all groups were at normal levels (150.000 – 400.000 platelets/mm^3). Microscopic analysis of platelet size and shape revealed a typical pattern (data not shown). Platelet integrity was determined by lactate dehydrogenase activity from control patients. The measurements of LDH showed that most cells (more that 90%) were intact after the isolation procedure and PRP was adequate (data not shown).

The different times of pre-incubation in the glucose curve *in vitro* did not interfere in ectonucleotidases activities ($p > 0.5$). The effect of glucose on NTPDase and 5'-nucleotidase is shown in Figure 1. The increase in ATP, ADP and AMP hydrolysis was observed in all pre-incubation times. Post-hoc comparisons by Duncan's test revealed that NTPDase and 5'-nucleotidase activities were significantly

higher with increasing glucose concentrations between 5 and 100 mM ($p < 0.001$). The effect of fructose on NTPDase and 5'-nucleotidase was similar to that of glucose (data not shown).

NTPDase and 5'-nucleotidase activities in all groups are shown in Figure 2. Post-hoc comparisons by Duncan's test determined that NTPDase activity was higher in the DM-2, HT and DM-2/HT groups when compared with the control group ($p < 0.001$), using ATP or ADP as substrate. There was an increase in the 5'-nucleotidase activity in the hypertensive and type 2 diabetes/hypertensive groups when compared with the control and in the type 2 diabetes groups ($p < 0.001$). There was a statistically significant correlation between serum glucose levels and ATP and ADP hydrolysis for both the DM-2 group (ATP ($r=0.90$, $p < 0.002$), ADP ($r=0.77$, $p < 0.02$)) and the DM-2/HT group (ATP ($r=0.75$, $p < 0.03$), ADP ($r=0.76$, $p < 0.02$)).

The evaluation of the content of CD39-positive cells by flow cytometry using labeled antibodies against NTPDase revealed that there was a difference in CD39 expression among the groups appraised. Post-hoc comparisons by Duncan's test demonstrated that DM-2, HT and DM-2/HT groups had a significant increase in the expression of NTPDase, when compared to the control group ($p < 0.004$). Results are shown in Figure 3. There was a statistically significant correlation between ATP and ADP hydrolysis and CD39 expression in platelets of DM-2, HT and DM-2/HT groups, as can be seen in Table 2.

4. Discussion

NTPDase and 5'-nucleotidase interfere in the modulation of platelet activation and thrombus formation [17,19,30]. Recent studies have demonstrated the connection between ectonucleotidases and processes of thrombus formation in

different diseases in humans [20-22,30]. Accordingly, our group has observed the interference of diabetes and pathologies associated in NTPDase and 5'-nucleotidase activities of human blood platelets and experimental models [20-22, 32-33]. In these studies were observed the participation of high glucose levels as a probable factor capable to interfere in ectonucleotidases activities.

Chronic hyperglycemia is a pro-thrombotic condition and when associated to other factors such as hypertension it constitutes a high risk for atherothrombotic disorders [34]. High glucose concentrations induce a series of metabolic changes that ultimately lead to the genesis of both microvascular complications and macrovascular damage [35]. Several mechanisms including platelet activation and aggregation as well as hypercoagulability are involved in the pathogenesis of thrombogenesis in diabetes [36].

In this study, the glucose and fructose curve *in vitro* with non-diabetic subjects was carried out with the objective of evaluating the carbohydrate concentration as a factor capable of modifying ectonucleotidases activities. The results demonstrated that the increase in NTPDase and 5'-nucleotidase activities was directly proportional to the increase in glucose and fructose concentrations. However, the time of pre-incubation did not alter ectonucleotidases activities. This data suggest that hyperglycemia should be considered an determinant factor in activities of enzymes that modulate platelet activation and thrombus formation. Similarly, another study showed that platelet reactivity is enhanced after the addition of glucose in the blood of patients with and without diabetes [37]. The formation of platelet micro aggregates is proportionally increased the concentration of glucose during acute hyperglycemia [38]. Therefore, glucose concentrations can have effect on the platelet reactivity.

The ectonucleotidases activities were increased in patients with diabetes and associated hypertension. Studies have shown that patients with chronic hyperglycemia frequently have hypercoagulable blood, as evidenced by increased plasmatic coagulators, reduced endothelial thromboresistance and platelet hyperactivity [39]. The positive correlation between serum glucose concentration and ATP and ADP hydrolysis in type 2 diabetic and type 2 diabetic/hypertensive patients demonstrates that hyperglycemia is an important factor capable of interfering in ectonucleotidases activities. Hypertension does not generally exist in isolation, but it occurs in the setting of concomitant risk factors. Platelet activation and fibrinolysis function are strongly associated with the level of blood pressure, which is associated with coexisting risk factors such as diabetes mellitus and dyslipidemia [40].

This study showed that NTPDase (CD39) expression in platelet membranes was great in patients of the pathological groups. This data confirmed the increase NTPDase activity in platelet membrane of patients with diabetes and hypertension. A previous study showed that acute hyperglycemia causes platelet hyperactivity to agonist stimulation [41]. There was a positive correlation between ATP and ADP hydrolysis and NTPDase expression in platelets of patients with diabetes and diabetes/hypertension. These data demonstrate that diabetes and hypertension may have involvement in the catalytic mechanism of the NTPDase in human blood platelets.

The chronic hyperglycemia presents multiple mechanisms involved in platelet hyperactivity as non-enzymatic glycation and sorbitol accumulation [42, 43]. Normally, these mechanisms require a long periods of elevated glucose levels. However, acute hyperglycemia may also alter platelet function. Short exhibition at elevated levels of glucose can to involve increase protein kinase C, enhances

collagen-induced platelet aggregation via increase mitochondrial superoxide production [44]. Previous study demonstrated that elevated osmolality may changes of platelet function [45].

Therefore the hyperglycemia may be promoting an excessive liberation of ATP and ADP of blood platelets. The platelet hyperactivity with increase in the hydrolysis of adenine nucleotides demonstrates a potential compensatory answer in patients with diabetes in function of elevated glucose levels. This compensatory mechanism at hyperglycemia may promote changes of platelet signaling.

In conclusion, our study demonstrated that diabetes and hypertension interfered in the NTPDase activity increasing the hydrolysis of adenine nucleotides in human platelets. We observed that an increasing glucose concentration had a direct effect on ectonucleotidases activities. These results allow us to suggest that hyperglycemia may be an important factor in platelet homeostasis and ATP, ADP and AMP hydrolysis are important parameters in the thromboregulation process.

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FIGURE LEGENDS

Figure 1. Effect *In vitro* of glucose (5-100 mM) on NTPDase-ATP (A), NTPDase-ADP (B) and 5'-nucleotidase-AMP (C) activities in platelets obtained from control patients (n=9), with different times of pre-incubation (10 min, 120 min and 24h). Activity is expressed as nmol Pi/min/mg protein. Values represent mean ∓ S.D. Groups not sharing the same latters, are different from each other (ANOVA, Duncan's test, $p < 0.05$).

Figure 2. NTPDase-ATP (A), NTPDase-ADP (B) and 5'-nucleotidase (C) activities from control (n=9), type 2 diabetes (DM-2, n=8), hypertensive (HT, n=9) and type 2 diabetes/hypertensive (DM-2/HT, n=9) groups. Values represent mean ± S.D. *****Different from the control group (ANOVA, Duncan's test, $p < 0.05$).

Figure 3. CD39 expression in platelets from control (n=9), type 2 diabetes (DM-2, n=8), hypertensive (HT, n=9) and type 2 diabetes/hypertensive (DM-2/HT, n=9) groups. Values represent mean ± S.D. *****Different from the control group (ANOVA, Duncan's test, $p < 0.05$).

Table 1. Clinical characteristics of the control, type 2 diabetes (DM-2), hypertension (HT) and type 2 diabetes/hypertension (DM-2/HT) groups.

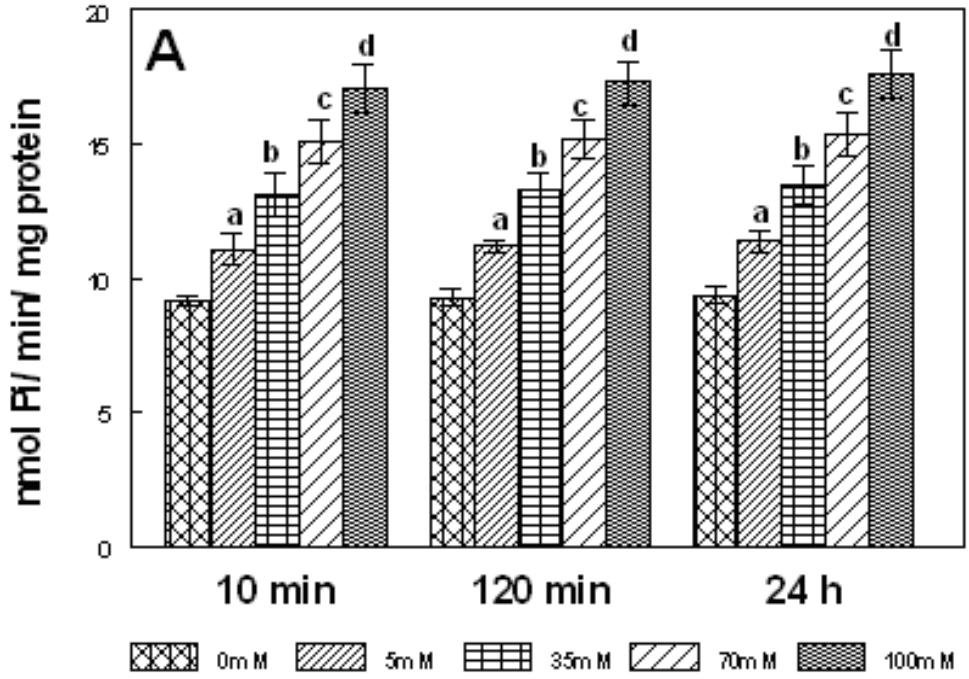
	Control (n = 9)	DM-2 (n = 8)	HT (n = 9)	DM-2/HT (n = 9)
Age (years)	41.3 ± 9	62.2 ± 4	51.6 ± 13	61.5 ± 6
Diabetes duration (years)	–	15.1 ± 2	–	18.3 ± 6
Hypertension duration (years)	–	–	12.3 ± 0.8	7.5 ± 0.7
Systolic blood pressure (mmHg)	112 ± 3	120 ± 4	149 ± 6 *	151 ± 7 *
Diastolic blood pressure (mmHg)	72 ± 2	78 ± 6	98 ± 9 *	99 ± 3 *
Serum glucose (mmol/L)	4.1 ± 0.4	9.9 ± 0.3 *	4.2 ± 0.5	9.7 ± 0.2 *
Serum cholesterol (mmol/L)	4.2 ± 0.2	5.5 ± 0.3 *	5.6 ± 0.1 *	6.2 ± 0.4 *
Serum triglycerides (mmol/L)	1.4 ± 0.2	3.3 ± 0.2 *	3.2 ± 0.2 *	3.4 ± 0.2 *
Drugs				
Glibenclamide	–	n = 3	–	–
Mettformin	–	n = 5	–	–
Captopril, propranolol, furosemide	–	–	n = 4	–
Captopril, acetylsalicylic acid, furosemide	–	–	n = 5	–
Glibenclamide, captopril, furosemide	–	–	–	n = 9

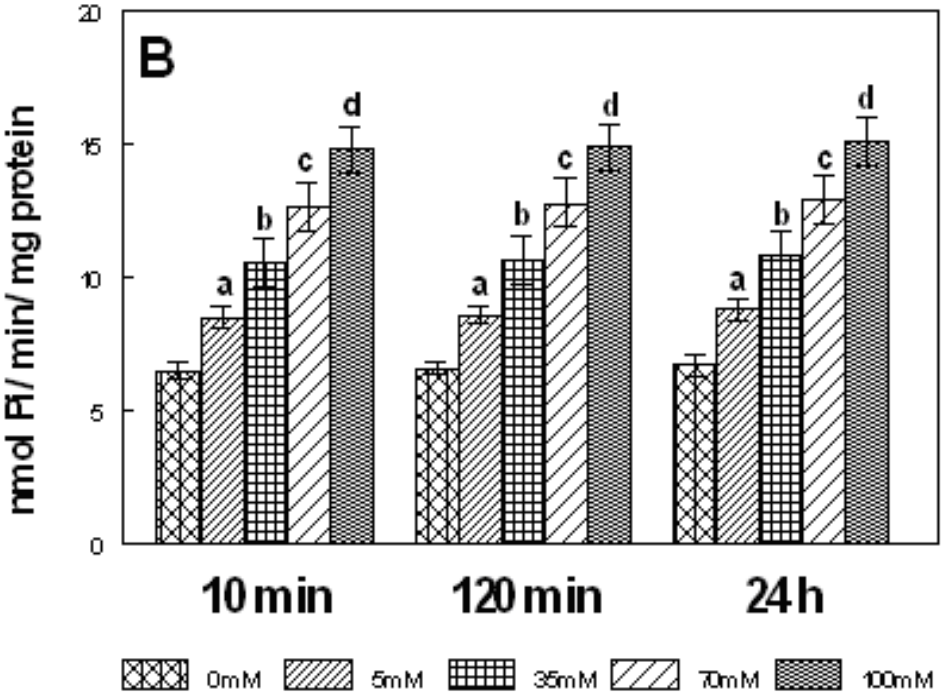
Values represent mean ± S.D. * Different from the others in the same line (ANOVA, Duncan's test, $p < 0.05$).

Table 2. Correlation between ATP and ADP hydrolysis and CD39 expression in platelets from type 2 diabetes (DM-2), hypertension (HT), type 2 diabetes/hypertension (DM-2/HT).

NTPDase (nmol Pi/min/mg protein)	CD39 positive cells (%)		
	DM-2 (n=8)	HT (n=9)	DM-2/HT (n=9)
ATP	r=0.83 *P* < 0.01	r=0.87 *P* < 0.005	r=0.84 *P* < 0.001
ADP	r=0.84 *P* < 0.01	r=0.90 *P* < 0.002	r=0.93 *P* < 0.001

Figure 1


A
nmol Pi/ min/ mg protein
20
15
10
5
0
a
b
c
d
10 min
120 min
24 h
0m M
5m M
35m M
70m M
100m M


B
nmol Pi/ min/ mg protein
20
15
10
5
0
a
b
c
d
10 min
120 min
24 h
0mM
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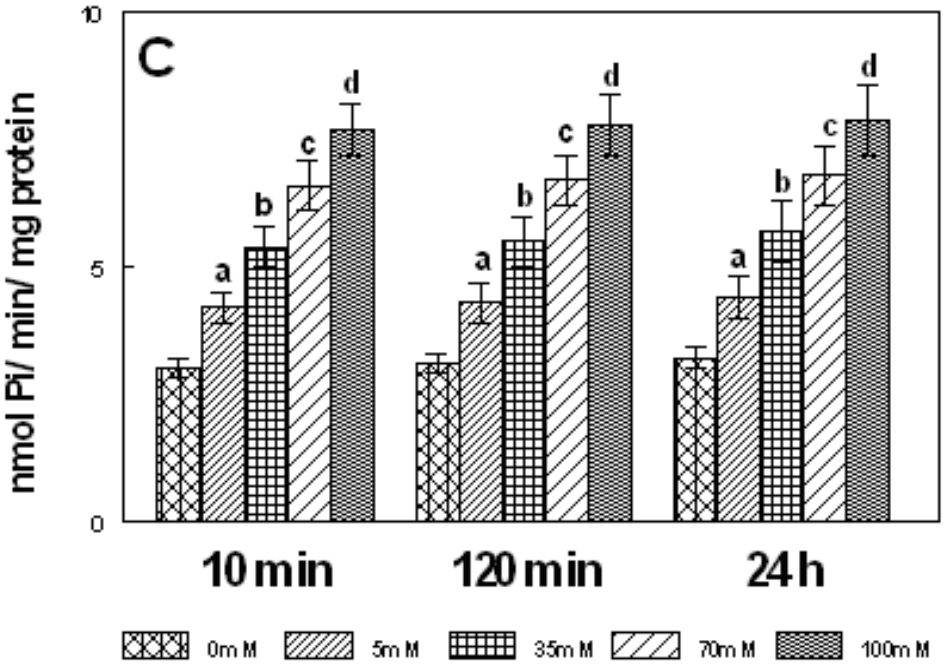
35mM
70mM
100mM


C
nmol Pi/ min/ mg protein
10
5
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b
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10 min
120 min
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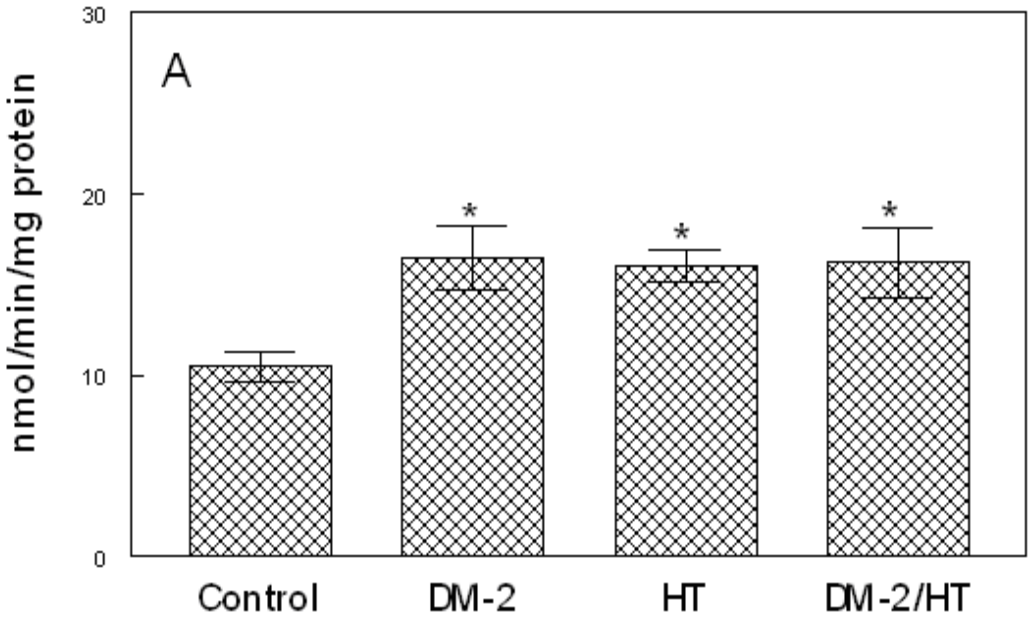
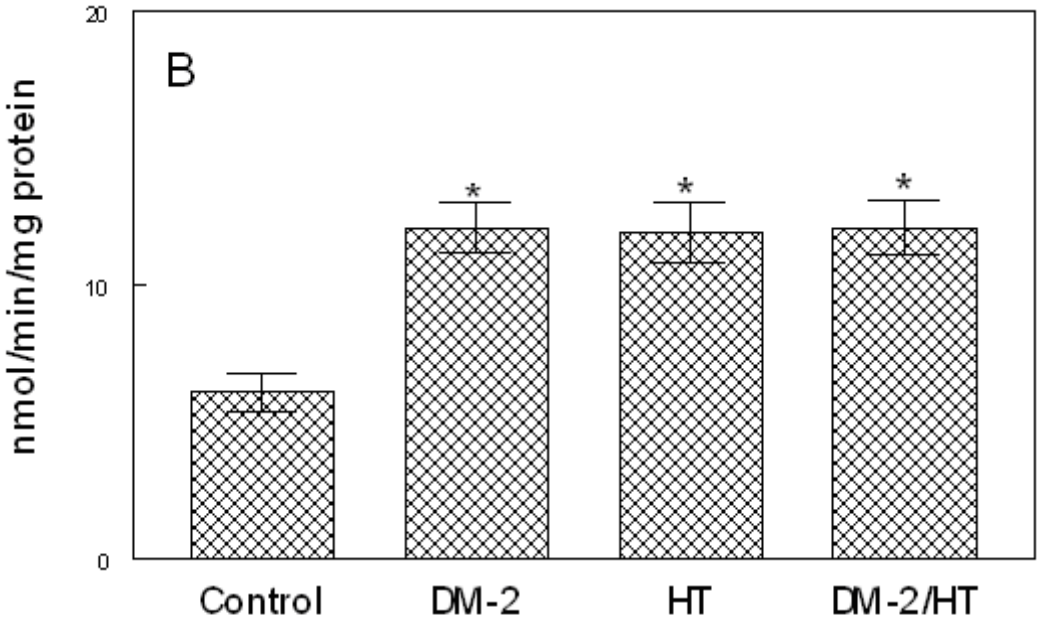
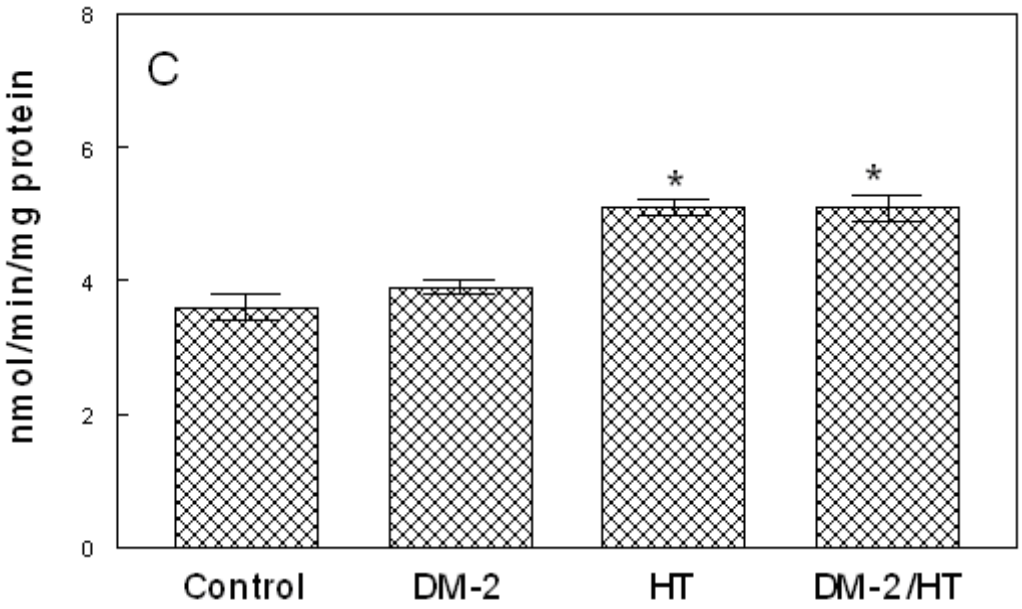
0m M
5m M
35m M
70m M
100m M

Figure 2




B
nmol/min/mg protein
20
10
0
*
*
*
Control
DM-2
HT
DM-2/HT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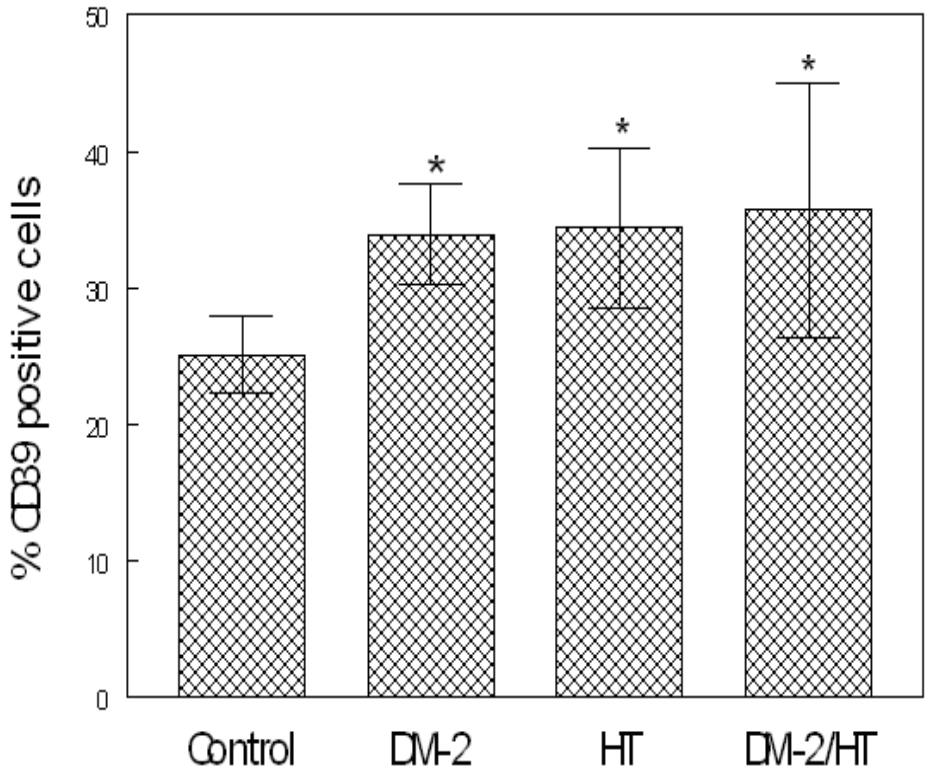
0
2
4
6
8
*
*
Control
DM-2
HT
DM-2/HT

Figure 3



3.3 Manuscrito 2

O manuscrito “Antioxidant status in platelet from patients with type 2 diabetes and hypertension” foi submetido ao periódico “Molecular and Cellular Biochemistry”.

Antioxidant status in platelet from patients with diabetes and hypertension

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Abstract

Diabetes and hypertension constitute risks factors that interfere and promote endothelial dysfunction. This study evaluated the oxidative status on platelets of patients with diabetes and hypertension alone or associated. The sample consisted of 90 patients and was divided into six groups, namely: control, hypertensive (HT), type 2 diabetic using oral hypoglycemic drugs (DM2-OHp), type 2 diabetic using insulin (DM2-Ins), type 2 diabetic with hypertension using oral hypoglycemic drugs (DM2/HT-OHp), and type 2 diabetic with hypertension using insulin (DM2/HT-Ins). The biochemical, lipid peroxidation, and ascorbic acid determinations were estimated in serum of all groups. There was an increase of TBARS in DM2-OHp, DM2-Ins, HT, DM2/HT-Ohp, and DM2/HT-Ins groups when compared to the control group ($p< 0.05$). The increase of superoxide dismutase (SOD), catalase (CAT), nonprotein thiols (NPSH) and protein carbonyl was observed in patients of DM2-OHp, DM2-Ins, HT, DM2/HT-Ohp, and DM2/HT-Ins groups when compared to the control group ($p< 0.05$). There was a significant and positive correlation between serum glucose levels with SOD, CAT, NPSH, and protein carbonyl. Low concentrations of serum ascorbic acid were observed in DM2-OHp, DM2-Ins, HT, DM2/HT-Ohp, and DM2/HT-Ins groups when compared to the control group ($p< 0.05$). In conclusion, the combination of hypertension with diabetes makes possible the maintenance of elevated levels of oxidative stress in platelets.

Keywords: Reactive oxygen species, platelet, type 2 diabetes, hypertension, human.

Introduction

The oxidative stress contributes to the development of different diseases, including vascular complications in chronic diseases as diabetes and atherosclerosis [1]. The reactive oxygen species (ROS) may behave as second messengers and may regulate platelet functions [2]. Oxidative stress is a factor associated with platelet activation in diabetic patients [3]. The levels of oxidants and antioxidant systems on platelets have significant balancing role in the homeostasis of vascular diseases [4].

Hyperglycemia in patients with type 2 diabetes can be related to many pathological states that involve disturbances metabolism, like disorders of oxidative-antioxidative balance [5]. Therefore, high glucose level is an important factor that may cause intensification of oxidative stress and etiopathogenesis of vascular complications in diabetes [6, 7].

The elevation of oxidative stress and associated oxidative damages are mediators of vascular injury in various cardiovascular pathologies, including hypertension and atherosclerosis [8]. Elevated levels of ROS in cases of hypertension may cause endothelial dysfunction and increased vascular resistance [9]. Hypertension increases pro-oxidant generation and can decrease antioxidant defense, and thereby induces oxidative stress in diabetes [10].

Platelets of diabetic patients are exposed to increased oxidative stress [11]. ROS modify both the adhesive and aggregatory responses of platelets, and free radical scavengers are therefore important regulators of platelet function [12]. The antioxidant system includes enzymatic and non-enzymatic components [13]. SOD and CAT are the enzymatic antioxidants which scavenge the ROS, while nonprotein thiols (NPSH) and ascorbic acid are non-enzymatic antioxidant systems which play

important roles in alleviating tissue damage due to the formation of ROS [14, 15]. Protein carbonyl content may used as marker of oxidative damage of proteins and correlates with the severity of protein oxidation in diabetes, hypertension, aging [16]. The malondialdehyde (MDA) possible to determinate the lipid peroxidation levels and it can be used as marker of oxidative stress [17].

Damage to endothelium, either by reactive oxygen species or by its oxidation products, with simultaneous platelet activation, results in alteration in the vascular permeability. Oxidative stress promotes alterations in the homeostasia of diabetes patients. The interactions of the hypertension with diabetes may increase the interference of the oxidative damage in vascular injury. However, the alterations ROS induced in platelets of diabetic patients with associated hypertension are few available in the literature. Hence, the aim of study was evaluated the antioxidant status in platelets of patients with diabetes and associated hypertension. Therefore, we also investigated the correlation between hyperglycemia and antioxidant system.

Material and Methods

Materials

Malondialdehyde (MDA), 2,4-dinitrophenylhydrazine (DNPH), 5-5'-dithio-bis-2-nitrobenzoic acid (DTNB), hydrogen peroxide, and adrenaline were purchased from Sigma (St. Louis, MO, USA). Glucose, cholesterol, HDL-cholesterol, triglycerides, glycated hemoglobin, and lactate dehydrogenase (LDH) commercial kits purchased from Labtest (Lagoa Santa, MG, Brazil). All other reagents used in the experiments were of analytical grade and of the highest purity.

Participants

The sample consisted of patients from the Assistance Program to diabetics and hypertensive patient assistance program linked with the Municipal Health Secretariat in Cruz Alta (RS, Brazil) as well as of healthy volunteers. All subjects gave written informed consent to participate in the study. The protocol was approved by the Human Ethics Committee of the Health Science Center of the Federal University of Santa Maria (Protocol number: 013/2004).

The sample was divided into four groups consisting of 50% males and 50% females. The control group consisted of 15 individuals with ages ranging from 36 to 55, who presented no disease and who had not been submitted to any pharmacological therapy during one month before the study began. Controls were carefully selected by clinical evaluation, matched by sex, age, and body mass index similar to that of the patients. The hypertensive (HT) group was formed by 15 patients with ages ranging from 38 to 58. The components of the HT group had different hypertension levels and were treated with captopril (25 mg/day), furosemide (40 mg/day), and acetylsalicylic acid (100 mg/day). Both type 2 diabetic groups, one of which used oral hypoglycemic drugs (DM2-OHp) and the other of which used insulin (DM2-Ins), consisted of 15 patients each with ages ranging from 39 to 65. The patients of DM2-OHp group were treated with metformin (850 mg/day), and the patients of DM2-Ins were treated with insulin NPH (35 UI/day). Both type 2 diabetic groups with hypertension, one of which used oral hypoglycemic drugs (DM2/HT-OHp) and the other of which used insulin (DM2/HT-Ins), were comprised of 15 patients each with ages ranging from 43 to 66. All patients of the type 2 diabetes mellitus with hypertension received appropriate medication for the associated diseases.

Sample collection

Blood was collected in vacutainer tubes without anticoagulant system. After the collection, the blood was centrifuged at 1400 X g for 10 min, the precipitate was discarded, and the serum was used for thiobarbituric acid reactive substances (TBARS), ascorbic acid (AA), and biochemical determinations. Then, blood was collected into citrate, centrifuged at 160 X g for 10 min, and the platelet-rich plasma (PRP) was used for superoxide dismutase, catalase, nonprotein thiols, and protein carbonyl determination.

The platelet-rich plasma sample was prepared from human donors by the methods of Pilla et al. [18]. Blood was collected into 0.129 M citrate and centrifuged at 160 X g for 10 min. The PRP was centrifuged at 1600 X g for 15 min and washed twice with 3.5 mmol/L Hepes isosmolar buffer containing 142 mmol/L NaCl, 2.5 mmol/L KCl, and 5.5 mmol/L glucose. The washed platelets were resuspended in Hepes isosmolar buffer and used for the protein carbonyl, NPSH, CAT and SOD determination.

Lipid peroxidation determination

Lipid peroxidation was estimated by the measurement of thiobarbituric acid reactive substances in serum samples by modifications of the method of Jentzsch et al. [19]. Briefly, 0.2 ml of serum was added to the reaction mixture containing 1 ml of 1% *ortho*-phosphoric acid, 0.25 mL alkaline solution of thiobarbituric acid-TBA (final volume 2.0 ml) followed by 45 min heating at 95ºC. After cooling, samples and standards of malondialdehyde were read at 532 nm against the blank of the standard curve. The results were expressed as nmol MDA/mL serum.

Carbonylation of protein determination

The carbonylation of platelet proteins was determined by modifications of the Goswami and Koner method [20]. Firstly, from 1 ml of PRP, the proteins were precipitated using 0.5 ml of 10% trichloroacetic acid (TCA) and centrifuged at 1600 X g rpm for 5 min discarding the supernatant. One half milliliter of 10 mmol/L 2,4-dinitrophenylhydrazine in 2 mol/L HCl was added to this precipitated protein and incubated at room temperature for 30 min. During the incubation time the samples were mixed vigorously every 15 min. After the incubation time, 0.5 mL of 10% TCA was added to the protein precipitated and centrifuged at 1600 X g for 5 min. After discarding the supernatant, precipitates were washed twice with 1 mL of ethanol/ethilacetate (1:1), each time centrifuging out the supernatant in order to remove the free DNPH. The precipitate was dissolved in 1.5 mL of protein dissolving solution (2 g SDS and 50 mg EDTA in 100 mL 80 mmol/L phosphate buffer, pH 8.0) and incubated at 37ºC water bath for 10 min. The color intensity of the supernatant was measured using spectrophotometer at 370 nm against 2 M HCl. Carbonyl content was calculated by using molar extinction coefficient (21 x 10^3 1/mol cm) and results were expressed as nmol carbonyl/mg protein.

Nonprotein thiols (NPSH) content

NPSH were determined in platelets by modifications of Sedlak and Lindsay method [21]. The PRP was precipitated with 20% trichloracetic acid and then centrifuged at 3500 rpm for 10 min. The reaction mixture contained 0.5 mL of supernatant, 2.0 mL of phosphate buffer, pH 8.9, and it was read at 412 nm after the addition of 0.1 mL of 0.01 mmol/L 5-5'-dithio-bis-2-nitrobenzoic acid (DTNB). The results were expressed as μmol/mg protein.

Catalase activity

CAT activity was determined in platelets by modifications of Luck method [22]. An aliquot (0.05 mL) of PRP was homogenized in potassium phosphate buffer, pH 7.0. The spectrophotometric determination was started by the addition of 0.07 mL of aqueous solution of hydrogen peroxide 0.3 mol/L. The change in absorbance at 240 nm was measured for 2 min. The catalase activity was calculated using molar extinction coefficient and the results were expressed as pmol/mg protein.

Superoxide dismutase activity

SOD activity in platelet was determined by modifications of Misra and Fridovich method [23]. The reaction mixture constituted of 1.0 mL Tris buffer 0.2 mol/L, pH 10.0, 0.2 mL aliquot of PRP and water to make up the volume to 2.8 mL. The reaction was started by the addition of 0.2 mL of 0.025 mol/L epinephrine. The change in absorbance was measured at 480 nm for 2 min. One unit enzyme inhibited the rate of autooxidation of 5 μmol of epinephrine by 50%, and the results were expressed as U SOD/mg protein.

Ascorbic acid content

Ascorbic acid levels were determined by the method of Jacques-Silva et al. [24]. An aliquot of 300 μL sample of serum was mixed with 2-4-dinitrophenylhydrazine (4.5 mg/mL), $CuSO_4$ (0.075 mg/mL) and trichloroacetic acid 13.3% (final volume 1 mL), and incubated for 3 h at 37°C. Then, 1 mL of H_2SO_4 65% (v/v) was added to the medium. The ascorbic acid levels were measured spectrophotometrically at 520 nm and calculated using a standard curve (1.5-4.5 μmol/L ascorbic acid freshly prepared in sulfuric acid).

Clinical parameters analysis

Serum glucose, cholesterol, HDL-cholesterol, triglycerides, glycated hemoglobin, and lactate dehydrogenase (LDH) were determined using commol/Lercial kits from Labtest (Lagoa Santa, MG, Brazil).

Statistical analysis

Data were analyzed statistically by two-way and one-way ANOVA, followed by Duncan's multiple range test. Differences between groups were considered to be significant when $p < 0.05$. All data were expressed as mean ± S.D. Correlation was evaluated with the Pearson test. Linear correlation between variables was also carried out.

Results

Clinical parameters

The clinical characteristics of the subjects are presented in Table 1. The patients of the control group had never smoked. However, 40% of the hypertensive patients had smoked for 22 years, 33% of the diabetes patients had smoked for 25 years, and 15% of the patients with diabetes and hypertension had smoked for 38 years.

The biochemical determinations of glucose, glycated hemoglobin, triglycerides, total cholesterol, and HDL-cholesterol from all patients are presented in Table 2. The serum glucose was increased in DM2-OHp (82.3%), DM2-Ins (81.1%), DM2/HT-OHp (74.5%), and DM2/HT-Ins (75.4%) groups, and these groups were different from the control and hypertensive groups ($p<0.05$). Glycated haemoglobin was increased in DM2-OHp (48.4%), DM2-Ins (47.1%), DM2/HT-OHp (55.7%), and

DM2/HT-Ins (54.1%) groups, and these groups were different from the control and hypertensive groups (p<0.05). Triglycerides in the control group were significantly different from other groups (p< 0.05) and the difference was in DM2-OHp (66.7%), DM2-Ins (68.1%), HT (60%), DM2/HT-OHp (106.7%), and DM2/HT-Ins (107.5%) groups. All the groups were significantly different from the control group for total cholesterol (p< 0.05) and the difference was in DM2-OHp (41.9%), DM2-Ins (42.7%), HT (44.2%), DM2/HT-OHp (46.5%), and DM2/HT-Ins (47.3%). HDL-cholesterol was not significantly different among the analyzed groups (p>0.05).

Cellular integrity

Quantitative analysis demonstrated that platelets count obtained from all groups were at normal levels (150.000 – 400.000 platelets/mmol/L^3). Microscopic analysis of platelet size and shape revealed a typical pattern (data not shown).

Platelet integrity was determined by lactate dehydrogenase activity. The measurements of LDH showed that most cells (more than 90%) were intact after the isolation procedure, and PRP was adequate (data not shown).

Lipid peroxidation

The patients of the DM2-OHp, DM2-Ins, HT, DM2/HT-Ohp, and DM2/HT-Ins groups had a significant increase (p< 0.05) in TBARS when compared to the control group (Fig.1). There was not difference among the DM-2 and DM-2/HT groups, independent of the medication administrated to the patients.

Antioxidant system

The antioxidant enzymes responsible for the scavenger of ROS were increased in platelets of all patients. The increase of superoxide dismutase (Fig. 2) and catalase (Fig. 3) was observed in patients of DM2-OHp, DM2-Ins, HT, DM2/HT-Ohp, and DM2/HT-Ins groups when compared to the control group ($p< 0.05$). The positive correlation between superoxide dismutase and serum glucose concentration was observed with DM2-OHp (r=0.72, $p < 0.045$), DM2-Ins (r=0.71, $p < 0.041$), DM2/HT-OHp (r=0.79, $p < 0.02$), and DM2/HT-Ins (r=0.76, $p < 0.018$) groups. The high glucose level action in the activity of catalase demonstrated a positive correlation with DM2-OHp (r=0.72, $p < 0.045$), DM2-Ins (r=0.69, $p < 0.043$), DM2/HT-OHp (r=0.79, $p < 0.02$), and DM2/HT-Ins (r=0.76, $p < 0.019$) groups.

The increase in NPSH (Fig.4) and carbonylation of protein (Fig. 5) indicates the high oxidative stress level, which was larger in DM2-OHp, DM2-Ins, HT, DM2/HT-Ohp, and DM2/HT-Ins groups when compared to the control group ($p< 0.05$). There was a significant and positive correlation between serum glucose levels with protein carbonyl in DM2-OHp (r=0.79, $p < 0.019$), DM2-Ins (r=0.75, $p < 0.016$), DM2/HT-OHp (r=0.82, $p < 0.011$) and DM2/HT-Ins (r=0.80, $p < 0.010$). Nonprotein thiols had also a positive correlation with hyperglycemia in DM2-OHp (r=0.74, $p < 0.036$), DM2-Ins (r=0.71, $p < 0.031$), DM2/HT-OHp (r=0.71, $p < 0.047$), and DM2/HT-Ins (r=0.73, $p < 0.045$) groups.

The antioxidant actions containing ascorbic acid (Fig. 6) in pathological groups were significantly decreased in serum by 28 % in relation to the control group. The low concentrations of serum ascorbic acid demonstrated high oxidative stress level in DM2-OHp, DM2-Ins, HT, DM2/HT-Ohp, and DM2/HT-Ins groups when compared to the control group ($p< 0.05$).

Discussion

Diabetes and hypertension constitute diseases that characterized by the chronicity and for the continuous administration of medicines. The results of glycemia and glycated hemoglobin demonstrated that the mean of patients with diabetes and hypertension associated presented poor glycemic control. The decrease of glycemic control may contribute to the generation of ROS with increase of protein oxidation [25].

Chronic smoking is a risk factor for the development of atherothrombosis [26]. The smoke compounds promote significant changes in initiating physiologic coagulation process and platelet adhesiveness, and aggregation increases as a result of smoking [27, 28]. Therefore, the patients with diabetes and associated pathologies that also smoke increase even more the oxidative damage and alterations in platelets activation.

The oxidative stress results of an imbalance between pro-oxidants and antioxidants systems. Lipid peroxidation is an important biological consequence of oxidative cellular damage [29], and MDA is considered as a marker of oxidative stress [30]. Therefore, the data of serum TBARS indicate an increase in oxidative damage in patients with hypertension, diabetes, and associated hypertension. The hyperglycemia contributes to increased lipid peroxide formation through auto-oxidation and non-enzymatic glycation and lipids as well as increased sorbitol pathway activity [31, 32]. The increase in the production of ROS causes oxidative stress that in platelets leads to chemical changes and may regulate platelet functions [33].

The results of the SOD and CAT activities demonstrated that in patients with diabetes the hyperglycemia was an important factor in the increment of the oxidative

stress. The hypertension when associated with diabetes seems to have contributed to maintenance of the high levels of ROS production. The antioxidant enzyme activities in platelets of patients with diabetes present different behavior [34, 35]. The glucose level had a straight interference in the antioxidant enzyme activities in the platelets as well as in blood. The increase in SOD and CAT activities may represent a response to stimulation by accumulating oxidative stress in the presence of chronic hyperglycemia. The metabolic control is associated with a significant reduction in both lipid peroxidation and platelet activation. This comportment suggest that enhanced lipid peroxidation may provide an important biochemical link between impaired glycemic control and persistent platelet activation [6]. These results suggest that antioxidant enzymatic defense in blood platelet has an important role in the modifications of homeostasis.

The elevation of NPSH in platelet of pathological patients may be sustained by a more active biosynthesis through the g-glutamylcysteine pathway [34]. The high sanguine glucose concentrations constitute one more factor to promote the increase of NPSH in diabetic patients. The hypertension constitutes a factor equally capable to promote an increase of NPSH. Therefore the association of the hypertension to the diabetes allows the maintenance of the high NPSH levels in the patients.

The elevation of protein carbonyl indicates the severity of oxidative damage in platelet of pathological patients. The same comportment has been found in platelet investigation [19]. Carbonyl stress may result from hyperglycemia and impaired detoxication of reactive carbonyl compounds [36]. This condition was observed through of the positive correlation between hyperglycemia and protein carbonyl demonstrating a direct interference in ROS production in platelets.

The administration of the drugs metformin and insulin did not differentiate the increase of oxidative stress levels among the groups. The short-term metformin administration promotes activation of oxidative stress together with alterations of the antioxidant system [37]. Studies with metformin in vitro and with animals showed reactive oxygen species production may have been reduced [38].

The low concentrations of ascorbic acid in serum, observed in pathological patients, suggest an exhibition to oxidative damages. The low concentrations ascorbic acid has been described as a biomarker in pathogenesis of hypertension [39, 40] and diabetes [401, when increase the oxidative stress. Therefore, low concentrations of ascorbic acid in serum can cause pro-oxidative effects [42, 43]. These results suggested that ascorbic acid levels constitute a factor in the control of oxidative stress.

In conclusion, we can observe that patients with diabetes and hypertension associated had the oxidative damage increased in platelets. The acid ascorbic was an important factor capable to modulate oxidative stress. The hyperglycemia constituted an important factor capable to provide an increase in reactive oxygen species production and in consequence to modify the antioxidant system status. Therefore, the combination of Diabetes mellitus and hypertension possible to maintain an elevated level of oxidative stress in platelets and potentially may promote more critical alterations in platelet homeostasia.

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FIGURE LEGENDS

Figure 1. TBARS level in serum from control (n=15); hypertensive (HT; n=15); type 2 diabetes mellitus – oral hypoglycemic drugs (DM2-OHp; n=15); type 2 diabetes mellitus – insulin (DM2-Ins; n=15); type 2 diabetes mellitus with hypertensive – oral hypoglycemic drugs (DM2/HT-OHp; n=15); type 2 diabetes mellitus with hypertensive – Insulin (DM2/HT-Ins; n=15) groups. Activity is expressed as nmol MDA/ml serum. Values represent mean ± S.D. *****Different from the control group (ANOVA, Duncan's test, $p < 0.05$).

Figure 2. SOD activity in platelets from control (n=15); hypertensive (HT; n=15); type 2 diabetes mellitus – oral hypoglycemic drugs (DM2-OHp; n=15); type 2 diabetes mellitus – insulin (DM2-Ins; n=15); type 2 diabetes mellitus with hypertensive – oral hypoglycemic drugs (DM2/HT-OHp; n=15); type 2 diabetes mellitus with hypertensive – Insulin (DM2/HT-Ins; n=15) groups. Activity is expressed as U SOD/mg protein. Values represent mean ± S.D. *****Different from the control group (ANOVA, Duncan's test, $p < 0.05$).

Figure 3. CAT activity in platelets from control (n=15); hypertensive (HT; n=15); type 2 diabetes mellitus – oral hypoglycemic drugs (DM2-OHp; n=15); type 2 diabetes mellitus – insulin (DM2-Ins; n=15); type 2 diabetes mellitus with hypertensive – oral hypoglycemic drugs (DM2/HT-OHp; n=15); type 2 diabetes mellitus with hypertensive – Insulin (DM2/HT-Ins; n=15) groups. Activity is expressed as *p* moles/mg protein. Values represent mean ± S.D. *****Different from the control group (ANOVA, Duncan's test, $p < 0.05$).

Figure 4. NPSH level in platelets from control (n=15); hypertensive (HT; n=15); type 2 diabetes mellitus – oral hypoglycemic drugs (DM2-OHp; n=15); type 2 diabetes mellitus – insulin (DM2-Ins; n=15); type 2 diabetes mellitus with hypertensive – oral hypoglycemic drugs (DM2/HT-OHp; n=15); type 2 diabetes mellitus with hypertensive – Insulin (DM2/HT-Ins; n=15) groups. Activity is expressed as μmol/mg protein. Values represent mean ± S.D. *****Different from the control group (ANOVA, Duncan's test, $p < 0.05$).

Figure 5. Protein carbonyl level in platelets from control (n=15); hypertensive (HT; n=15); type 2 diabetes mellitus – oral hypoglycemic drugs (DM2-OHp; n=15); type 2 diabetes mellitus – insulin (DM2-Ins; n=15); type 2 diabetes mellitus with hypertensive – oral hypoglycemic drugs (DM2/HT-OHp; n=15); type 2 diabetes mellitus with hypertensive – Insulin (DM2/HT-Ins; n=15) groups. Activity is expressed as nmol carbonyl/mg protein. Values represent mean ± S.D. *****Different from the control group (ANOVA, Duncan's test, $p < 0.05$).

Figure 6. Ascorbic acid concentration in serum from control (n=15); hypertensive (HT; n=15); type 2 diabetes mellitus – oral hypoglycemic drugs (DM2-OHp; n=15); type 2 diabetes mellitus – insulin (DM2-Ins; n=15); type 2 diabetes mellitus with hypertensive – oral hypoglycemic drugs (DM2/HT-OHp; n=15); type 2 diabetes mellitus with hypertensive – Insulin (DM2/HT-Ins; n=15) groups. Activity is expressed as μg ascorbic acid/ml serum. Values represent mean ± S.D. *****Different from the others groups (ANOVA, Duncan's test, $p < 0.05$).

Table 1. Clinical characteristics of the control, hypertension (HT), type 2 diabetes (DM2), and type 2 diabetes/hypertension (DM2/HT) groups.

	Control	HT	DM2		DM2/HT	
			OHp	Ins	OHp	Ins
	(*n* = 15)	(*n* = 15)	(*n* = 15)	(*n* = 15)	(*n* = 15)	(*n* = 15)
Age (years)	46 ± 9	48 ± 10	52 ± 13		55 ± 11	
Diabetes (years)	_		10 ± 5		10 ± 4	
Hypertension (years)	_	11 ± 9	_		7 ± 5	
Drugs						
Metformin	_	_	*n = 15*	_	*n = 15*	_
Insulin NPH	_	_	_	*n = 15*		*n = 15*
Captopril, furosemide, acetylsalicylic acid	_	*n* = 4	_	_	*n = 15*	*n = 15*

Values represent mean ± S.D. Oral hypoglycemic drug (OHp) and insulin (Ins).

Table 2. Biochemical characteristics of the control, hypertension (HT), type 2 diabetes (DM2), and type 2 diabetes/hypertension (DM2/HT) groups.

	Control	HT	DM2		DM2/HT	
			OHp	Ins	OHp	Ins
	(*n* = 15)	(*n* = 15)	(*n* = 15)	(*n* = 15)	(*n* = 15)	(*n* = 15)
Serum glucose (mmol/Lol/L)	5.1 ± 0.5	5.2 ± 0.5	9.3 ± 1.1*	9.1 ± 0.9*	8.9 ± 0.2*	8.6 ± 0.3*
Glycated Hemoglobin (%)	6.1 ± 0.5	6.2 ± 0.4	9.2 ± 0.3*	9.3 ± 0.1*	9.5 ± 0.2*	9.7 ± 0.3*
Serum total cholesterol (mmol/Lol/L)	4.3 ± 0.4	6.1 ± 0.7*	6.2 ± 0.5*	6.0 ± 0.2*	6.3 ± 0.8*	6.4 ± 0.7*
Serum HDL-cholesterol (mmol/Lol/L)	1.6 ± 0.2	1.3 ± 0.4	1.3 ± 0.4	1.3 ± 0.2	1.4 ± 0.2	1.5 ± 0.3
Serum triglycerides (mmol/Lol/L)	1.5 ± 0.1	2.5 ± 0.8*	2.5 ± 0.8*	2.7 ± 0.6*	3.1 ± 0.8*	3.3 ± 0.5*

Values represent mean ± S.D. Oral hypoglycemic drug (OHp) and insulin (Ins).

*Different from the others in the same line (ANOVA, Duncan test, $p < 0.05$).

Figure 1

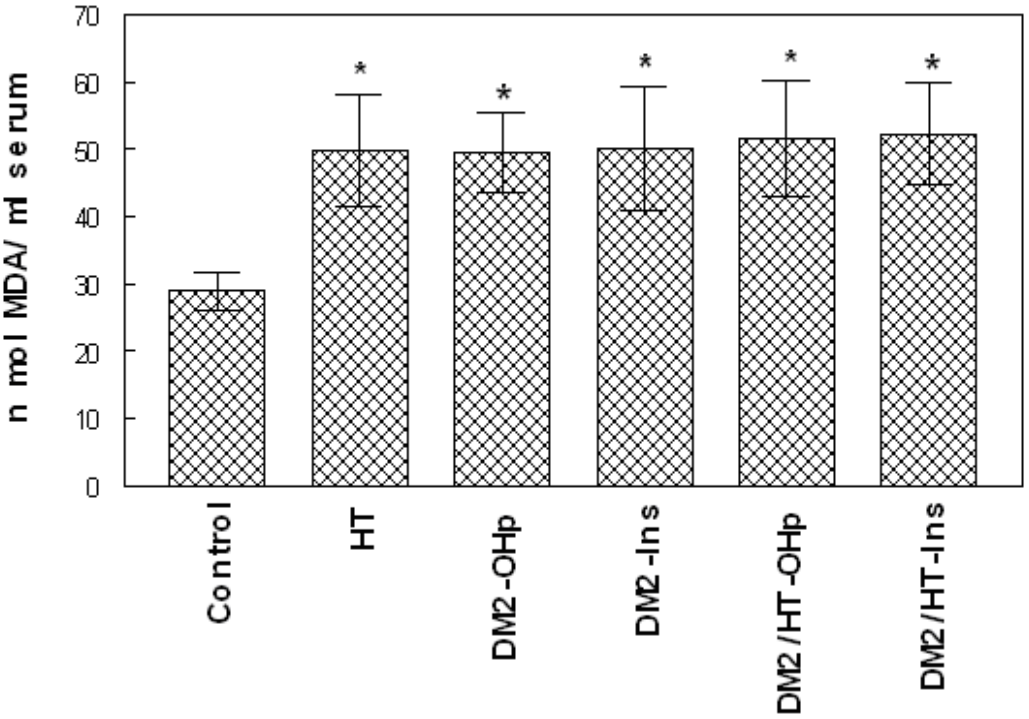

n mol MDA/ ml serum
70
60
50
40
30
20
10
0
*
*
*
*
*
Control
HT
DM2 -OHp
DM2 -Ins
DM2 /HT -OHp
DM2 /HT -Ins

Figure 2

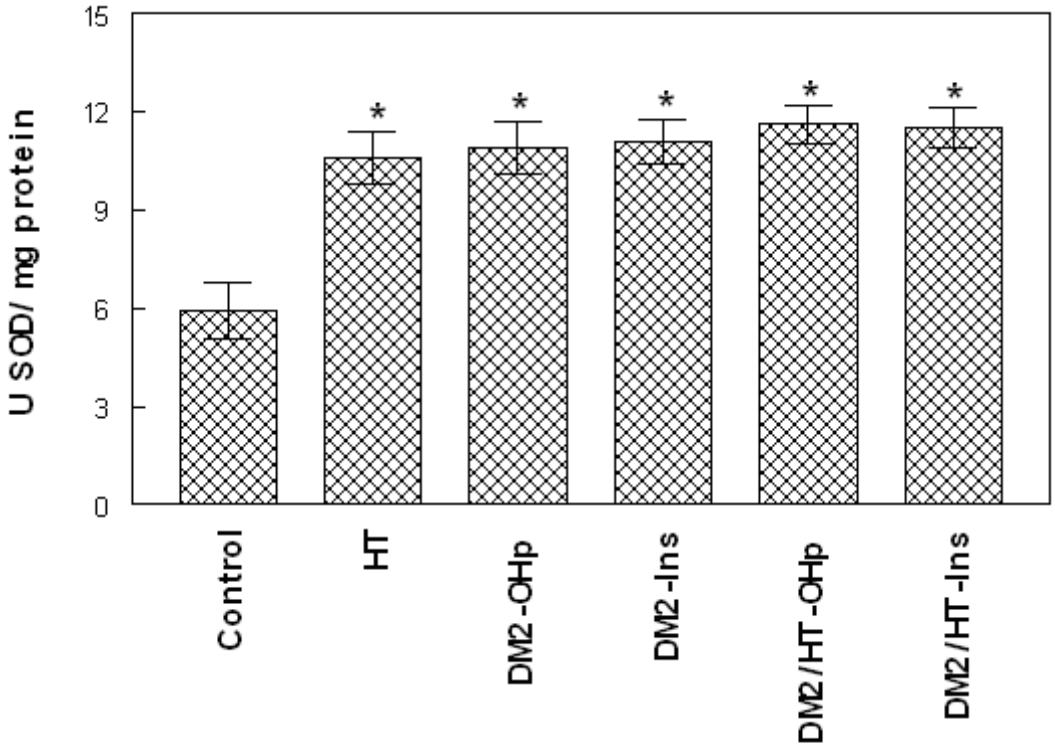
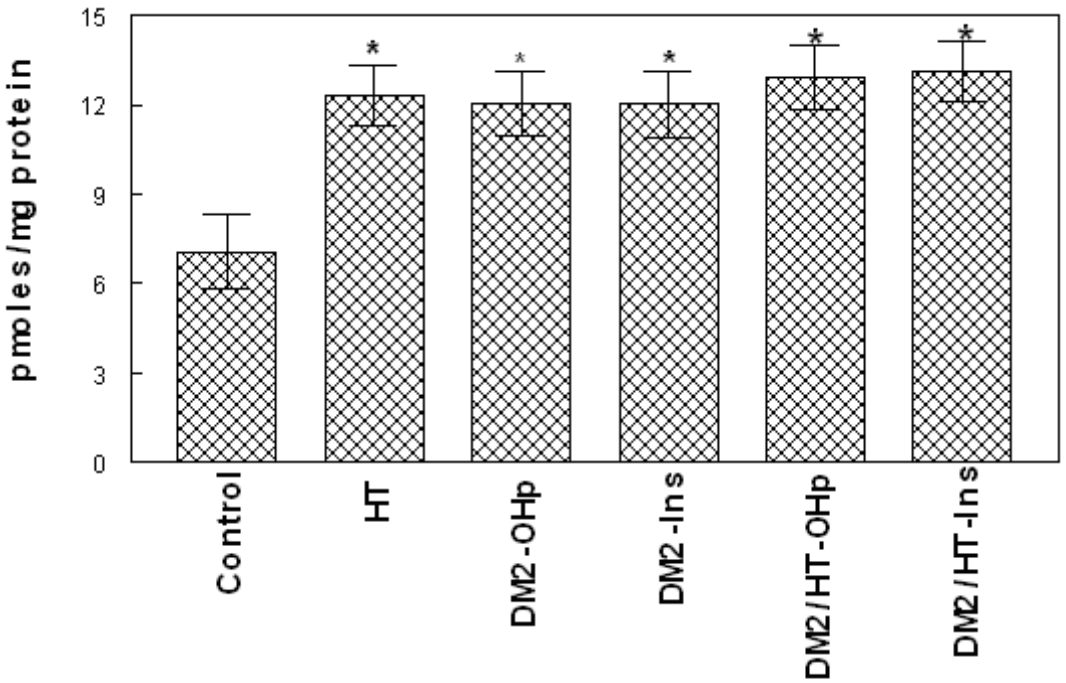

U SOD/ mg protein
15
12
9
6
3
0
*
*
*
*
*
Control
HT
DM2 -OHp
DM2 -Ins
DM2 /HT -OHp
DM2 /HT -Ins

Figure 3


pmoles/mg protein
15
12
9
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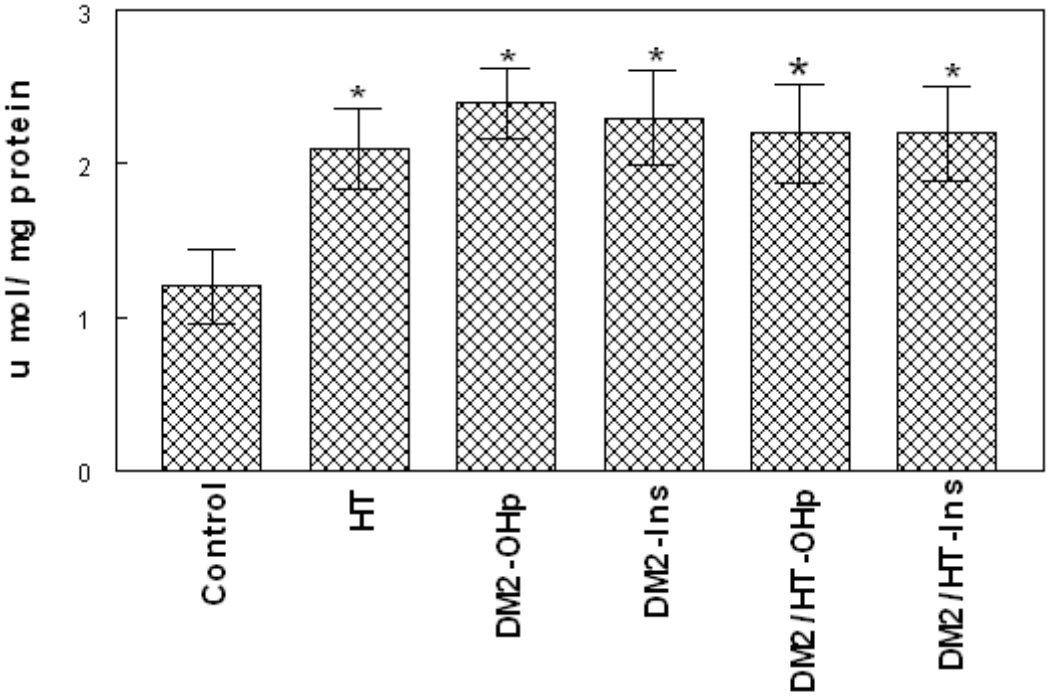
Control
HT
DM2-OHp
DM2-Ins
DM2/HT-OHp
DM2/HT-Ins

Figure 4


u mol/ mg protein
3
2
1
0
*
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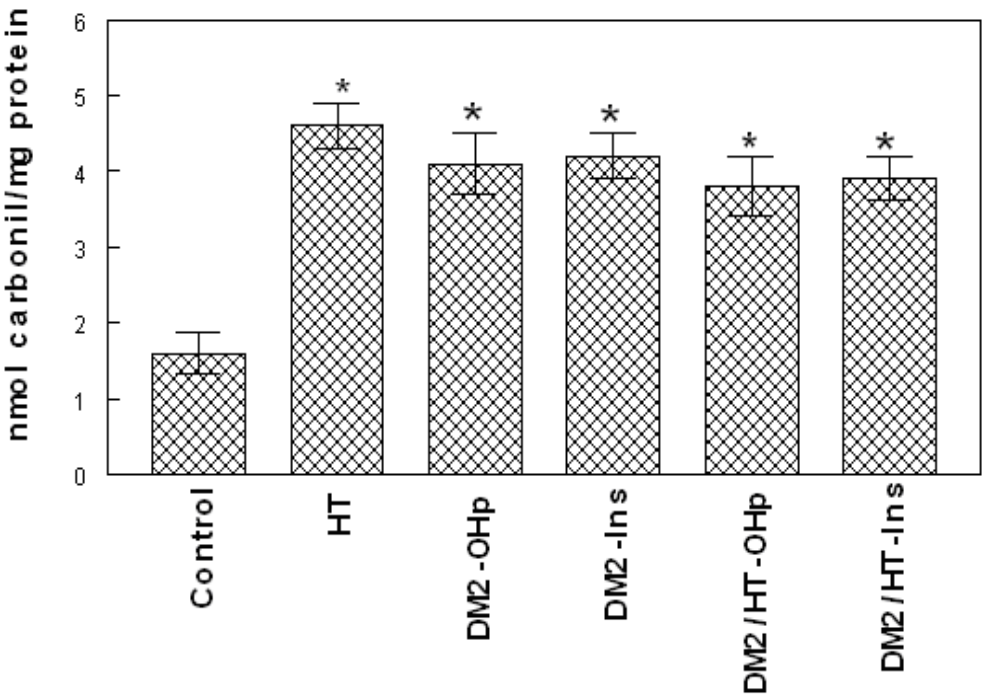
*
*
*
Control
HT
DM2-OHp
DM2-Ins
DM2/HT-OHp
DM2/HT-Ins

Figure 5


nmol carbonil/mg protein
0
1
2
3
4
5
6
*
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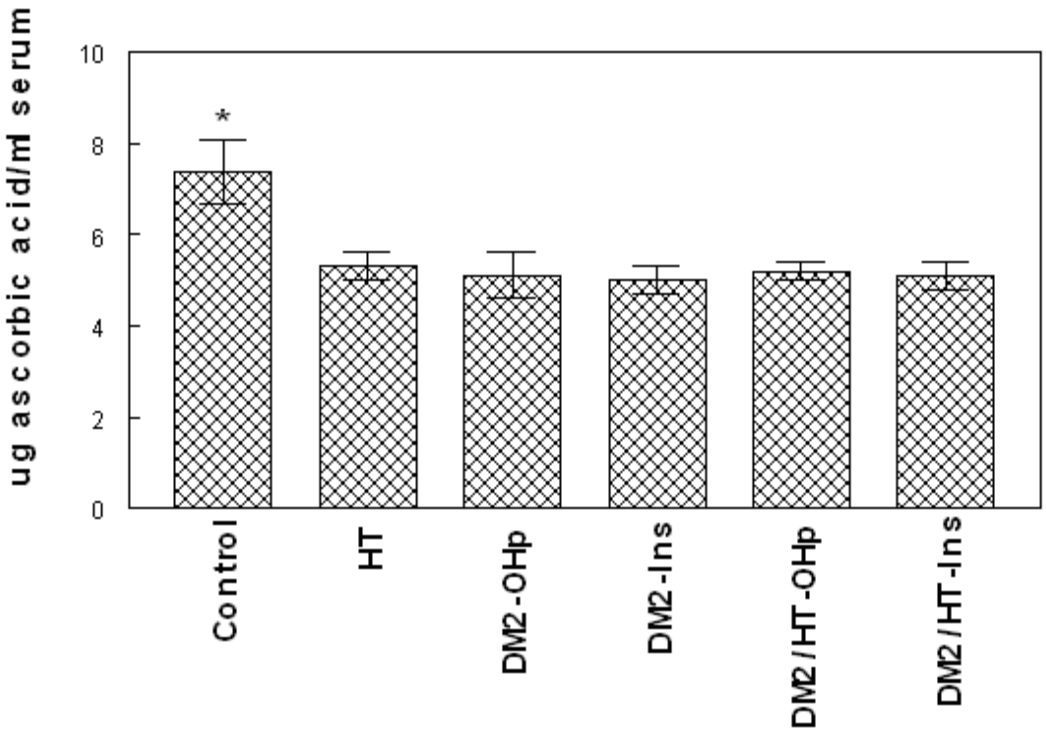
Control
HT
DM2 -OHp
DM2 -Ins
DM2 / HT -OHp
DM2 / HT -Ins

Figure 6


ug ascorbic acid/ml serum
10
8
6
4
2
0
*
Control
HT
DM2-OHp
DM2-Ins
DM2/HT-OHp
DM2/HT-Ins

3.4 Manuscrito 3

O manuscrito “Oxidative stress and antioxidant profile in serum from patients with type 2 diabetes and hypertension” está na fase de redação.

Oxidative stress and antioxidant profile in patients with diabetes and hypertension

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Abstract

This investigation analyzed the action of the hyperglycemia and micronutrient in antioxidant defense system in patients with diabetes. The sample composed of 80 patients was divided into four groups. The antioxidant enzymatic and non-enzymatic systems were estimated in patients of control, type 2 diabetes (DM2), hypertension (HYP) and type 2 diabetes/hypertension (DM2/HYP) groups. There was an increase in TBARS, CAT, SOD, NPSH and protein carbonyl in patients of pathological groups when compared to control group ($p< 0.05$). There was significant and positive correlation between serum glucose levels with SOD, CAT, NPSH and protein carbonyl. The serum calcium and magnesium levels were lower while copper and zinc levels had increased in patients of DM2, HYP and DM2/HYP groups when compared to the control group ($p< 0.05$). The micronutrient concentration demonstrated an exhibition the oxidative damage in the pathological patients. The hyperglycemia constitutes a factor that promotes the increase of oxidative stress in patients with diabetes. The hypertension associated with diabetes possible to maintain elevated levels of oxidative stress and, consequently these patients can develop vascular alterations.

Keywords: Diabetes mellitus, hypertension, hyperglycemia, antioxidant system.

1. Introduction

Diabetes mellitus is a syndrome with chronic hyperglycemia. The diabetic patients have significant alteration on antioxidant protections and increase in generation of reactive oxygen species and nitrogen [1]. Elevated free radicals levels can develop endothelial vascular dysfunction specially in type 2 diabetes [2, 3, 4]. The oxidative stress can promote pro-atherogenic events in diabetic patients and their relationships with atherosclerosis could potentially identify molecular targets of therapy [5]. Hyperglycemia may induce reactive oxygen species and nitrogen production through glucose metabolism, auto-oxidation, and formation of advanced glycation end product [6].

The production of reactive oxygen species has been linked to certain diseases of the cardiovascular system including hypertension. The oxidative stress may constitute a major pathogenic factor in the development of hypertension [7]. The hypertension increases pro-oxidant generation and the combination of hypertension with diabetes can exacerbate oxidative stress [8].

Catalase (CAT), superoxide dismutase (SOD), nonprotein thiols (NPSH) are endogenous antioxidant system defense. The imbalance between pro-oxidants and antioxidant gives rise to cellular oxidative damage. The ROS in diabetes was greatly increased due to prolonged exposure to hyperglycemia and impairment of oxidant/antioxidant equilibrium [9].

The protein carbonyl and lipid peroxidation determination can be used as markers of oxidative stress. Protein carbonyl content is an indicator and marker of protein oxidation, and accumulation of protein carbonyl has been observed in several human diseases including diabetes [10]. TBARS assay let to evaluate the lipid

peroxidation level that refers to the oxidative degradation of lipids that result in cell damage [11].

Some minerals, as copper, zinc, and magnesium can participate in the reduction of oxidative stress in diabetic patients and can be proposed as adjunctive therapy [12]. Deficiency in copper levels increases reactive oxygen species and it can be involved in complications in diabetes [13]. The treatment with zinc decreases the cardiovascular involvement in type 2 diabetes mellitus patients [14]. Calcium regulates several mechanisms that generate oxidative or nitrosative stress and the oxidative stress modifies the calcium level interfering in the vascular reactivity [15]. The magnesium modulates free radicals, malondialdehyde and nitric oxide production. Thus, this mineral may be involved in the regulation of lipid peroxidation [16].

Thus, the present studies propose to available the antioxidant defense system in patients with diabetes and hypertension. Then we investigate the interference of the hyperglycemia and micronutrient in antioxidant system.

2. Patients and Methods

2.1. Patients

A total of 80 subjects participated in the study. The patients were divided into four groups with similar age and they were separate with the same proportion between men and women. The patients participate in the Program of Attendance to Diabetic, Hypertensive and Diabetic-Hypertensive patients, associated with the Municipal Secretary's Office of Health of Cruz Alta (RS, Brazil) and health volunteers.

All subjects gave written informed consent to participate in the study. The protocol was approved by the Human Ethics Committee of the Health Science Center of the Federal University of Santa Maria (Protocol number: 013/2004).

The sample was divided into four groups consisting of 50% males and 50% females. The control group (n=26) consisted of individuals with ages ranging from 28 to 52 years, who did not present any disease and who had not been submitted to any pharmacological therapy during the last month. Controls were carefully selected by clinical evaluation and presented sex, age and body mass index similar to those of the patients. The type 2 diabetic (DM2, n=16) group consisted of patients with ages ranging from 56 to 68 years. The patients of the DM2 group had type 2 diabetes mellitus and were treated with glibenclamide (10 mg/day) or chlorpropamide (250 mg/day). The hypertensive (HYP, n=12) group was made up of patients with ages ranging from 30 to 70 years. The patients of the HYP group had different hypertension levels and were treated with captopril (25 mg/day), furosemide (40 mg/day) or propranolol (40 mg/day). The type 2 diabetic/hypertensive (DM2/HYP, n=26) group consisted of patients with ages ranging from 51 to 69 years. All patients of the DM2/HYP had type 2 diabetes mellitus plus hypertension and received appropriate medication for the associated diseases. Ten milliliters of blood was obtained from each participant and used for platelet-rich plasma preparations, biochemical determinations and hematological determinations.

2.2. Sample collection

The blood was collected in vacutainer tubes without anticoagulant system. After the collection, the blood was centrifuged at 1400 X g for 10 min, the precipitated

was discarded and the serum was used to make thiobarbituric acid reactive substances (TBARS), protein carbonyl and biochemical determinations. The second aliquot was obtained with anticoagulant EDTA. This blood was used for the catalase and superoxide dismutase assays and the plasma was used for nonprotein thiols and micronutrient determination. All samples were analyzed in the same day of collection.

2.3. Reagents

The hydrogen peroxide, adrenaline, acid 5,5-ditio-bis-2-nitrobenzoic (DTNB), malondialdehyede, and 2,4-dinitrophenylhydrazine (DNPH) were purchased from Sigma (St. Louis, MO, USA). The glucose, cholesterol, HDL-cholesterol and triglycerides commercial kits purchased from Labtest (Lagoa Santa, MG, Brazil) and calcium from Bioclin (Belo Horizonte, MG, Brazil). All other reagents used in the experiments were of analytical grade and highest purity.

2.4. Catalase activity

Catalase (CAT, EC 1.11.1.6) assay involves the change in absorbance at 240 nm due to the catalase dependent decomposition of H_2O_2 by Nelson & Kiesov [17]. An aliquot (20 μL) of blood was mixed with potassium phosphate buffer 50 mM, pH 7.0, and 70 μL of 30 mM H_2O_2 was added to each sample. The change in absorbance at 240 nm was measured for 2 min and the slope of the curve at linearity was calculated. The values were expressed in *p*moles/mg protein.

2.5. Superoxide dismutase activity

Superoxide dismutase (SOD, EC 1.15.1.1) assay was analyzed from the inhibition of reaction of the radical superoxide with adrenaline, as described by Boveris [18]. Aliquots of blood were mixed with glycine buffer 5 mM, and adrenaline 60 mM, pH 2.0 were added to each sample. The change in absorbance at 320 nm was measured for 5 min and the slope of the curve at linearity was calculated. The results were expressed in U SOD/mg protein.

2.6. Nonprotein thiols content

Nonprotein thiols (NPSH) were determined by the method of Ellman [19]. An aliquot of plasma was mixed with potassium phosphate buffer 1 M, pH 7.4, and 5-5'-dithio-bis,2-nitrobenzoic acid 10 mM. NPSH was estimated in absorbance at 412 nm. The results were expressed as μmol/ml plasma.

2.7. Protein carbonyl levels

The carbonylation of serum proteins was determined by modifications method of Levine [20]. Firstly, from 1 mL of serum, the proteins were precipitated using 0.5 mL of 10% trichloroacetic acid (TCA) and centrifuged at 1600 X g for 5 min discarding the supernatant. One half milliliter of 10 mmol/L 2,4-dinitrophenylhydrazine in 2 mol/L HCl was added to this precipitated protein and incubated at room temperature for 30 min. During the incubation time the samples were mixed vigorously every 15 min. After the incubation time, 0.5mL of 10% TCA

was added to the protein precipitated and centrifuged at 1600 X g for 5 min. After discarding the supernatant, precipitates were washed twice with 1 mL of ethanol/ethilacetate (1:1), each time centrifuging out the supernatant in order to remove the free DNPH. The precipitate was dissolved in 1.5 mL of protein dissolving solution (2 g SDS and 50 mg EDTA in 100 ml 80 mmol/L phosphate buffer, pH 8.0) and incubated at 37ºC water bath for 10 min. The color intensity of the supernatant was measured using spectrophotometer at 370 nm against 2 mol/L HCl. Carbonyl content was calculated by using molar extinction coefficient (21 x 10^3 1/mol cm) and results were expressed as nmol carbonyl/mg protein.

2.8. Lipid peroxidation determination

The malondialdehyede (MDA) levels in serum were determined by the method described by Jentzsch et al. [21]. An aliquot (20 μL) of serum was added to 0.250 mL of 0.11 mol/L 2-thiobarbituric-acid and 1 mL of 0.2 M phosphoric acid. The mixture was heated at 90ºC for 45 min and read at 532 nm. The results were expressed as nmol MDA/ml serum.

2.9. Micronutrients determination

The sample used in determination of copper, zinc and magnesium was plasma. After sample digestion all determinations of metals were carried out using a Model 3030 graphite furnace atomic absorption spectrometer (Perkin Elmer, Norwalk, USA) equipped with an autosampler (Model AS-40), and a deuterium background correction system. Hollow cathode lamps for lead, copper, zinc and

magnesium were operated at 5 mA. The correspondent wavelength and spectral bandpass were 283.3 nm/0.7 nm, 324.8 nm/0.7 nm, 213.9 nm/0.7 nm, and 285.2 nm/0.7 nm. Pyrolytic coated graphite tubes with platforms were used throughout the analysis. Chemical modifiers were used whenever necessary [22]. The injection volume was 20 ml and integrated absorbance (peak area) was used for signal evaluation.

The serum calcium was measured by colorimetric method, using commercial kit from Bioclin (Belo Horizonte, MG, Brazil). The results were expressed as mg/dL serum.

2.10. Biochemical determination

The serum glucose, cholesterol, HDL-cholesterol and triglycerides were measured by enzymatic method, using commercial kits from Labtest (Lagoa Santa, MG, Brazil). The results were expressed as nmol/mL serum.

2.11. Protein concentration analysis

Protein concentrations were estimated using bovine serum albumin as standard, as described by Bradford [23].

2.12. Statistical analysis

Data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range test. Differences between groups were considered to be significant

when $p < 0.05$. All data were expressed as mean ± S.D. Correlation was evaluated with the Pearson test. Linear correlation between variables was also carried out.

3. Results

3.1. Clinical characteristics of the patients

The biochemical determinations of glucose, triglycerides, total cholesterol, and HDL-cholesterol from all patients are presented in Table 1. The serum glucose was increased in type 2 diabetes (116.05%) and type 2 diabetes/hypertensive (109.87%) groups and these groups were different from control and hypertensive groups ($p< 0.05$). Triglycerides in the pathological groups were significantly different from control group ($p< 0.05$) and the difference was: type 2 diabetes (172.1%), hypertensive (150.9%), type 2 diabetes/hypertensive (189.4%). The total cholesterol was not significantly different among the analyzed groups ($p> 0.05$). HDL-cholesterol was decreased in type 2 diabetes (43.3%), hypertensive (38.3%), type 2 diabetes/hypertensive (35%) groups when compared to the control group ($p< 0.05$).

3.2. Micronutrients concentrations

The copper, zinc, magnesium and calcium concentration were expressed in Table 2. The DM2, HYP and DM2/HYP had an increase in the copper and zinc concentration when compared to the control group ($p< 0.05$). However, serum

calcium and magnesium levels were lower in the DM2, HYP and DM2/HYP ($p< 0.05$) when compared to the control group.

3.3. Lipid peroxidation

There was an increase in the blood levels of lipid peroxidation in serum (Fig. 1). The patients of the DM2, HYP, DM2/HYP groups had a significant increase in TBARS, when compared to the control group ($p< 0.05$).

3.4. Catalase and superoxide dismutase activities

The antioxidant enzymes were change in the activities in pathological patients. The catalase (Fig. 2) and superoxide dismutase (Fig. 3) activities had an increased in patients of DM2, HYP, DM2/HYP groups, when compared to the control group ($p< 0.05$). The glucose curve in vitro was developed with antioxidant enzymes, using concentrations ranging from 5 to 100 mM from control subjects. The results demonstrated an increase in catalase and superoxide dismutase activities. The elevation in antioxidant enzymes activities was proportional the elevation of glucose concentration (data not show). The high glycemia had the positive correlation with catalase activity in DM2 ($r=0.58$, $p < 0.028$) and DM2/HYP ($r = 0.40$, $p < 0.039$) groups. The elevated glucose concentrations had positive correlation with superoxide dismutase activity in DM2 ($r=0.57$, $p < 0.018$) and DM2/HYP ($r = 0.42$, $p < 0.028$) groups.

3.5. nonprotein thiols content

There was an increase in the blood content of NPSH in serum (Fig. 4). The patients of the DM2, HYP and DM2/HYP groups had a significant increase in nonprotein thiols, when compared to the control group ($p< 0.05$). The hyperglycemia had positive correlation with nonprotein thiols in DM2 (r=0.56, $p < 0.036$) and DM2/HYP (r = 0.610, $p < 0.033$) groups.

3.6. Protein carbonyl content

Protein oxidation, determined by protein carbonyl content in serum, is shown in Fig. 5. The elevated protein carbonyl content indicates a high oxidative stress in patients of the DM2, HYP and DM2/HYP groups when compared to the control group ($p< 0.05$). There was significant and positive correlation between serum glucose levels with protein carbonyl in DM2 (r=0.59, $p < 0.027$), DM2/HYP (r=0.5182, $p < 0.021$).

4. Discussion

Antioxidant defense mechanisms are important in the protection of tissues from oxidative damage [24]. There are many ways through which hyperglycemia may increase the generation of oxygen free radicals, such as glycoxidation process, polyol pathway, and prostanoid biosynthesis and protein glycation [25]. The elevation in the production of the reactive oxygen species has the potential to initiate changes in the endothelial function.

Previous studies have suggested that cardiovascular complications in diabetes can be controlled by therapeutic strategies that focus on a good glycemic control and loosely bound systemic Cu(II) [26]. The reduction in the copper and zinc concentrations in diabetic patients can promote an elevation in the oxidative stress levels [13]. The zinc deficiency in rats demonstrated that this state produces low resistance to oxidant injury, and it produces high vulnerability of lipoproteins to oxidation [27]. Magnesium deficiency has recently been related with age-related diseases through free-radical mechanism [28]. The existence of oxidative stress has been well documented in diabetes and late diabetic complications. The calcium concentration can be modified by oxidative stress levels. This mechanism of calcium regulation can interfere in the vascular reactivity [15].

The hyperglycemia appears to play a major role in ROS production and lipid hydroperoxides to yield lipid-associated free radicals. This condition permits the propagation of free radical-mediated reactions that produced extensive lipid peroxidation as a marker of oxidative stress. Our data demonstrated that oxidative damage in patients of DM2, HYP and DM2/HYP groups was large, because there was an increase in TBARS concentration in these groups. The increased MDA concentration has been reported in patients with diabetes [29]. The hypertension appears to be an important factor in the increase of MDA and investigations showing increased serum TBARS in hypertensive patients suggest an association between increased oxidative stress with higher blood pressure [30].

The results obtained in this study showed an increase in CAT, SOD activities and NPSH content in diabetes patients as it was also demonstrated in other studies [31, 32]. The development of diabetes complications is associated with an increase in ROS and alterations on antioxidant enzymes induced by chronic hyperglycemia [33,

34]. Conflicting reports on CAT, SOD activities and NPSH content in diabetic patients have appeared in others studies [35, 36]. Our results suggested that erythrocyte anti-oxidant enzymes may point to an adaptive reaction to oxidative stress reflecting free radical overproduction and increased enzyme biosynthesis. A possible explanation for this phenomenon could be a compensatory mechanism by the body to prevent tissue damage.

The level of protein oxidation may indicate the oxidative damage and it is associated with increased in patients with diabetes [37]. Our data demonstrated that oxidative damage in patients of DM2, HYP and DM2/HYP groups was intense, because there was an increase in protein carbonylation concentration in these groups. Therefore, we can observe that hyperglycemia was predominant factor in the increase of the protein carbonylation in patients with diabetes and associated pathologies.

5. Conclusion

The hyperglycemia was an important factor in the increase of oxidative stress in patients with type 2 diabetes. The micronutrients levels constituted another factor able to modify oxidative stress status. The hypertension associated with diabetes demonstrated an elevated maintenance of the oxidative damage. Therefore, we can observe that decrease in glycemia control can aggravate oxidative stress and in consequence vascular alterations.

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FIGURE LEGENDS

Figure 1. TBARS level in serum from control (n = 26); type 2 diabetes (DM2; n = 16); hypertensive (HYP; n = 12); diabetic and hypertensive (DM2/HYP; n = 26) groups. Activity is expressed as nmol MDA/ml serum. Values represent mean ± S.D.

Figure 2. Catalase activity in serum from control (n = 26); type 2 diabetes (DM2; n = 16); hypertensive (HYP; n = 12); diabetic and hypertensive (DM2/HYP; n = 26) groups. Activity is expressed as pmoles/mg protein. Values represent mean ± S.D.

Figure 3. Superoxide dismutase activity in serum from control (n = 26); type 2 diabetes (DM2; n = 16); hypertensive (HT; n = 12); diabetic and hypertensive (DM2/HT; n = 26) groups. Activity is expressed as U SOD/mg protein. Values represent mean ± S.D.

Figure 4. Nonoprotein thiols content in serum from control (n = 26); type 2 diabetes (DM2; n = 16); hypertensive (HT; n = 12); diabetic and hypertensive (DM2/HT; n = 26) groups. Activity is expressed as μmol/ml plasma. Values represent mean ± S.D.

Figure 5. Protein carbonyl concentration in serum from control (n = 26); type 2 diabetes (DM2; n = 16); hypertensive (HT; n = 12); diabetic and hypertensive (DM2/HT; n = 26) groups. Activity is expressed as nmol carbonyl/mg protein. Values represent mean ± S.D.

Table 1: Clinical characteristics of the control, type 2 diabetes (DM2), hypertension (HYP) and type 2 diabetes/hypertension (DM2/HYP) groups.

	Control (n = 26)	DM 2 (n = 16)	HYP (n = 12)	DM 2/HYP (n = 26)
Gender (Male/Female)	12/14	7/9	6/6	11/15
Age (years)	47.4 ± 6.6	51.3 ± 2.5	52.1 ± 4.1	54.5 ± 3.3
Diabetes duration (years)	–	5.9 ± 0.2	–	8.3 ± 0.6
Hypertension duration (years)	–	–	12.3 ± 0.8	4.5 ± 0.7
Systolic blood pressure (mmHg)	118 ± 2	120 ± 4	145 ± 2*	148 ± 3*
Diastolic blood pressure (mmHg)	76 ± 2	80 ± 4	98 ± 2*	99 ± 6*
Serum glucose (mmol/L)	4.5 ± 0.6	9.7 ± 0.5*	4.7 ± 0.7	9.2 ± 0.8*
Serum cholesterol (mmol/L)	4.7 ± 0.1	5.7 ± 0.2*	5.3 ± 0.2*	5.8 ± 0.3*
Serum HDL-cholesterol (mmol/L)	1.6 ± 0.1	0.8 ± 0.1*	0.9 ± 0.1*	0.9 ± 0.1*
Serum triglycerides (mmol/L)	1.2 ± 0.1	3.1 ± 0.5*	2.9 ± 0.1*	3.3 ± 0.4*
Drugs				
Chlorpropamide	–	n = 8	–	–
Glibenclamide	–	n = 8	–	–
Captopril, propranolol, furosemide	–	–	n = 12	–
Glibenclamide, captopril, furosemide	–	–	–	n = 26

Values represent mean ± S.D. *Different from the others in the same line (ANOVA, Duncan's test, $p < 0.05$).

Table 2: Micronutrients concentrations from control, type 2 diabetic (DM2), hypertensive (HYP) and type 2 diabetic/hypertensive patients (DM2/HYP).

Groups	Copper (mg/L)	Zinc (mg/L)	Magnesium (mg/L)	Calcium (mg/dL)
CONTROL	1.08 ± 0.04	1.45 ± 0.05	17.72 ± 0.1	7.42 ± 0.03
DM2	1.36 ± 0.04*	1.68 ± 0.11*	14.51 ± 0.15*	6.05 ± 0.04*
HYP	1.30 ± 0.01*	1.89 ± 0.14*	15.45 ± 0.16*	6.17 ± 0.08*
DM2/HYP	1.32 ± 0.01*	1.80 ± 0.15*	15.89 ± 0.25*	6.29 ± 0.02*

Values represent mean ± S.D. from individual experiments. *Different from the others in the same column (ANOVA, Duncan's test, $p < 0.05$).

Figure 1

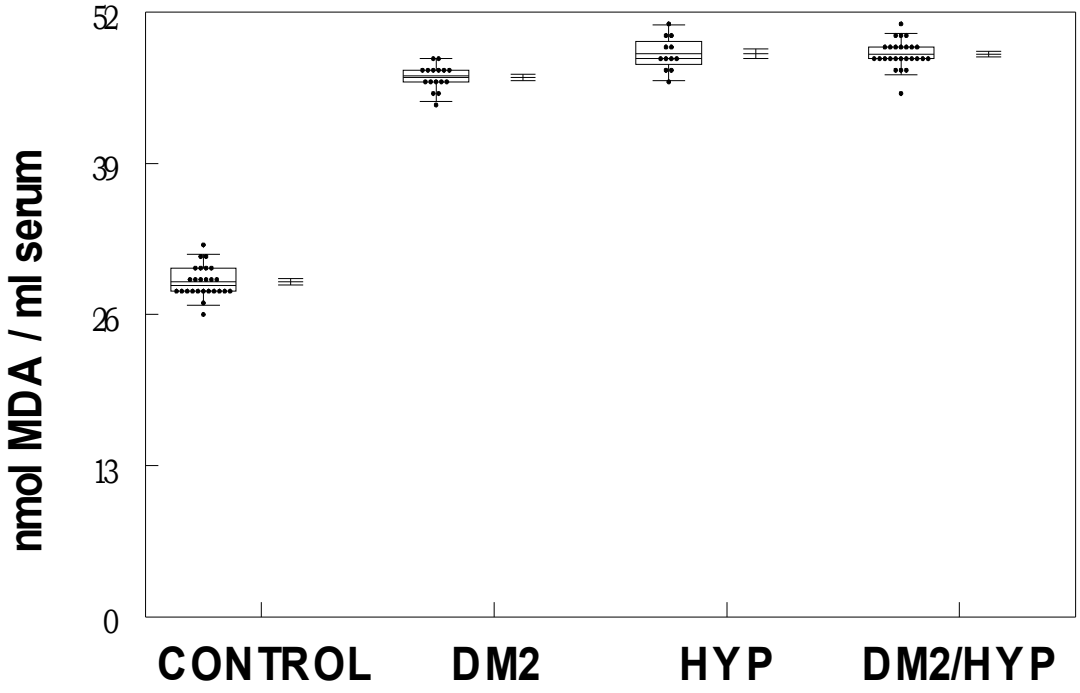


Figure 2

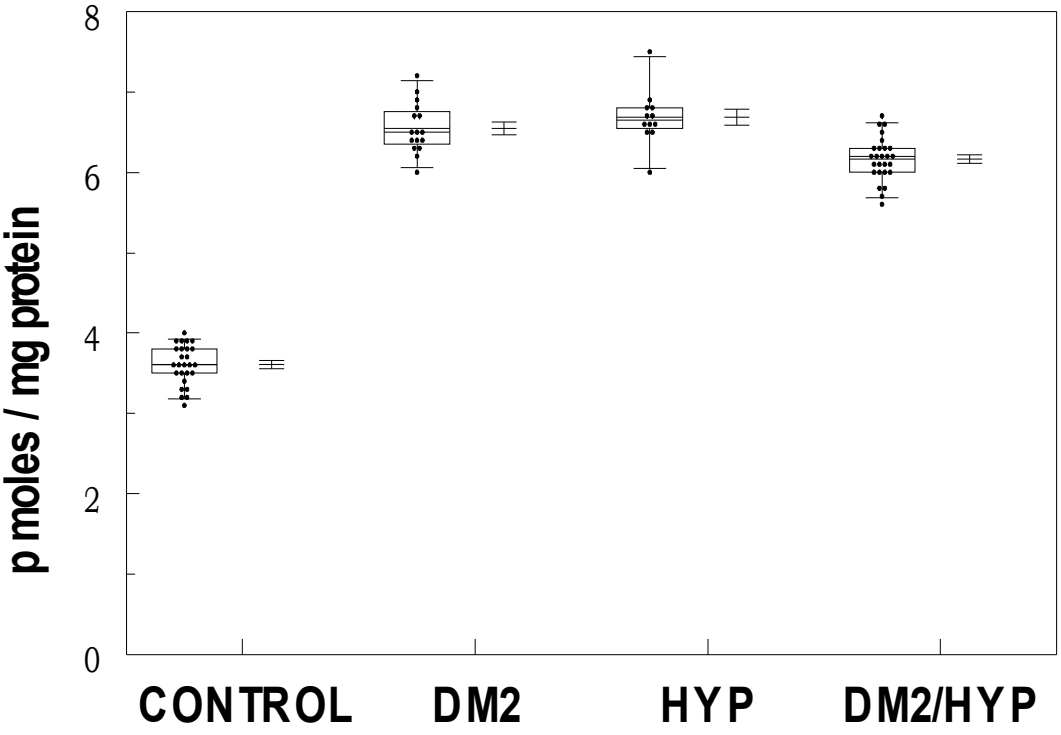


Figure 3

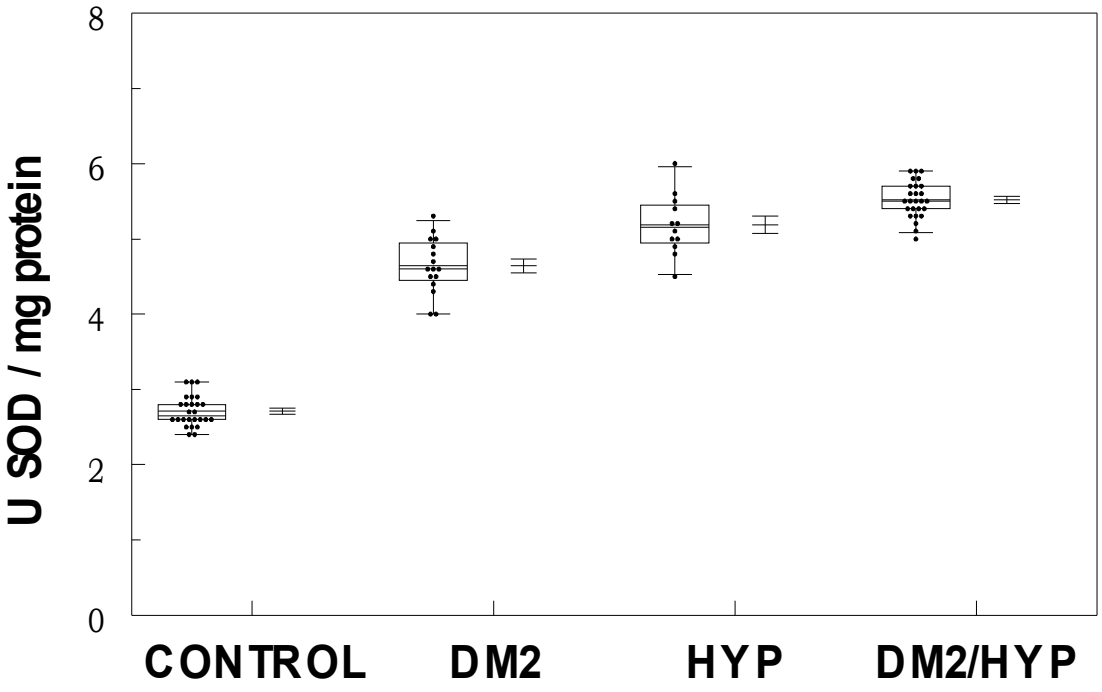


Figure 4

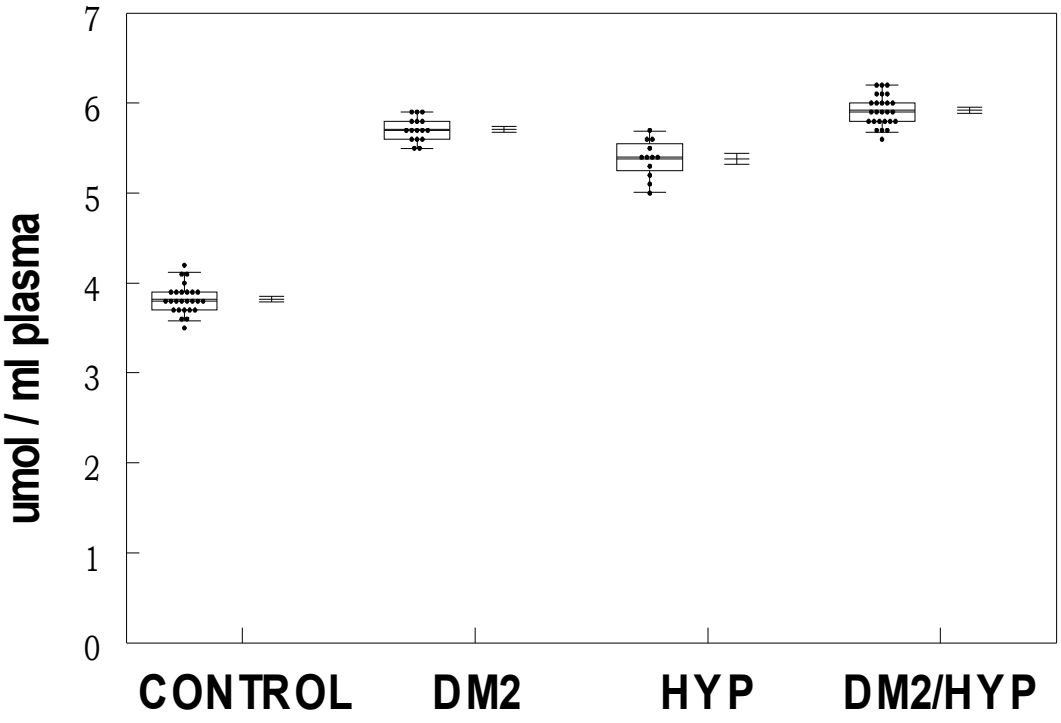
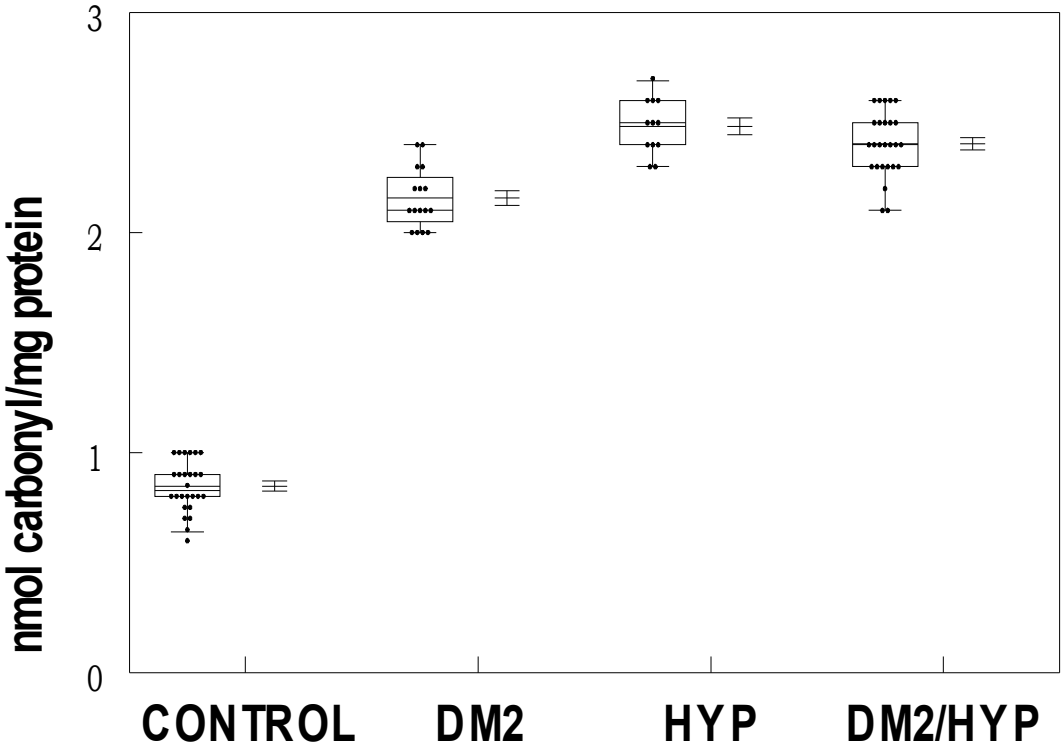


Figure 5



4 DISCUSSÃO DOS RESULTADOS

O Diabetes melito corresponde a um grupo de doenças metabólicas caracterizadas por hiperglicemia, que pode resultar na alteração da secreção de insulina, ação da insulina ou ambas (MINISTÉRIO DA SAÚDE, 2006). A presença de hiperglicemia persistente em pacientes com diabetes está associada a alterações macro e microvasculares (YUAN et al., 2007). As alterações macrovasculares em paciente com diabetes são causa de mortalidade e morbidade, assim como, podem estar associadas com doenças ateroscleróticas obstrutivas (RAHMAN et al., 2007).

A hiperglicemia tem sido indicada como a responsável por induzir efeitos pró-coagulantes e antifibrinolíticos no sangue, que podem contribuir para com um grande risco de trombose arterial (HANSEN et al., 2007). O desenvolvimento de disfunção endotelial, com supressão da síntese de óxido nítrico e prostaciclinas, associado com a hiperglicemia promovem um aumento da ativação plaquetária. As alterações na homeostasia plaquetária criam um risco trombótico que permite o desenvolvimento de doença cardiovascular (GRANT, 2007). A interferência de hiperglicemia/hiperinsulinemia, em pessoas saudáveis, é capaz de induzir um aumento na ativação das plaquetas, promovendo um estado pró-coagulante que pode contribuir para eventos vasculares agudos e aterogênicos (VAIDYULA et al., 2006).

Alterações na atividade da enzima colinesterase no soro têm sido investigadas em diabéticos e um incremento em sua atividade pode estar associado com complicações vasculares (RAGOOBIRSINGH et al., 1992; ABBOTT et al., 1993). Outros estudos têm relatado uma associação entre alterações na atividade da colinesterase sérica com hipertensão e distúrbios encontrados no Diabetes melito (RUSTEMEIJER et al., 2001; ALCANTARA et al., 2002; KÁLMÁN et al., 2004). O aumento na atividade da enzima colinesterase sérica, em pacientes com diabetes e hipertensão associada, sugere que estas doenças poderiam alterar o mecanismo catalítico da enzima. Estes resultados demonstram que diabetes e hipertensão podem ser importantes no aumento na síntese de colinesterase ou liberação pelo tecido hepático. A interferência dos níveis de glicose na atividade da colinesterase

sérica foi demonstrada por uma curva *in vitro*, que indicou um incremento na atividade enzimática proporcional a elevação nas concentrações de glicose. A interferência do metabolismo dos lipídios na atividade da colinesterase sérica foi demonstrada pela positiva correlação entre os níveis de triglicerídios e colesterol total com o aumento na sua atividade enzimática (IWASAKI et al., 2007). Portanto, o perfil enzimático da colinesterase sérica, em pacientes com diabetes, pode constituir um importante fator na etiopatogenia através da influência da resistência à insulina e metabolismo de lipídios (SRIDHAR et al., 2006).

Estudos prévios de nosso grupo já haviam determinado um aumento na atividade das ectonucleotidases em pacientes diabéticos e patologias associadas (LUNKES et al., 2003). Em virtude desses dados, uma curva *in vitro* de glicose e frutose frente às enzimas tromboreguladoras, NTPDase e 5'-nucleotidase, foi realizada em plasma rico em plaquetas de pacientes saudáveis para diabetes e hipertensão. Este estudo determinou que o aumento na atividade das enzimas foi proporcional à elevação na concentração de glicose e frutose. Estes resultados demonstraram que elevadas concentrações de glicose e frutose constituem um relevante fator capaz de modificar a atividade das ectonucleotidases, que são enzimas capazes de modular ativação plaquetária e formação de trombo (MARCUS et al., 2005). Dessa forma, pode-se sugerir que o diabetes *per se* é capaz de promover o aumento na atividade da NTPDase e 5'-nucleotidase. As investigações demonstram que a hiperglicemia e baixo controle glicêmico podem aumentar a agregabilidade das plaquetas (WATALA et al., 2006). Os nossos dados demonstram que a hipertensão associada ao diabetes constitui um importante fator para manutenção do aumento na atividade das ectonucleotidases.

As plaquetas quando expostas a condições hiperosmolar têm apresentado igualmente um aumento da reatividade das plaquetas. Estudos prévios *in vitro* com manitol sugerem que os efeitos osmóticos da glicose constituem um importante mecanismo pelo qual a hiperglicemia pode aumentar a reatividade plaquetária (KEATING et al., 2003). Portanto, a exposição das plaquetas a uma elevação de osmolaridade, aumenta a propensão de agregação plaquetária e em conseqüência promover complicações tromboembólicas. Dessa forma, pode-se sugerir que um aumento de osmolaridade associado hiperglicemia pode aumentar a reatividade plaquetária.

O tempo de pré-incubação não constituiu um fator capaz de interferir na atividade das ectonucleotidases. Esse comportamento enzimático demonstra que a hiperglicemia, mesmo que em processo agudo, é capaz de influenciar os processos de tromboregulação. Assim, a supressão da hiperglicemia transitória constitui uma medida preventiva para diminuir alterações coronárias associadas com quadros de hiper agregabilidade plaquetária (SAKAMOTO et al., 2000). A hiperglicemia pós-prandial tem sido demonstrada como um importante preditor de mortalidade em pacientes com diabetes tipo 2 (DECODE, 1999). Dessa forma, os níveis glicêmicos pós-prandias são de extrema relevância na patogênese das complicações crônicas do diabetes (AFFONSO et al., 2003).

Posteriormente, a análise da expressão da enzima NTPDase (CD39), por citometria de fluxo, demonstrou uma intensa correlação da hidrólise de ATP e ADP frente a pacientes diabéticos e hipertensão associada. Esses dados indicam que o incremento na expressão de CD39 pode interferir em processos de ativação plaquetária. Assim, pode se observar que a proeminente interferência da glicemia na homeostasia das plaquetas e na hidrólise dos nucleotídeos representa um importante parâmetro nos processos de tromboregulação (FRIEDMAN et al., 2007).

O desenvolvimento de disfunção vascular, em diabéticos tipo 2, pode estar associado a uma elevação na geração de espécies reativas de oxigênio (WEIDIG et al., 2004), assim como, em hipertensos (de CHAMPLAIN et al., 2004). Os elevados níveis de TBARS, em pacientes com diabetes e hipertensão associada, indicam que a hiperglicemia está interferindo na peroxidação dos lipídios, que constitui um biomarcador de estresse oxidativo.

O sistema de defesa antioxidante elevado no soro, em pacientes com diabetes e hipertensão associada, pode estar relacionado a um mecanismo compensatório para prevenir o dano oxidativo. A interferência dos níveis de glicose no sistema enzimático antioxidante foi demonstrada por uma curva *in vitro*, onde o aumento na atividade de CAT e SOD foi proporcional ao incremento nas concentrações de glicose. O sistema não-enzimático antioxidante teve uma correlação positiva com os elevados níveis de glicose sangüínea em pacientes com o diabetes e hipertensão associada. Estudos demonstram que a hipertensão aumenta a formação de pró-oxidantes e a sua combinação com diabetes pode exacerbar o estresse oxidativo (BISWAS et al., 2008). Portanto, a manifestação de

diabetes e hipertensão associados pode aumentar a produção de espécies reativas de oxigênio e promover alterações vasculares.

A geração de espécies reativas de oxigênio está fortemente relacionada com ativação das plaquetas em pacientes diabéticos. Portanto, a relação entre os níveis de sistemas oxidantes e antioxidantes em plaquetas é extremamente relevante no balanço da homeostasia de doenças vasculares (KRÖTZ et al., 2004). O incremento na atividade das enzimas catalase e superóxido dismutase e dos níveis de carbonilação de proteínas e grupos tióis não protéicos representam uma resposta nas plaquetas à estimulação pela geração de espécies reativas de oxigênio resultante da hiperglicemia crônica. A presença da hipertensão constitui mais um fator de estimulação dos níveis de estresse oxidativo e a combinação com quadro de hiperglicemia persistente podem estimular um aumento na ativação plaquetária (SUDICA et al., 2006). As baixas concentrações séricas de ácido ascórbico, em pacientes com diabetes e hipertensão associada, aumentam a exposição aos danos oxidativos. Tem sido demonstrado que os baixos níveis de ácido ascórbico podem causar efeitos pró-oxidativos (WILKINSON et al., 1999). A administração dos medicamentos metformina e insulina não comprometeram o comportamento dos sistemas enzimático e não-enzimático antioxidantes de defesa.

As enzimas NTPDase e 5'-nucleotidase participam ativamente no processo de tromboregulação. O diabetes exerceu uma interferência direta na atividade das ectonucleotidases. Neste estudo, pode se sugerir que as elevadas concentrações de glicose modularam as enzimas tromboreguladoras. A presença concomitante de hipertensão, nos pacientes diabéticos, tenha sido um fator de manutenção do aumento da atividade das ectonucleotidases.

5 CONCLUSÃO

a. O incremento na atividade da enzima colinesterase sérica está diretamente relacionado com as concentrações de glicose e com o metabolismo dos lipídios. Há igualmente, uma intensa relação com diabetes e hipertensão, podendo estar associada com as complicações vasculares em pacientes com diabetes e hipertensão associada.

b. O aumento na atividade das enzimas NTPDase-ATP, NTPDase-ADP e 5'-nucleotidase-AMP foi proporcional ao aumento na concentração de glicose e frutose, enquanto que os tempos de pré-incubação não modificaram as respostas enzimáticas, demonstrando que a hiperglicemia pode interferir na atividade enzimática independente do tempo de exposição.

c. O aumento na expressão da enzima NTPDase-ATP e NTPDase-ADP indica que a hidrólise dos nucleotídeos de adenina representam um importante parâmetro no processo de reatividade plaquetária e que a patologia causou o aumento da expressão da enzima.

d. O aumento nos níveis de biomarcadores de dano oxidativo, como TBARS e proteína carbonil, indicam um incremento nos níveis de estresse oxidativo. Em conseqüência, há um estímulo na geração de espécies reativas de oxigênio que desencadearam de forma compensatória um aumento na atividade dos sistemas antioxidantes tanto no sangue como nas plaquetas, com exceção do ácido ascórbico. Essas alterações nos sistemas enzimáticos podem interferir na homeostasia das plaquetas. Os níveis elevados de glicose constituíram um fator com uma correlação direta com a elevação dos níveis de estresse oxidativo. A concentração dos micronutrientes demonstrou uma exposição aos danos oxidativos e uma tentativa de compensação ao aumento na produção de espécies reativas de oxigênio.

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ANEXOS

ANEXO A

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

1. Título

Avaliação do incremento de enzimas que degradam nucleotídeos da adenina, ésteres de colina e de espécies ativas de oxigênio em diabetes e patologias associadas.

2. Objetivos

a. Verificar a interferência de diferentes concentrações de glicose e frutose na atividade das ectonucleotidases em plaquetas de voluntários humanos;
b. Verificar se há uma alteração na expressão da enzima NTPDase em plaquetas de pacientes com diabetes, hipertensão e diabéticos hipertensos;
c. Avaliar os sistemas anti-oxidantes enzimáticos e não-enzimáticos em pacientes com diabetes, hipertensão e diabéticos hipertensos;
d. Verificar a interferência de diferentes concentrações de glicose na atividade enzimática da butirilcolinesterase em pacientes com diabetes, hipertensão e diabéticos hipertensos.
e. Observar a atividade das ectonucleotidases frente extrato bruto de *Wedelia paludosa* em modelo experimental.

3. Registro

O estudo será desenvolvido no Centro de Ciências Naturais e Exatas, Departamento de Química, Programa de Pós-Graduação em Bioquímica Toxicológica, no Laboratório de Enzimologia Toxicológica, da Universidade Federal de Santa Maria. O presente estudo envolverá pacientes diabéticos, hipertensos e diabéticos hipertensos vinculados ao Programa de Assistência aos Pacientes Diabéticos, Hipertensos e Diabéticos-Hipertensos da Secretaria Municipal de Saúde do município de Cruz Alta, RS. Esse estudo com voluntários humanos obteve a aprovação junto a Comissão de Ética do Centro de Ciências da Saúde da Universidade Federal de Santa Maria, com protocolo n° 013/2004.

4. Procedimento

Os pacientes serão submetidos a uma punção venosa com sistema vacutainer. O material biológico, sangue, será destinado para análise de plaquetas, soro e plasma para determinações bioquímicas e de atividade enzimática. As plaquetas serão analisadas no Laboratório de Enzimologia Toxicológica, Centro de Ciências Naturais e Exatas, Departamento de Química, Programa de Pós-Graduação em Bioquímica Toxicológica, da Universidade Federal de Santa Maria, RS.

5. Riscos individuais

Os pacientes que voluntariamente se submeterem as punções venosas, poderão em casos de coleta com procedimento errôneo desenvolver flebite, flebotrombose, hematoma local, petéquias.

6. Identificação do paciente voluntário

Nome:_____

Identidade:_____

Assinatura:_____

7. Cruz Alta, _____ de _____ de 200__.

Anexo B

---------- Mensagem Original ----------
Assunto: Diabetes Research and Clinical Practice Submission Confirmation
De: "CLB (ELS)" <clbi@elsevier.com>
Data: Dezembro 12, 2007
Para: mariaschetinger@gmail.com

Ms. Ref. No.: DIAB-D-07-00782
Title: Effect of high glucose levels in human platelet NTPDase and 5'-nucleotidase activities
Diabetes Research and Clinical Practice

Dear Dr. Schetinger,

Your submission entitled "Effect of high glucose levels in human platelet NTPDase and 5'-nucleotidase activities" assigned the following manuscript number: DIAB-D-07-00782.

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Kind regards,

Abi Robinson
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ANEXO C

Submitting – **Antioxidant status in platelet from patients with diabetes and hypertension.**

---------- Forwarded message ----------
From: **.Molecular and Cellular Biochemistry** <mcb@sbrc.ca>
Date: 21 Jan 2008 16:33:55 -0500
Subject: Acknowledgement of Receipt
To: mariaschetinger@gmail.com

Dear Maria:

Thank you for submitting your manuscript, "Antioxidant status in platelet from patients with diabetes and hypertension", to Molecular and Cellular Biochemistry.

During the review process, you can keep track of the status of your manuscript by accessing the following web site:

http://mcbi.edmgr.com/

Your username is:mariarosa
Your password is:schetinger463

Sincerely,

The Editorial Office
Molecular and Cellular Biochemistry