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CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:
BIOQUÍMICA TOXICOLÓGICA

AVALIAÇÃO DA ATIVIDADE DE ECTONUCLEOTIDASES E ACETILCOLINESTERASE EM RATOS EXPOSTOS À FUMAÇA DE CIGARRO E NICOTINA

TESE DE DOUTORADO

Gustavo Roberto Thomé

Santa Maria, RS, Brasil.

2012

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Gustavo Roberto Thomé

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

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elaborada por
Gustavo Roberto Thomé

Como requisito parcial para obtenção do grau de
Doutor em Ciências Biológicas: Bioquímica Toxicológica

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Prof. Dr. Ricardo Brandão (UFSM)

Santa Maria, 30 de janeiro de 2012.

Essa tese é dedicada a “Nossa Família”, a qual sempre procurou viver em harmonia e muita fé, composta por pessoas que acreditam no trabalho e na sinceridade: o Pai Euclides (*in memorian - 2008*), a Mãe Leni, os filhos: Fernando (*in memorian - 2004*), César e Tânia (esposa), Gustavo e Maríndia (namorada); Netos: Vanessa e Héctor.

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Hoje tenho a absoluta certeza que incorporar sentimentos ruins das pessoas, em nossas próprias vidas, não muda nada como as coisas são. Quando a concorrência está em jogo, o amor a própria vida não significa falta de colocar-se incondicionalmente ao lado do outro, sem qualquer tipo de julgamento quanto à situação que ele está vivenciando, sem nenhum outro sentimento que não seja o de propiciar alívio à situação na qual aquele ser se encontra. Mas, acredito que muitas vezes, isso é deixado de lado na disputa pela vida mesmo que essa pessoa não seja uma pessoa má! O tempo passa e não tenho piedade com os culpados. Entretanto, a generosidade dos sorrisos me fortalece a cada dia, mesmo que o território conquistado jamais seja totalmente meu.

O que se leva da vida é a vida que se leva!

RESUMO

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

AVALIAÇÃO DA ATIVIDADE DE ECTONUCLEOTIDASES E ACETILCOLINESTERASE EM RATOS EXPOSTOS À FUMAÇA DE CIGARRO E NICOTINA
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Local e data da defesa: Santa Maria, 30 de janeiro de 2012.

A fumaça de cigarro é um grave problema de saúde e a mais importante causa de morte evitável no mundo. Possui uma complexa mistura com mais de 4000 constituintes, incluindo a nicotina e compostos que geram espécies reativas de oxigênio (EROs) no organismo. A causa mais comum de sua toxicidade é a aterosclerose e a peroxidação lipídica sendo a nicotina um composto imunossupressor. Entre as alterações celulares causadas pela nicotina, destacam-se os distúrbios na agregação de plaquetas e estas participam da regulação nos processos tromboembólicos, pela liberação de nucleotídeos tais como a adenosina difosfato (ADP). Uma vez exercido os eventos de sinalização os nucleotídeos são degradados pela ação das enzimas NTPDases, 5´-nucleotidase e adenosina desaminase (ADA). Esta cascata enzimática de hidrólise das purinas também está presente nos linfócitos e fornece um grande leque de sinalizações modulando a resposta imune. Outra molécula importante de sinalização é a acetilcolina (ACh) que é rapidamente hidrolisada pela acetilcolinesterase (AChE) e está associada ao processo de cognição. A primeira hipótese a ser testada neste estudo foi investigar a atividade das ectonucleotidases em plaquetas e a ADA no plasma de ratos expostos à fumaça de cigarro por quatro semanas. Os resultados em plaquetas demonstraram um aumento para a hidrólise de adenosina trifosfato (ATP), ADP, adenosina monofosfato (AMP) e adenosina. Esses resultados sugerem uma resposta orgânica compensatória com objetivo de manter os níveis de adenosina, um potente inibidor da agregação plaquetária e importante modulador do tônus vascular. A segunda hipótese foi realizar estudos *in vivo e in vitro* da hidrólise de nucleotídeos e nucleosídeos em linfócitos de ratos submetidos a exposição à nicotina *per se*. Os resultados *in vivo* de linfócitos demonstraram um decréscimo da hidrólise de ATP, ADP e adenosina nas concentrações de nicotina de 0,25 e 1,0 mg/kg. A expressão da proteína NTPDase e a contagem de linfócitos em ratos também foram diminuídas pela nicotina. A determinação dos níveis de nucleotídeos e nucleosídeos no soro de ratos tratados com nicotina na dose de 0,25 mg/kg aumentou para o ATP (39%), o ADP (39%) e a adenosina (303%). Para o estudo *in vitro* a hidrólise de ATP-ADP-adenosina foi diminuída pelas concentraçoes de nicotina (1 mM, 5 mM, 10 mM e 50 mM). Estes resultados sugerem que alterações na atividade e expressão destas enzimas nos linfócitos pode contribuir para a compreensão dos mecanismos envolvendo a supressão da resposta imune causada pela nicotina. Por fim, a terceira hipótese, foi investigar a atividade da AChE e o nível de peroxidação lipídica no estriado (ES), no córtex cerebral (CC), no hipocampo (HC) e no cerebelo (CE) de ratos expostos à fumaça de cigarro e tratados com vitamina E (50 mg/kg/dia) por quatro semanas. Os resultados demonstraram um aumento na atividade da AChE e no nível de peroxidação lipídica no ES, no CC e no CE, sugerindo que este tipo de exposição pode afetar a funcionalidade do sistema colinérgico e aumentar os danos oxidativos no sistema nervoso central (SNC). Em adição, a Vitamina E foi capaz de reverter estes efeitos, o que sugere uma boa escolha de terapia com este antioxidante neste tipo de exposição. Alem disso, este estudo também pode sugerir que a fumaça de cigarro e a nicotina modulam o sistema purinérgico de plaquetas e linfócitos, o que pode levar a propensão de doenças tromboembólicas e imunossupressoras, com a mobilização das defesas do organismo para uma resposta compensatória.

Palavras-chave: Fumaça de Cigarro. Nicotina. Ectonucleotidases. Acetilcolinesterase. Vitamina E. Peroxidação Lipídica.

ABSTRACT

Doctoral Thesis
Graduate Program in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

EVALUATION OF THE ACTIVITY OF ECTONUCLEOTIDASES AND ACETYLCHOLINESTERASE IN RATS EXPOSED TO CIGARETTE SMOKE AND NICOTINE

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Place and date of defense: Santa Maria, 30 of january of 2012.

Cigarette smoke is a serious health problem and the most important cause of avoidable death in the world. It has a complex mixture with more than 4700 constituents, including nicotine and reactive oxygen species (ROS). The most common cause of its toxicity is the atherosclerosis and lipid peroxidation being nicotine an immunosuppressant compound. Among the major cellular changes promoted by nicotine is the disorder in the aggregation of platelets, which participate in the regulation of thromboembolic processes releasing nucleotides such as adenosine diphosphate (ADP). Once exerted the signaling events the nucleotides are degraded by the action of the enzymes NTPDase, 5´-nucleotidase and adenosine deaminase (ADA). This purinergic cascade of enzymatic hydrolysis is also present in lymphocytes and supplies a great amount of signaling modulating the immune system. Another important signaling molecule is acetylcholine (ACh) which is quicklys hydrolyzed by acetylcholinesterase (AChE) and is associated with the cognition process. The first hypothesis to be tested in this study was to investigate the activity of ectonucleotidases in platelets and the ADA from plasma of rats exposed to cigarette smoke during four weeks. The results in platelets demonstrated an increase for adenosine triphosphate (ATP), ADP, adenosine monophosphate (AMP) hydrolysis and adenosine deamination. These results suggest a compensatory organic response to regulate the evels of adenosine, a powerful inhibitor of platelet aggregation and important modulator of vascular tone. The second hypothesis was studied in vivo and in vitro the hydrolysis of nucleotides and nucleosides in lymphocytes of rats submitted to nicotine exposure per se. The *in vivo* results of lymphocytes demonstrated a decrease in the hydrolysis of ATP, ADP and adenosine in the concentrations of 0.25 and 1.0 mg/kg of nicotine. The expression of NTPDase protein and the counting of lymphocytes in rats were also diminished after nicotine exposure. The quantification of nucleotides and nucleoside in serum of rats treated with nicotine in the dose of 0.25 mg/kg showed an increase in the ATP (39%), ADP (39%) and adenosine (303%) levels. For the in vitro study the ATP-ADP-adenosine hydrolysis were diminished by nicotine in the nicotine concentrations tested (1 mM, 5 mM, 10 mM and 50 mM). These results suggest that alterations in the activity and expression of these enzymes in lymphocytes can contribute for the understanding of the mechanisms involving the suppression of the immune system caused by nicotine. Finally, the third hypothesis was to investigate the activity of AChE and the level of lipid peroxidation in striatum, cerebral cortex, hippocampus, and cerebellum of rats exposed to cigarette smoke and treated with vitamin E (50 mg/kg/day) during four weeks. The results had demonstrated an increase in the activity of AChE and in the levels of lipid peroxidation in the striatum, cerebral cortex and cerebellum, suggesting that this type of exposition can affect the functionality of the cholinergic system and increase the oxidative damage in the central nervous system (SNC). In addition, vitamin E was capable to reverse these increases, suggesting the use of this antioxidant compound in this type of exposition. Moreover, this study also suggests that cigarette smoke and nicotine modulate the purinergic system of platelets and lymphocytes, which may lead the propensity of thromboembolic and immunosuppressant illnesses, with mobilization of the phisiological defenses for a compensatory response.

Keywords: Cigarette Smoke. Nicotine. Ectonucleotidases. Acetylcholinesterase. Vitamin E. Lipid Peroxidation.

LISTA DE TABELAS

ARTIGO 1

ARTIGO 2

ARTIGO 3

LISTA DE ILUSTRAÇÕES

INTRODUÇÃO

ARTIGO 1

ARTIGO 2

ARTIGO 3

LISTA DE ABREVIATURAS

ACh - Acetilcolina

AChE - Acetilcolinesterase

ACRS - Regiões Conservadas da Apirase

ADA - Adenosina Desaminase

ADP - Adenosina Difosfato

AFT - Ambiente com Fumaça de Tabaco

AMP - Adenosina Monofosfato

ATP - Adenosina Trifosfato

EROs - Espécies Reativas de Oxigênio

ERNs - Espécies Reativas de Nitrogênio

FCDE - Fumaça de Cigarro Diluída e Envelhecida

HbCo - Carboxihemoglobina

MDA - Malondialdeído

nAChRs - Receptores de acetilcolina nicotínico

OMS - Organização Mundial da Saúde

pCO_2 - Pressão Parcial de Dióxido de Carbono

PRP - Plasma Rico em Plaquetas

RL - Radical Livre

SO_2 - Saturação de Oxigênio

TBA - Ácido Tiobarbitúrico

TBARS - Substâncias Reativas ao Ácido Tiobarbitúrico

TCA - Ácido Tricloroacético

tCO_2 - Dióxido de Carbono total

SUMÁRIO

APRESENTAÇÃO

Os resultados dessa tese estão apresentados sob a forma de artigos os quais encontram-se no item "**ARTIGOS**". As seções Materiais e Métodos, Resultados, Discussões e Referências Bibliográficas encontram-se nos próprios artigos e representam a íntegra desde estudo. Os itens **DISCUSSÃO** e **CONCLUSÕES**, encontrados no final desta tese, apresentam interpretações e comentários gerais a respeito dos resultados demonstrados nos artigos contidos neste trabalho. As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem no item **INTRODUÇÃO** e **DISCUSSÃO** desta tese.

1 INTRODUÇÃO

O tabagismo é um grave problema de saúde e a mais importante causa de morte evitável do mundo (JHA et al., 2008). O tabagismo tem sido fortemente implicado como um fator de risco para a doença pulmonar obstrutiva crônica, câncer e aterosclerose entre outras (GUPTA et al., 1997; RANI et al., 2003). A Organização Mundial da Saúde (OMS) estima que as mortes por tabaco somente na Índia podem superar 1,5 milhões por ano até 2020 (RANI et al., 2003). Hoje, pelo menos 15% de todos os principais problemas de saúde são atribuídos ao tabagismo (RANI et al., 2003; PASUPATHI et al., 2009).

Ainda de acordo com a OMS, a mortalidade causada pelo uso do tabaco é de aproximadamente 4,9 milhões de óbitos anuais em todo o mundo, ou seja, 10 mil falecimentos por dia. Se o atual padrão de consumo do tabaco não for revertido, o número de mortes poderá alcançar a cifra de 10 milhões em 2020, sendo que a metade ocorrerá em indivíduos em idade produtiva, na faixa etária de 35 a 69 anos (JÚNIOR et al., 2007).

No território nacional, as mortes causadas pelo uso do tabaco correspondem a 200 mil a cada ano (JÚNIOR et al., 2007). No Brasil, apesar do conhecimento sobre o tabagismo como fator de risco para várias doenças e a existência de política para o seu controle como uma legislação que proíbe o ato de fumar em local fechado, ainda não é o suficiente para combatê-lo (CAVALCANTE, 2005).

O consumo anual de cigarros por adultos no mundo aumentou da década de 1970 para 1980, estabilizando-se na década de 90 (THE WORD BANK AND WORD HEALTH ORGANIZATION, 2000). No Brasil, a prevalência do tabagismo caiu de 35% para 18% no período de 1989 a 2003, sendo ainda mais reduzida para 16% em 2006 (MINISTÉRIO DA SAÚDE, 2007). O uso de outros produtos do tabaco no Brasil é algo que ocorre entre as pessoas mais velhas (maiores de 45 anos de idade), com menor grau de instrução, e entre habitantes de pequenas áreas urbanas (MINISTÉRIO DA SAÚDE 2006, 2007).

As estratégias das grandes indústrias de cigarro superam as campanhas contra o tabagismo fazendo com que haja cada vez mais fumantes, em especial entre os jovens e mulheres (CAVALCANTE, 2005). No Brasil, 30 milhões de

indivíduos com 15 ou mais anos de idade são fumantes regulares. De acordo com os dados do Instituto Nacional do Câncer (INSTITUTO NACIONAL DO CÂNCER, 2011), cerca de 370 mil jovens entre 10 e 14 anos e 2,3 milhões entre 15 e 18 anos, fumam com regularidade. Um estudo populacional realizado em uma cidade do sul do Brasil revelou que há uma idade precoce para o início do tabagismo em que 55% dos adolescentes começam a fumar entre 13 e 15 anos. Dos adolescentes e jovens que se iniciaram no tabagismo, 90% tornaram-se “nicotino-dependentes” ao atingir 19 anos (MALCON et al., 2003).

O cigarro é constituído principalmente pela folha seca da planta *Nicotiana tabacum* (Figura 1). A folha do tabaco contém uma complexa mistura de componentes químicos: hidrocarbonetos, fenóis, ácidos graxos, isopropenos, ésteres e minerais inorgânicos.

Figura 1 – Planta *Nicotiana tabacum*.

Foto tirada em 04/12/2011 na cidade de Restinga Seca – RS.

A combustão da folha de tabaco e seus aditivos formam à fumaça de cigarro que é uma mistura heterogênea de gases, vapores e partículas líquidas (BATISTA,

1980; FISHER, 1999; NORMAN, 1999). Quando a fumaça de cigarro é tragada, ou seja, no momento de inalação da fumaça, entra para as vias aéreas respiratórias um aerossol tóxico concentrado com milhões de partículas por centímetro cúbico. O tamanho médio das partículas é de aproximadamente 0,5 µm. Para fins de estudos da composição química e propriedades biológicas, a fumaça é separada em fase particulada e fase gasosa. A análise da composição química é realizada em máquinas de fumar, nas quais um grande número de cigarros é aspirado simultaneamente, de forma que simule os hábitos de fumantes (BATISTA, 1980; FISHER, 1999; NORMAN, 1999). A toxicidade da fumaça de cigarro é determinada pelo conjunto complexo das características do produto, incluindo a mistura do tabaco, aditivos e a estrutura do mesmo demonstrada na figura 2 (KOZLOWSKI et al., 2001).

A fumaça do cigarro é um aerossol complexo, produzido pela destilação e combustão dos componentes do tabaco e no qual foram identificadas cerca de 4700 substâncias químicas (DUBE & GREE, 1982; CHURG & PRIOR, 1985). Aproximadamente 10% desta fumaça é constituída pela fase particulada, composta principalmente de alcatrão, água e nicotina e os 90% restantes constituem a fase gasosa, que contém monóxido de carbono, dióxido de carbono, óxidos de nitrogênio e cianetos (ARMITAGE et al., 1975; ABEL, 1980; NASH & PERSAUD, 1988; BYRD, 1992). O alcatrão contém toda a fase particulada da fumaça, assim como os componentes condensáveis da fase gasosa. A quantidade média de alcatrão da fumaça de cigarro é 20 mg, variando essa quantidade de acordo com as condições de queima e de condensação, tamanho do cigarro, uso ou não de filtro, porosidade do papel e filtro, peso e tipo de tabaco (BATISTA, 1980; BORGERDING & KLUS, 2005).

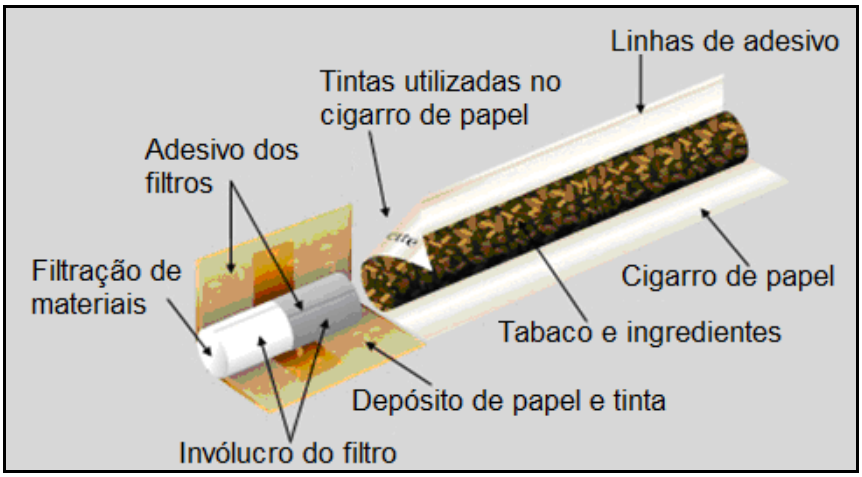

Figura 2 – Estrutura do cigarro de papel.

Adaptado de PHILIP MORRIS INTERNATIONAL (2009).

A fumaça de cigarro, gerada durante a aspiração, possui dois fluxos, os quais são denominados de via (corrente) principal (central) e via secundária (periférica, lateral) de fumaça. A fumaça que sai diretamente do cigarro via boca do tabagista e inalada para o sistema respiratório, período da “tragada”, é chamada “via principal da fumaça de cigarro”. É produzida em altas temperaturas (acima de 950ºC), e polui o ambiente após ter sido aspirada através do cigarro, entrado em contato com os pulmões e, em seguida, exalada (WEISS et al., 1983; SCHERER et al., 1990). Constitui a fonte predominante de exposição dos indivíduos “fumantes ativos”. Alguns dos componentes do tabaco são estáveis o suficiente para sofrer destilação e não se modificar, porém outros sofrem várias reações envolvendo oxidação, desidrogenação, quebra, rearranjo e condensação (BATISTA, 1980; BORGERDING & KLUS, 2005).

Já a “via secundária da fumaça de cigarro” é, sobretudo, a fumaça emergente do ambiente a partir do cigarro aceso e inalada através do nariz. Esta fumaça é produzida em temperaturas mais baixas (cerca de 350ºC), durante a queima lenta do cigarro que ocorre entre as tragadas. Oitenta e cinco por cento da fumaça de cigarro presente no ambiente é resultante da fumaça periférica produzida a partir da queima espontânea da extremidade do cigarro. Fumaça via secundária difere da fumaça via principal inalada pelo tabagista ativo pelo fato de não ser filtrada e ter diferenças quantitativas, sendo que a composição química da fumaça via secundária é similar à da fumaça principal (WEISS et al., 1983; SOPORI & KOZAK, 1998). A fumaça via

secundária apresenta concentrações de alcatrão, nicotina, monóxido de carbono, dióxido de carbono, benzopireno, e substâncias cancerígenas duas a dez vezes mais elevadas que a fumaça via principal, muito embora o fumante passivo inale uma concentração menor destas substâncias devido à diluição que ocorre no ar ambiente. A fumaça da via secundária é a fonte de exposição de todos aqueles que inalam a fumaça presente no meio ambiente, os chamados “fumantes passivos” (LAW & HACKSHAW, 1996).

A fumaça exalada da via principal e a fumaça da via secundária difusa para a atmosfera tornam-se diluídas pelo ambiente do ar e, após várias alterações físicas e químicas, incluindo reações com substâncias químicas não geradas pelo tabaco, tornam-se ambiente com fumaça de tabaco (AFT). Antes de ser exalada do trato respiratório a fumaça da via principal é filtrada, a qual contribui entre 15% a 43% das partículas do AFT e entre 1% a 13% da fase de vapor, sendo o restante proveniente da fumaça da via secundária (BAKER & ROBINSON, 1990; BAKER & PROCTOR, 1990; EATOUGH et al., 1990; GUERIN & JENKINS, 1992; BORGERDING & KLUS, 2005).

O grande número e variedade de componentes na fumaça de cigarro são oriundos da composição do alcatrão, formado na carbonização da brasa, os quais em alguns casos podem acontecer em temperaturas menores que aquelas da queima do cigarro. Cerca de 5.000 (cinco mil) componentes diferentes já foram identificados tanto na fase particulada como na fase gasosa da fumaça. Em estudos com cigarros regulares (70mm comprimento, 1g peso) sem filtro, produz-se de 17 a 40 mg de alcatrão por cigarro (BATISTA, 1980). Em outra investigação com 174.000 cigarros regulares americanos, foram encontrados 4 kg de alcatrão, em média 23 mg, além de 1,04 mg de nicotina por cigarro (BATISTA, 1980; FISHER, 1999; NORMAN, 1999; BORGERDING & KLUS, 2005).

Algumas substâncias da fumaça de cigarro servem como indicadores de exposição em sistemas que mimetizam a exposição passiva ao tabaco. Os marcadores de exposição ao tabagismo permitem uma avaliação objetiva do grau de tabagismo e uma estimativa da quantidade inalada de fumaça de cigarro (BARLOW et al., 1987). Os marcadores mais utilizados são: percentual de carboxihemoglobina no sangue; monóxido de carbono no ar expirado; as medidas das concentrações de tiocianato, nicotina e cotinina no plasma, saliva e urina (HAUFROID & LISON, 1998). A nicotina apresenta meia vida curta (cerca de 1 a 2 horas), o que dificulta a

detecção desta substância nos fluidos corporais para diagnóstico e quantificação da exposição tabágica, tanto ativa quanto passiva (FEYERABEND et al., 1986). Por ser mais polar e menos lipofílica que a nicotina, a cotinina apresenta volume de distribuição relativamente restrito em comparação à nicotina (BENOWITZ, 1983). Devido à baixa taxa de metabolismo e excreção renal, sua meia-vida na circulação é 10 vezes mais longa - 19 a 40 horas, (comparado com 30 a 110 minutos da nicotina) e sua concentração é relativamente estável durante todo o dia de um tabagista (BENOWITZ, 1983; ETZEL et al., 1985; JACOB et al., 1988). Assim, por estar presente no sangue em maior concentração e por mais tempo que a nicotina (LANGONNE et al., 1973), e ainda, por ser específica para a exposição ao tabaco (BENOWITZ, 1983), e possuir distribuição nos fluidos corporais mais restrita, a cotinina tem sido o marcador mais utilizado para avaliar o consumo de tabaco, bem como para controle de sua abstinência.

Tanto o fumante ativo quanto o fumante passivo podem sofrer com as ações tóxicas da exposição à fumaça de cigarro e o efeito acumulativo desta intoxicação é devido às pessoas passarem 80% de seu tempo em locais fechados como no trabalho, nas residências ou em locais de lazer, onde há grande risco de exposição excessiva (Fundação Faculdade Federal de Ciências Médicas de Porto Alegre, 2007). As principais manifestações clínicas em fumantes passivos adultos são sintomas respiratórios, exacerbação de efeitos irritativos em pacientes alérgicos e um aumento da taxa de mortalidade por doenças cardiovasculares, além de câncer de pulmão, esôfago e encéfalo (MINISTÉRIO DA SAÚDE, 2008).

Na queima dos cigarros são liberadas centenas de substâncias com propriedades irritantes e várias são carcinogênicas em humanos e animais. A fumaça de cigarro contém carcinógenos que incluem hidrocarbonetos aromáticos policíclicos, aminas heterocíclicas e N-nitrosaminas (HOFFMANN & HOFFMANN, 1997; HOOKER et al., 2007). Além disso, esta fumaça contém uma complexa mistura de mais de 4700 substâncias incluindo a nicotina, aldeídos reativos, cádmio, além de compostos que induzem a produção de espécies reativas de oxigênio (EROs) e de nitrogênio (ERNs), conhecidas genericamente como radicais livres (RL), os quais são capazes de iniciar ou promover dano oxidativo (RAHMAN et al., 1996; PANDA et al., 1999; GENBACEV-KRTOLICA, 2005; LUCHESE et al., 2007). As principais EROs distribuem-se em dois grupos, os radicalares: hidroxila ($HO^{\bullet}$), superóxido ($O2^{\bullet -}$), peroxila ($ROO^{\bullet}$) e alcoxila ($RO^{\bullet}$); e os não-radicalares: oxigênio,

peróxido de hidrogênio e ácido hipocloroso. Dentre as ERNs incluem-se o óxido nítrico ($NO^{\bullet}$), óxido nitroso (N_2O_3), ácido nitroso (HNO_2), nitritos (NO_2^-), nitratos (NO_3^-) e peroxinitritos ($ONOO^-$) (DRÖGE, 2002).

Estudos demonstraram que a fumaça de cigarro pode alterar diversos sistemas biológicos como, por exemplo, o tecido encefálico e deste modo causar dano irreparável no organismo, como o aumento da peroxidação lipídica (ANBARASI et al., 2005a, 2005b; MANNA, et al., 2006). Os lipídeos da membrana do encéfalo contêm altos níveis de ácidos graxos poliinsaturados e, portanto são particularmente sensíveis à oxidação (VAJRAGUPTA et al., 2000; SCHOLPP et al., 2004). A membrana celular é o alvo mais atingido em decorrência da peroxidação lipídica (lipoperoxidação), que culmina na formação de malondialdeído (MDA), hidroxinonenal (HNE) e isoprostanos (VASCONCELOS et al., 2007; LEOPOLD & LOSCALZO, 2009). Consequentemente ocorre liberação do conteúdo de organelas, entre outros eventos, culminando com a morte celular (FERREIRA & MATSUBARA, 1997).

A peroxidação lipídica causada pelos radicais livres no organismo é combatida por antioxidantes produzidos pelo corpo ou absorvidos da dieta. Antioxidante é qualquer substância que, quando presente em baixa concentração comparada à do substrato oxidável, regenera o substrato ou previne significativamente a oxidação do mesmo (HALLIWELL, 2007). As proteções conhecidas do organismo contra as EROs e ERNs abrangem a proteção enzimática ou por macromoléculas, que podem ter origem no próprio organismo ou são adquiridas através da dieta. São conhecidos três sistemas enzimáticos antioxidantes: o primeiro é composto por dois tipos de enzimas SOD, que catalisam a destruição do radical ânion superóxido ($O_2^{\bullet -}$), convertendo-o em oxigênio e peróxido de hidrogênio. O segundo é a catalase (CAT) que atua na dismutação do peróxido de hidrogênio (H_2O_2) em oxigênio e água. O terceiro sistema é composto pela glutationa (GSH) em conjunto com duas enzimas Se-glutationa peroxidase (GPx) e glutationa redutase (GR). Esse sistema também catalisa a dismutação do peróxido de hidrogênio em água e oxigênio, sendo que a glutationa opera em ciclos entre sua forma oxidada e sua forma reduzida (BABIOR & BRAZ, 1997; BARREIROS & DAVID, 2006). A GSH reduz o H_2O_2 a H_2O em presença de GPx, formando uma ponte dissulfeto e, em seguida, a GSH é regenerada.

Entre os antioxidantes biológicos de baixo peso molecular podem ser destacados os carotenóides (vitamina A), a bilirrubina, a ubiquinona e o ácido úrico. Porém, as mais importantes macromoléculas no combate ao estresse oxidativo são os tocoferóis (vitamina E) e o ácido ascórbico (vitamina C) (BABIOR & BRAZ, 1997; BARREIROS & DAVID, 2006). A vitamina C, na sua forma ascorbato no organismo, é solúvel em água e está localizado em compartimentos aquosos dos tecidos orgânicos (MAYNE, 2003). O ascorbato desempenha papéis metabólicos fundamentais no organismo humano, atuando como agente redutor, reduzindo metais de transição (em particular Fe^{3+} e Cu^{2+}) presentes nos sítios ativos das enzimas ou nas formas livres no organismo (MAYNE, 2003). Por ser um bom agente redutor, o ascorbato pode ser oxidado pela maioria das EROs e ERNs que chegam ou são formadas nos compartimentos aquosos dos tecidos orgânicos (BENZIE & STRAIN, 1999).

A vitamina E é constituída principalmente por quatro tocoferóis, e secundariamente por quatro tocotrienóis, sendo o α-tocoferol o mais ativo (MACHLIN & BENDICH, 1987; MAYNE, 2003). No entanto a vitamina E é um eficiente inibidor da peroxidação de lipídios *in vivo*. Estas substâncias agem como doadores de H para o radical peroxila, interrompendo a reação radicalar em cadeia. Cada tocoferol pode reagir com até dois radicais peroxila e, neste caso, o tocoferol é irreversivelmente desativado. Para que eles não se desativem, necessitam do mecanismo de regeneração sinergético com o ascorbato nas membranas celulares e com a ubiquinona na membrana mitocondrial (BARREIROS & DAVID, 2006). A deficiência de vitamina E é uma das características da patogênese de várias doenças associadas à fumaça de cigarro, com aumento dos marcadores inflamatórios em todas as áreas do encéfalo (MANNA et al., 2006; REITER et al., 2007), além de aumentar a viscosidade das plaquetas do sangue, predispondo à formação de coágulos potencialmente fatais (STAMPFER & RIMM, 1995; BONITHON-KOOP et al., 1997). No entanto, em ambientes com fumaça de cigarro está contida a nicotina, um alcalóide natural encontrado nos membros das famílias de plantas solanácea tais como: o tomate, a batata, o pimentão verde e o tabaco (DOOLITLE et al. 1995). É a principal substância que causa dependência na fumaça de cigarro e possui um centro quimicamente ativo e ocorre como isômero estéreo com estrutura [1-metil-2-(3-piridil-pirrolidina),C10H14N2] (Figura 3) (DANI &

HEINEMANN, 1996; DI CHIARA, 2000; KARAN et al., 2003; BALFOUR, 2004; Di CHIARA et al., 2004; YILDIZ, 2004; DANI & HARRIS, 2005).

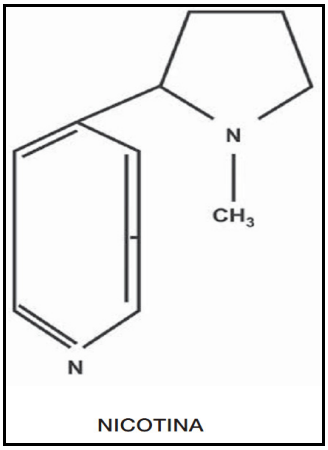

Figura 3 – A estrutura da nicotina.

Adaptado ROSEMBERG (2005).

Muitas questões importantes sobre o efeito da nicotina no encéfalo ainda permanecem desconhecidas. A migração adequada de neurotransmissores e a localização de populações de células específicas dentro de cada região encefálica faz parte do desenvolvimento normal do sistema nervoso central (SNC) (SLOTKIN, 2004). Porém, a atividade neural pode ficar vulnerável devido a agentes químicos, que podem diminuir as respostas ou bloquear neurotransmissores, cuja extensão de alterações pode alcançar todas as fases de desenvolvimento do encéfalo, desde o estágio embrionário inicial até a adolescência (SLOTKIN, 2004). A exposição da nicotina no período pré-natal, ou no ambiente com fumaça de cigarro podem ser os meios de perturbação trófica na sinalização de neurotransmissores (SLOTKIN, 2004) nesta fase de desenvolvimento. A nicotina produz uma variedade de efeitos complexos sobre algumas regiões do encéfalo que são essenciais para a manifestação do comportamento de dependência (DANI et al., 2001; DANI & HARRIS, 2005; HYMAN, 2006). Este alcalóide é um dos componentes chaves que causa neuroteratogênese com efeitos adversos no número de células do encéfalo, no desenvolvimento sináptico e na função neurocomportamental (LEVIN & SLOTKIN, 1998; SLOTKIN, 1998, 1999; MANNA et al., 2006). Os efeitos danosos no SNC são causados por fumantes ativos e seguramente persistem por meio da

exposição passiva à fumaça de cigarro, promovendo alterações na atividade sináptica, entre elas a colinérgica (ABREU-VILLAÇA et al., 2003a, 2003b; SLOTKIN, 2004). A nicotina sozinha é um protótipo agonista do receptor de acetilcolina nicotínico (nAChR) que pode aumentar a cognição pelos seus efeitos diretos sobre a atenção e pela interação com os nAChR pré-sinápticos para facilitar a liberação de acetilcolina (ACh), glutamato, dopamina, norepinefrina, serotonina e ácido δ-aminobutírico, neurotransmissores implicados com os processos de aprendizado e memória (SAMUELS & DAVIS 1998).

As alterações promovidas pela nicotina também se estendem a outras células do sangue tais como os linfócitos. Estudos demonstraram que a nicotina pode danificar o DNA de linfócitos do sangue periférico de ratos *in vitro* (SUDHEER et al., 2007), inibir a expressão de moléculas de adesão, a produção de citocinas e a proliferação de linfócitos (TAKAHASHI et al., 2007). Não somente fumantes ativos, mas também não fumantes e ex-fumantes expostos à fumaça de cigarro em ambiente confinado demonstraram aumento significativo de danos ao DNA dos linfócitos, afetando assim as funções desta célula (FRACASSO et al., 2006).

A fumaça de cigarro e a nicotina também podem acelerar o risco de hipertensão sanguínea contribuindo para a vasoconstrição ou nas interações entre plaquetas e o revestimento de células endoteliais (DOWNEY et al., 1981; GREEN et al., 1986), além de apresentar efeitos trombogênicos por sua interação com as plaquetas. Em fumantes, a agregabilidade das plaquetas aumenta fortemente, e numa exposição de longo tempo à fumaça de cigarro pode aumentar a sensibilidade das plaquetas pela epinefrina ou ADP (FUSEGAWA & HANDA, 2000). Devido a esse fato, inúmeros estudos indicam que a fumaça de cigarro é um dos fatores de risco que leva às doenças ateroscleróticas, particularmente em pacientes com hipertensão, hiperlipidemia e doenças cardíacas (FUSEGAWA et al., 1999; FUSEGAWA & HANDA, 2000). Pesquisas são ainda conduzidas para verificar qual a influência da nicotina sobre as plaquetas. Entretanto, estudos *in vitro* e alguns estudos com “gomas de mascar” e adesivos trans-dérmicos de nicotina não mostraram evidência da ativação de plaquetas (BENOWITZ et al., 1993; MUNDAL et al., 1995). É postulado que o estreito lúmen arterial, promovido pela ação da nicotina, pode causar um fluxo turbulento que danifica as células vermelhas do sangue e plaquetas, levando a liberação de ADP, o qual promove agregação de plaquetas (PFUELLER et al., 1988). A exposição aguda à fumaça de cigarro

aumenta os níveis de epinefrina do plasma e induz à reação de liberação de plaquetas em indivíduos hipertensos, o que exibe determinado aumento no grau de hiper-reatividade de plaquetas (MUNDAL et al., 1998). Posteriormente, foi demonstrado que a administração intravenosa de nicotina promove aumento de catecolaminas no plasma pela direta estimulação à liberação de epinefrina da medula adrenal, e esta por sua vez, estimula a agregação plaquetária e formação de trombo pelo mecanismo alfa-adrenérgico (KILARU et al., 2001). Além disso, foi relatado que a agregação espontânea de plaquetas continua aumentada após 10 horas da abstinência do fumo em fumantes habituais, e esta cronicidade de agregabilidade plaquetária pode ter efeito sobre o aparecimento e progressão das desordens aterosclerótica e trombogênica (FUSEGAWA et al., 1999).

As plaquetas são fragmentos citoplasmáticos derivados de megacariócitos, envolvidos por membranas e destituídas de núcleo (Figura 4). Nas plaquetas se reconhecem três zonas: (1) zona externa ou periférica que condiciona a propriedade de adesão, nessa porção encontra-se antígenos, glicoproteínas e vários tipos de enzimas, mais internamente existe a membrana plaquetária, onde estão localizadas glicoproteínas que são receptores específicos para determinados fatores de coagulação; (2) Uma zona sol-gel ou citosol com proteínas actinomiosina e tubulina, formando microtúbulos e microfilamentos, responsáveis pelo esqueleto da plaqueta; (3) Uma zona de organelas contendo corpúsculos densos (Ca^{2+}, ADP, ATP, Serotonina, Pirofosfato), grânulos alfa (fatores de crescimento, fatores de coagulação e proteínas de adesão) e um sistema de membrana, local de síntese de prostaglandinas e tromboxano A2 (LORENZI et al., 2003).

As plaquetas em média circulam no sangue de 9-10 dias. Durante esse tempo de sobrevida, o seu metabolismo se baseia na liberação de energia, representada pela transformação do ATP em ADP, a partir da glicólise e do mecanismo oxidativo das mitocôndrias. Esta energia é utilizada para as funções básicas das plaquetas, que são a adesão, a agregação e a secreção de substâncias contidas nos grânulos citoplasmáticos (LORENZI et al., 2003).

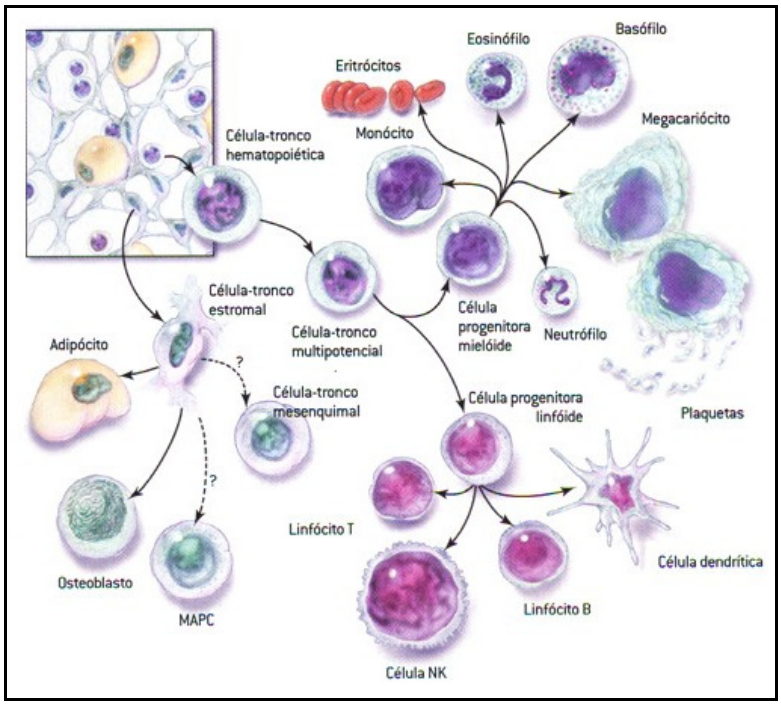

Figura 4 – Origem das plaquetas.

Adaptado de VILELA (2012).

Quando um vaso sanguíneo é lesado, ocorre exposição da plaqueta ao colágeno. Neste momento ocorre a ativação plaquetária, iniciando-se uma série de fenômenos que tem por finalidade evitar a hemorragia. Após a adesão das plaquetas ao subendotélio, novas plaquetas são ativadas e acabam aderindo a essas plaquetas. Durante esta ativação, as plaquetas mudam sua forma passando de discóide a irregular, emitindo pseudópodes a partir da membrana e se agregam. Os grânulos plaquetários são liberados. O primeiro sinal de ativação plaquetária é sentido na membrana externa, onde os fatores capazes de promover esta ativação como a trombina, a adrenalina, o colágeno e o ADP se ligam aos seus receptores específicos. A seguir, ocorre a ligação das plaquetas aos receptores das glicoproteínas e ativação das moléculas de integrinas plaquetárias levando ao processo de agregação plaquetária (LEE et al., 1998; LORENZI et al., 2003).

A agregação plaquetária ocorre devido à formação de pontes de fibrinogênio, pois este se liga ao receptor na membrana plaquetária, GP IIb/IIIa, que na presença do Ca^{2+} forma um complexo estável na superfície das plaquetas ativadas. Essa também ocorre devido ao metabolismo do ácido araquidônico, sendo o mesmo liberado a partir da membrana fosfolipídica das plaquetas pela ativação da enzima

fosfolipase A2 e, subsequentemente, a enzima cicloxigenase (COX-1) converte o ácido araquidônico em endoperóxidos cíclicos. Esses são então convertidos pela tromboxano sintetase em tromboxano A2 (TXA2), funcionando como um potente agonista que induz a agregação (LEE et al., 1998; ANDREWS et al., 1999; BECKER, 1999; GAETANO, 2001). A amplificação e a propagação contínua da agregação plaquetária é ativada pela formação de agregados plaquetários e pela expulsão de ADP e de outras substâncias ativas das organelas plaquetárias (LEE et al., 1998).

A principal função das plaquetas é a hemostasia, na qual elas desempenham atividade mecânica e bioquímica. As plaquetas aderem-se as estruturas subendoteliais expostas e tornam-se ativadas; ocorrendo a adesão plaquetária (Figura 5) (HARKER, et a., 1997). Posteriormente, ocorre uma intensa atividade metabólica, havendo a síntese de mensageiros intracelulares e formação de agonistas plaquetários. Na sequência as plaquetas sofrem transformações morfológicas, iniciando a formação de agregados plaquetários também denominados de tampão plaquetário, que vão constituir a barreira plaquetária inicial. A liberação de ADP e outras substâncias ativas amplificam o processo de ativação plaquetária. Segue-se a ativação com a liberação do fator III plaquetário e tem início o processo de coagulação sanguínea, promovendo à consolidação da rolha plaquetária pela fibrina e a consequente retração do coágulo (LEE et al., 1998; LORENZI et al., 2003; RAMASAMY, 2004; DAVÍ AND PATRONO, 2007).

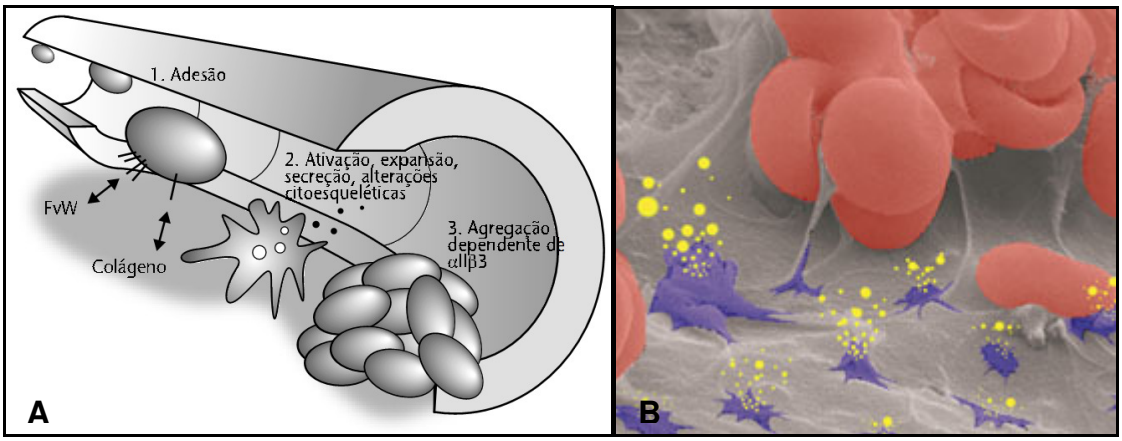

Figura 5 – Três componentes da função plaquetária para hemostasia na injúria vascular: adesão, atividade e agregação (A). Liberação de substâncias ativas e agregação ao eritrócito para formação do tampão plaquetário (B).

Adaptado BIOMET (2011) e VANNI (2007).

Além da sinalização do ADP em plaquetas os nucleotídeos extracelulares de adenina (ATP e AMP) e seu derivado nucleosídeo adenosina também são importantes moléculas sinalizadoras que medeiam diversos processos biológicos e patológicos (RATHBONE et al., 1999; RIBEIRO et al., 2003; ILLES AND RIBEIRO, 2004). Os nucleotídeos e nucleosídeos podem ter suas concentrações alteradas, hidrolisados por enzimas do sistema purinérgico como as ectonucleotidases. Deste modo, esses nucleotídeos ATP, ADP e AMP podem estimular células ou órgãos alvos conferindo determinado efeito tanto no SNC como no sistema vascular (RATHBONE et al., 1999; KANNAN, 2002; REMIJIN et al., 2002).

No sistema vascular, a sinalização purinérgica está associada com o controle do tônus vascular mediado por ATP liberado de células endoteliais (BURNSTOCK, 1990; RALEVIC, 2001). Durante as doenças arteriais como a aterosclerose, eventos de migração, proliferação de células endoteliais tem um importante papel no desenvolvimento do espessamento intimal (SCHACHTER, 1990; GIBBONS, 1993). Estudos indicam que o ATP e o ADP e a adenosina também desempenham papel central de sinalização destes acontecimentos e do mecanismo de tromborregulação (SOSLAU AND YOUNGPRAPAKORN, 1997; ZIMMERMANN, 1999; BURNSTOCK, 2002; REMIJIN et al., 2002).

O ATP possui um papel complexo nos mecanismos de regulação de agregação plaquetária, sendo que em baixas concentrações ativa e em altas concentrações inibe a agregação de plaquetas induzida pelo ADP (BIRK et al., 2002; ROZALSKI et al., 2005). Por sua vez, o ADP constitui-se no principal agonista envolvido no recrutamento e agregação das plaquetas em locais de injúria vascular, enquanto que a adenosina possui efeitos inibitórios sobre esta agregação (ROZALSKI et al., 2005).

Em linfócitos, o papel do ATP extracelular na modulação da imunidade teve uma aceitação lenta através dos anos (DI VIRGILIO, 2006). Três grandes avanços derrubaram a descrença deste conceito: a clonagem e identificação de células do sistema imune às quais possuem receptores P2 e subtipos; a demonstração que o ATP é liberado pelas células do sistema imune; e que os mediadores podem ser inativados por específicas ectonucleotidases. Nos dias de hoje está claro que a rede de sinalização purinérgica é componente fundamental do ambiente imuno-regulatório (DI VIRGILIO, 2006). Altos níveis de ATP podem ser liberados por células T $CD4^+$ e $CD8^+$ sob estimulação (LUTHJE, 1989), e com isso estimular outras células tais

como os monócitos, os linfócitos e a célula endotelial que induzem uma grande resposta pró-inflamtória como a liberação de interleucinas (IL-1, IL-8) (LUTHJE, 1989; IMAI et al., 2000; WARNY et al, 2001; LA SALA et al., 2003). Sobre as células dendríticas a exposição do ATP extracelular induz a migração e a diferenciação dirigindo a resposta imune celular (LA SALA et al., 2001).

O produto de quebra do ATP, a adenosina, regula as funções dos linfócitos indiretamente pela estimulação dos receptores de adenosina sobre as células do sistema imune inato e células dendríticas. A adenosina também pode afetar diretamente as respostas de linfócitos pela ligação e ativação de seus receptores nestas células (HASKÓ et al., 2008). Recentes estudos revelaram um importante papel para adenosina a qual medeia propriedades de supressão imune de células T regulatórias, um tipo de célula que possui papel chave para manter o sistema imunitário em compartimentos, evitando assim a lesão tecidual excessiva (HASKÓ et al., 2008). Em adição, as células T regulatórias expressam altos níveis de NTPDase (CD39) e 5'-nucleotidase (CD73) que convertem ATP e ADP para adenosina, e esta por sua vez, estimula os receptores A_{2A} que aumenta a expressão de moléculas co-estimulatórias negativas como o antígeno 4 associado ao linfócito T-citotóxico e a morte celular programada (KOBIE et al., 2006; DEAGLIO et al., 2007).

No SNC os nucleotídeos e nucleosídeos de adenina possuem diversas funções como a neurotransmissão, a neuroproteção (CUNHA & RIBEIRO, 2000; DUNWIDDIE & MASINO, 2001; CUNHA, 2001) e a proliferação de células glias (FIELDS & BURNSTOCK, 2006). Também, podem estar envolvidos na formação e regulação da sinaptogênese, na plasticidade neuronal e na diferenciação de células progenitoras de oligodendrócitos (RATHBONE et al., 1999; FIELDS AND STEVENS, 2000; CICARRELLI et al., 2001; STEVENS et al., 2002; WINK et al., 2003, ARESTI et al., 2005). O ATP é um importante neurotransmissor excitatório nas sinapses nervosas purinérgicas, age como neuromodulador e pode ser armazenado e liberado no meio extracelular juntamente com outros neurotransmissores, tais como acetilcolina, glutamato e noradrenalina através das vesículas pré-sinápticas dependentes de cálcio (GIBB AND HALLIDAY, 1996; SPERLÁGH AND VIZI, 1996; ZIMMERMANN, 1996; ILLES AND RIBEIRO, 2004).

A adenosina tem papel relevante na neuromodulação regulando a liberação de vários neurotransmissores e age tanto pré quanto pós-sinapticamente (CUNHA, 2001; DUNWIDDIE AND MASINO, 2001; RIBEIRO, et al., 2003). A regulação da

liberação de neurotransmissores excitatórios, por esta molécula, tem se tornado importante em muitos processos patológicos, pois a adenosina pode limitar o dano causado pela excitotoxicidade destes neurotransmissores, exercendo assim uma ação protetora no SNC (ZIMMERMANN et al., 1998; DUNWIDDIE AND MASINO, 2001). Geralmente, este nucleosídeo é uma molécula sinalizadora inibitória, protege os sistemas do miocárdio e cerebrovascular contra o dano induzido pela isquemia (ELY & BERNE, 1992; OBATA, 2002) e diminuição do débito cardíaco.

A adenosina também limita o grau da injúria vascular depois da isquemia e reperfusão reduzindo ROS liberadas e a inibição da agregação de plaquetas (ELY & BERNE, 1992). Além da atividade antiinflamatória (CRONSTEIN, 1994), possui propriedades analgésicas (SAWYNOK & SWEENEY, 1989) e pode influenciar na proliferação, sobrevivência ou apoptose de várias diferentes células (JACOBSON et al., 1999), bem como trabalhar como um potente supressor do sistema imune (SPYCHALA et al., 1997).

Cada nucleotídeo ou nucleosídeo, uma vez presente no meio extracelular, desempenha sua ação pela ligação a determinado receptor localizado na superfície de vários tipos de células (BURNSTOCK, 2007). Os receptores purinérgicos foram primeiramente definidos em 1976 (BURNSTOCK, 1972), e nos anos seguintes foram classificados em dois tipos de receptores: P1 (adenosina) e P2 (ATP/ADP) (BURNSTOCK, 1978). P2X são receptores acoplados a canais iônicos com seus domínios carboxi e aminoterminal voltados para o meio intracelular e compreendem sete subtipos nomeados de P2X1-7 (DI VERGÍLIO et al., 2001). P2Y são receptores acoplados a proteína G apresentando sete regiões transmembrana com a porção aminoterminal voltada para meio extracelular e a porção carboxiterminal voltada para o meio citoplasmático compreendendo 14 subtipos os quais foram nomeados de P2Y1-14 (BURNSTOCK, 2007; YEGUTKIN, 2008). A sinalização purinérgica destes receptores P2 está envolvida em muitos mecanismos tais como neuronais e não neuronais, incluindo secreções exócrinas e endócrinas, respostas imune, inflamação, dor, agregação de plaquetas e mediadores endoteliais de vasodilatação (BURNSTOCK & KNIGHT, 2004; BURNSTOCK, 2006). Os receptores para adenosina incluem quatro tipos: A1, A2A, A2B e A3, os quais são proteínas transmembrana acoplados aproteína G (HASKO et al., 2008; YEGUTKIN, 2008) que possuem diversos efeitos fisiológicos tais como a modulação cardiovascular, o sistema imune e o SNC (LEDENT et al., 1997; SUN et al., 2001).

Os nucleotídeos de adenina são hidrolisados pelas enzimas ecto-nucleotidases, entre elas destacam-se a NTPDase (E-NTPDase, CD39), a 5'-nucleotidase (CD73) e a adenosina desaminase (ADA) as quais são capazes de alterar os níveis de ATP, ADP, AMP e adenosina (Figura 6) (ZIMMERMANN, 2001; GODING et al., 2003; ROBSON et al., 2006).

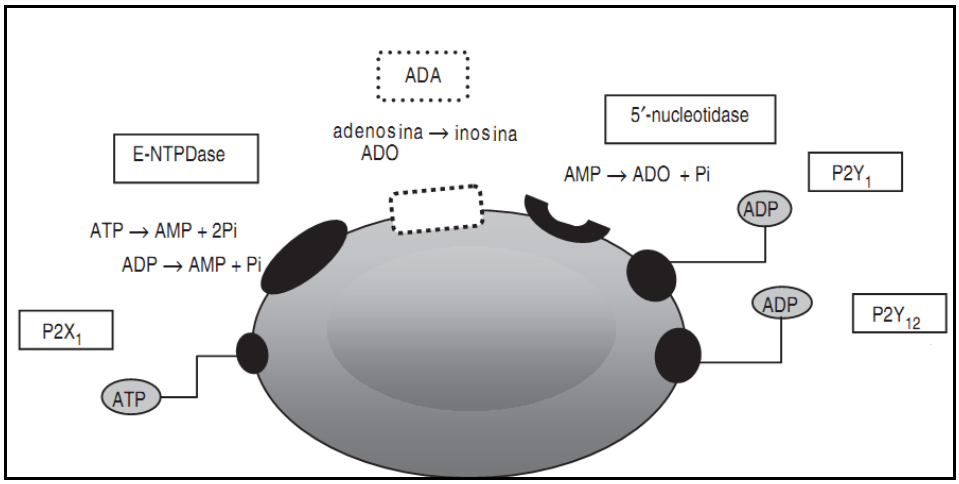

Figura 6 – Esquema ilustrativo do sistema múltiplo (cascata enzimática) da hidrólise de nucleotídeos (ATP, ADP e AMP) e desaminação de nucleosídeo (ADO) na superfície celular.

Adaptato de FÜRSTÜRSTENAU (2006).

A família das NTPDases (Ecto-nucleosídeo trifosfato difosfoidrolases) possui oito membros (Figura 7), os quais são responsáveis pela hidrólise de nucleotídeos tri e difosfatados (ZIMMERMANN, 2001) e diferem quanto à especificidade ao substrato, distribuição tecidual e localização celular (SHI et al., 2001; ZIMMERMANN, 2001; BIGONNESE et al., 2004). Quatro NTPDases são típicas da superfície celular, onde seus sítios catalíticos estão voltados para o meio extracelular (NTPDase 1, 2, 3 e 8). NTPDases 4, 5, 6 e 7 apresentam localização intracelular e sitio catalítico voltado para face interna da célula (ROBSON et al., 2006).

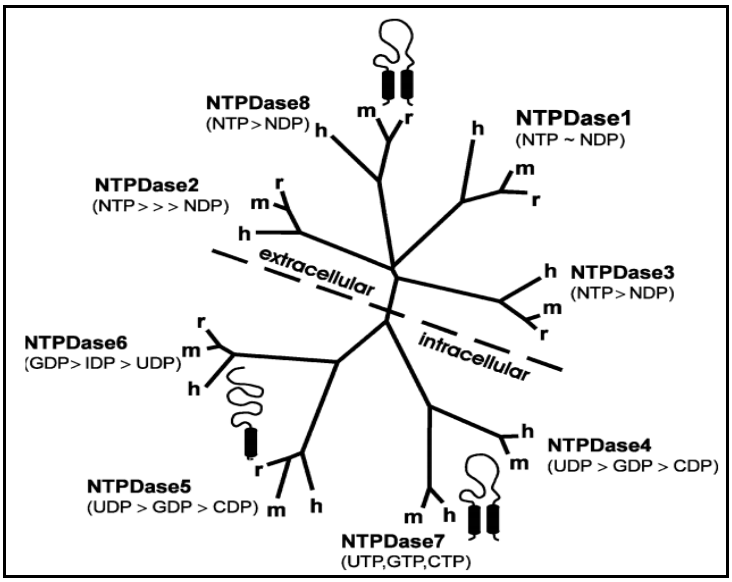

Figura 7 – Família das enzimas E-NTPDases.

Adaptado de ROBSON (2006).

Estas enzimas apresentam um alto grau de similaridade na sua seqüência de aminoácidos, particularmente dentro de cinco regiões que são conhecidas como “regiões conservadas da apirase - ACRS”, as quais são de extrema importância para a atividade catalítica (ZIMMERMANN, 1999).

A NTPDase-1 (ecto/CD 39) hidrolisa tanto ATP como ADP formando AMP na presença de íons Ca^{2+} e Mg^{2+} (ZIGANSCHIN et al., 1994). Na família das NTPDases, a enzima NTPDase-1 foi a primeira a ser descrita e encontram-se ancorada na superfície celular através de duas regiões transmembranas, próximas ao grupamento amino e carboxi terminal com o seu sítio catalítico voltado para o meio extracelular (Figura 7) (ZIMMERMANN, 2001).

Já foi identificado em sinaptossomas isolados que a NTPDase-1 é expressa neste meio, bem como em cultura de neurônios primários de córtex cerebral e astrócitos (BATTASTINI et al., 1991; WANG AND GUIDOTTI, 1998). As pesquisas em imunohistoquímica revelaram que esta enzima é amplamente distribuída no encéfalo de ratos, tal como em neurônios de córtex cerebral, hipocampo, cerebelo, células gliais e endoteliais (WANG AND GUIDOTTI, 1998).

As NTPDases possuem funções fisiológicas e fisiopatológicas em vários tecidos (ROBSON et al., 2006; SCHETINGER et al., 2007). Em plaquetas intactas de humanos, a NTPDase-1 pode estar envolvida na regulação da concentração dos

nucleotídeos, na circulação e no tônus vascular. Alguns estudos indicam que o uso de NTPDase-1 solúvel constitui-se num potencial agente terapêutico para inibição de processos trombóticos mediados por plaquetas.

Uma solução purificada de NTPDase-1 bloqueou *in vitro* a agregação plaquetária induzida por ADP e inibiu a reatividade plaquetária induzida pelo colágeno (GAYLE et al., 1998; ENJYOJI, 1999). Nos últimos anos, esta enzima está sendo amplamente estudada em condições patológicas de modelos experimentais (BONAN et al., 2000; LUNKES et al., 2003; ARAÚJO et al., 2005). A função geral desta enzima é atribuída à hidrólise extracelular dos nucleotídeos ATP e ADP e, portanto, dependendo da localização tecidual, da atividade enzimática, possui diferentes papéis fisiológicos (SARKIS et al., 1995; ZIMMERMANN, 1999; BONAN et al., 2001). NTPDase é uma enzima que possui importante papel na adesão celular e no controle das funções dos linfócitos, incluindo o reconhecimento e a ativação das células T citotóxicas (DOMBROWSKI et al. 1995; LEAL et al., 2005a). A atividade da NTPDase nos linfócitos T tem um efeito protetor contra a lise celular causada pelo ATP extracelular o qual pode agir como composto sinalizador dos mecanismos citolíticos (DOMBROWSKI et al., 1995; LEAL et al., 2005a; ROBSON et al., 2006). O decréscimo na atividade da NTPDase inibe as funções efetoras de células T, B e Natural Killer (NK) (DOMBROWSKI et al., 1995; ROBSON et al., 2006).

A enzima 5'-nucleotidase (ecto-5'-nucleotidase, CD73) é uma enzima ancorada à membrana plasmática via uma molécula de GPI (glicosilfosfatidil inositol) com seu sítio catalítico voltado para o meio extracelular (Figura 8). Entretanto, formas solúveis e clivadas desta enzima também já foram descritas (ZIMMERMANN, 2001; HUNSUCKER et al., 2005). Esta enzima catalisa a desfosforilação de vários nucleotídeos 5'-monofosfatados como CMP, IMP, UMP, GMP e AMP a seus respectivos nucleosídeos (ZIMMERMANN, 1996). No entanto, foi demonstrado que a 5'-nucleotidase hidrolisa mais eficientemente o AMP, sendo por isto considerada a principal enzima responsável pela formação de adenosina (ZIMMERMANN, 1996; ZIMMERMANN et al., 1998; ZIMMERMANN, 2001).

A função da enzima 5'-nucleotidase correlaciona-se diretamente ao seu papel na produção de adenosina. Assim, de acordo com a sua localização tecidual, ela desempenha importantes funções como, por exemplo, no controle da agregação plaquetária, na regulação do tônus vascular e também na neuromodulação e

neuroproteção do sistema nervoso (ZIMMERMANN et al., 1998; KAWASHINA et al., 2000; DUNWIDDIE & MASINO, 2001).

No SNC, a 5'-nucleotidase está predominantemente associada à glia, mas várias evidências têm demonstrado que esta atividade também está associada a neurônios (ZIMMERMANN et aI, 1996, 1998, 2001; COGNATO & BONNAN, 2010; SILVA et al., 2012). A 5'-nucleotidase é transitoriamente expressa na superfície de células neuronais e nas sinapses durante o desenvolvimento sináptico (BRAUN et aI, 1995). A atividade da enzima 5'-nucleotidase encontra-se aumentada em astrócitos, células microgliais (BRAUN et aI, 1997) e em sinaptossomas de hipocampo após isquemia focal e reperfusão (SCHETINGER et al., 1998). Além disso, estudos demonstraram que a 5'-nucleotidase possui características de molécula de adesão (AIRAS et al, 1995).

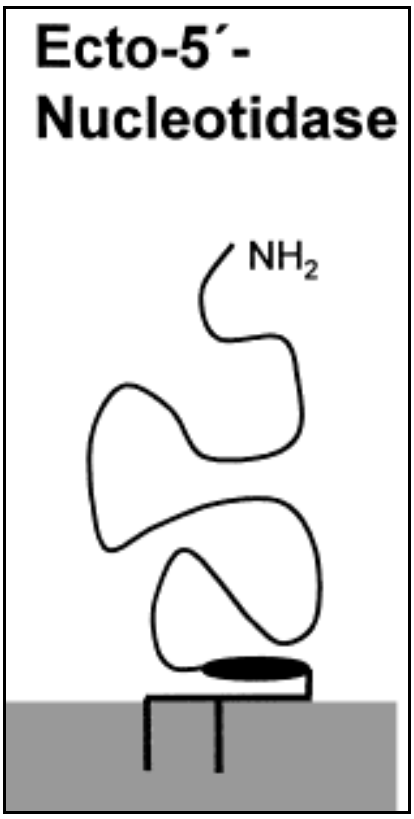

Figura 8 – Estrutura da enzima 5'-nucleotidase ancorada à membrana plasmática via uma molécula de GPI.

Adaptado de ZIMMERMANN (2001).

O produto final da quebra de nucleotídeos é realizado pela adenosina desaminase (ADA, E.C. 3.5.4.4; Figura 9 e 10), enzima chave no metabolismo de purinas. Esta enzima catalisa a hidrólise irreversível da adenosina ou deoxiadenosina em seus respectivos produtos, a inosina e a amônia. É altamente distribuída em quase todos os tecidos dos mamíferos e tem papel central na manutenção da competência imune (FRANCO et al., 2006). Em soro humano a ADA possui três formas moleculares: a forma monomérica de 35.000 g/mol (ADA1); a tetramérica de 280.000 g/mol formada pela combinação de dois monômeros da ADA1 e uma glicoproteína dimérica nomeada de proteínas combinadas; e a terceira forma de 100.000 g/mol (ADA2). Estas formas diferem em propriedades cinéticas e imunológicas (BOTA et al., 2000). A isoenzima ADA1 é encontrada em todas as células, e com maior atividade em linfócitos e monócitos, já a ADA2 é isoenzima predominante no soro de indivíduos saudáveis (UNGERER et al., 1992). Alguns estudos indicam que ADA pode ser uma enzima importante na modulação da insulina bioativa (RUTKIEWICZ et al., 1990) e na resposta inflamatória aguda e crônica (CONLON & LAW 2004; DESROSIERS et al., 2007).

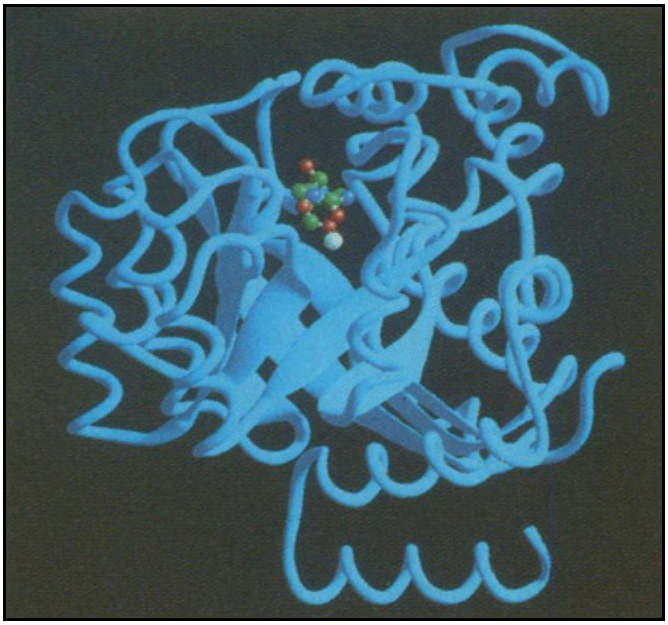

Figura 9 – Estrutura da enzima adenosina desaminase.

Adaptado WILSON (1991).

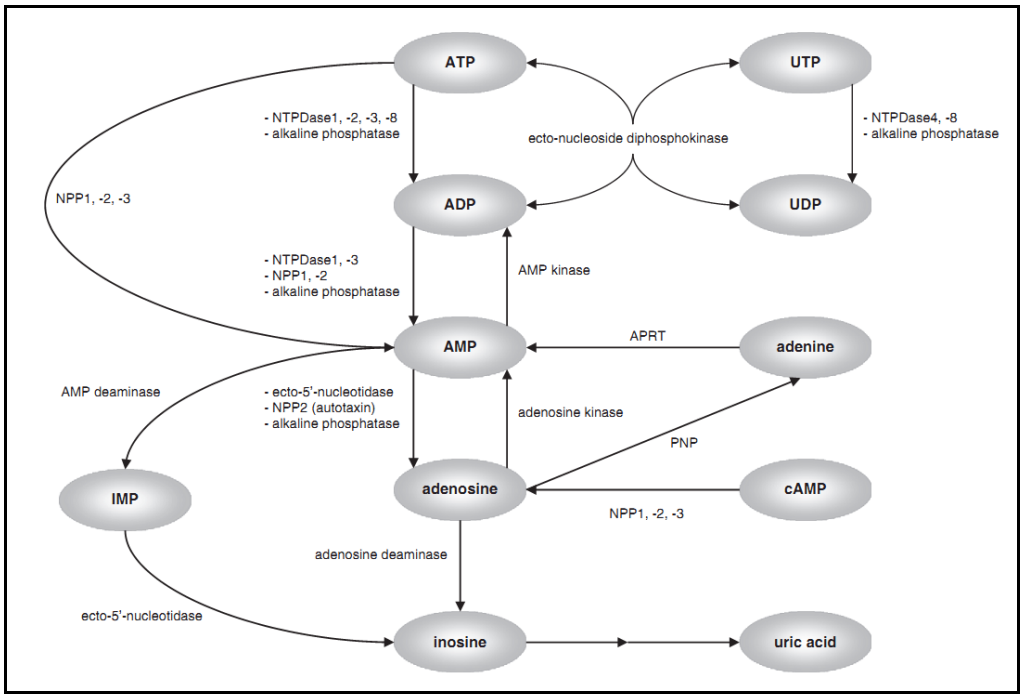

Figura 10 – Visão geral das vias de conversão do ATP, ADP, AMP e da adenosina.

Adaptado BOURS (2006).

Outro importante mediador químico é a acetilcolina (ACh), a qual possui ações no SNC, sistema nervoso periférico (SNP) e na junção neuromuscular (BRUNEAU & AKAABOUNE, 2006). Este neurotransmissor possui um papel essencial nas vias colinérgicas do SNC que controlam o aprendizado, a memória e as respostas psicoestimulantes (SLOTKIN, 2004). A ACh, seus receptores e o aparato enzimático responsável por sua síntese e degradação constituem o sistema de neurotransmissão colinérgica. Uma vez sintetizada, parte da ACh é transportada e armazenada em vesículas sinápticas. Este processo é realizado por um transportador vesicular de ACh, capaz de elevar em até 100 vezes sua concentração no interior destas vesículas. Após ser liberada inteiramente por exocitose, a ACh interage especificamente com os receptores colinérgicos (nicotínicos e muscarínicos) presentes nas membranas pré e pós-sinápticas (TAYLOR & BROWN 1994; DESCARRIES et al., 1997; FUJII et al., 2008). Até o momento cinco subtipos de receptores muscarínicos foram identificados (M1-M5) e agem via ativação de proteína G, sendo que os receptores M1 e M2 estão presentes em neurônios do SNC e SNP além de outros tecidos ganglionares (VAN DER ZEE & LUITEN, 1999).

Os receptores nicotínicos são estruturas pentaméricas que atuam como canais iônicos regulados por ligantes e localizam-se nas sinapses ganglionares e nas junções neuromusculares e são compostos por cinco subunidades conhecidas **α1, α2, β, γ, δ** (ARIAS, 1998). A ação da acetilcolina cessa quando é hidrolisada em acetato e colina pela enzima acetilcolinesterase (AChE), presente na fenda sináptica (MESULAM et al., 2002) (Figura 11).

A AChE pode apresentar as formas globulares ou assimétricas. A forma globular é composta por monômeros (G1), dímeros (G2) e tetrâmeros (G4) da subunidade catalítica. A forma G1 é citosólica e a G4 é ligada a membrana, sendo esta última encontrada em maior quantidade no tecido nervoso (DAS et al., 2001; ALDUNATE et al., 2004). A forma assimétrica consiste em um (A4), dois (A8) e três (A12), tetrâmeros catalíticos ligados covalentemente a uma subunidade estrutural colagênica chamada Q (ColQ). Essas formas estão associadas com a lâmina basal e são abundantes na junção neuromuscular (ALDUNATE et al., 2004).

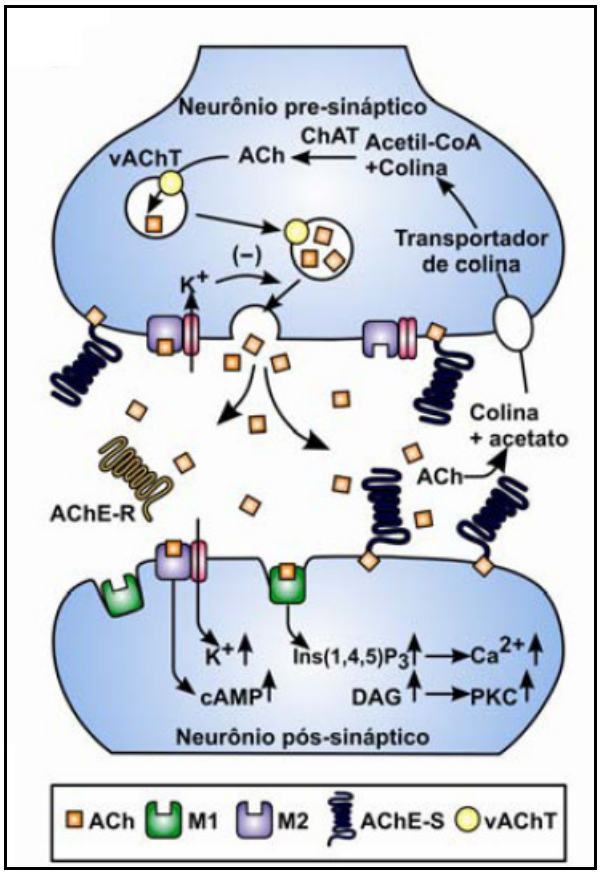

Figura 11 – Sinapse colinérgica.

Adaptado de SOREQ & SEIDMAN (2001).

A expressão da AChE tem sido encontrada em várias estruturas do encéfalo tais como o hipotálamo, estriado, hipocampo, cerebelo, substância nigra e também no fluído cérebroespinhal (PAXINOS, 1985). Por ser uma das mais eficientes e conhecidas catálises biológicas, a AChE tem sido investigada como um importante alvo terapêutico em várias doenças degenerativas (DAS et al., 2001). Na doença de Alzheimer e na esclerose múltipla estudos demonstraram o envolvimento do déficit cognitivo com o sistema colinérgico nestas patologias (FREO et al., 2002). A inibição na atividade da AChE pelo donepezil, a rivastigmina e a galantamina, inibidores clássicos desta enzima, melhora o aprendizado e a memória fornecendo melhor qualidade de vida em pacientes com doenças neurodegenerativas (GREENE et al., 2000; WERBER & RABEY, 2001; DOME et al., 2010). No modelo experimental de desmielinização no SNC, MAZZANTI et al. (2006, 2009) demonstraram que a neurotransmissão colinérgica é prejudicada em diferentes regiões do encéfalo de ratos, e a hipofunção do sistema colinérgico pode ser parcialmente restaurada pelo uso de antioxidantes tal como viamina E, o ebselen e o agente imunomodulador interferon beta. Em adição, a AChE também é alvo de intoxicações por inseticidas na agricultura e na exposição à fumaça de cigarro (GUBERT et al., 2011). A nicotina, oriunda da fumaça de cigarro, também inibiu a atividade da AChE num estudo *in vitro* (BENOWITZ 1996; JÓSÊ et al., 2009), além de ativar os receptores de acetilcolina e alterar as funções do sistema colinérgico com efeitos adversos na função neurocomportamental e desenvolvimento sináptico (ROWELL & WINKLER, 1984; SLOTKIN, 1998, DOME et al., 2010).

Algumas disfunções biológicas podem promover alterações em determinadas enzimas, tais como a AChE, as ectonucleotidases, e a ADA entre outras (FERREIRA & MATSUBARA, 1997; Schetinger et al., 2007). O envolvimento destas enzimas nos processos de doenças em alguns tecidos e tipos celulares tem sido investigado. A atividade da AChE pode ser alterada em processos patológicos tais como a doença de Alzheimer, a Esquizofrenia, a doença de Parkinson, nas desordens de ansiedade e atenção as quais demonstraram deficiência no sistema colinérgico central (NEWHOUSE et al., 2004; RIPOLL et al., 2004). As ectonucleotidases podem estar relacionadas aos eventos de tromboregulação no diabetes melito, na hipertensão, na hipercolesterolemia, no câncer e também em condições patológicas em que as plaquetas possuem alterada agregação, bem como na falência renal crônica (Schetinger et al., 2007) Na modulação imune e na interação célula-célula também

há o envolvimento das ectonucleotidases tanto para a hidrólise de ATP e ADP e a desaminação da adenosina os quais estão relacionados com alterações do sistema imune, tais como a leucemia linfobástica aguda e infecções associadas ao vírus da imunodeficiência humana (LEAL et al., 2005b). Porém, ainda não foram encontrados na literatura estudos referentes a exposição à fumaça de cigarro e da nicotina com as enzimas em questão em questão.

Neste contexto, considerando a que a fumaça de cigarro está envolvida em várias patologias tais como doenças vasculares, imunossupressão e desordens no SNC o objetivo deste estudo foi investigar a atividade das enzimas ectonucleotidases e AChE, bem como a peroxidação lipídica em animais submetidos ao modelo de exposição à fumaça de cigarro e de nicotina.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar a atividade das enzimas ectonucleotidases e acetilcolinesterase e o nível de peroxidação lipídica em ratos expostos à fumaça de cigarro e nicotina.

2.2 Objetivos específicos

- Avaliar a atividade das enzimas NTPDase e 5´-nucleotidase em plaquetas de ratos expostos à fumaça de cigarro.

- Avaliar a atividade da enzima ADA em plasma de ratos expostos à fumaça de cigarro.

- Avaliar a atividade das enzimas NTPDase, 5´-nucleotidase e ADA em linfócitos de ratos expostos à nicotina: estudo *in vivo* e *in vitro*.

- Determinar a atividade da AChE e a peroxidação lipídica em estriado, córtex cerebral, hipocampo e cerebelo de ratos expostos à fumaça de cigarro.

- Avaliar o potencial efeito terapêutico da vitamina E em ratos expostos à fumaça de cigarro.

3 ARTIGOS

3.1 Artigo 1

Atividade das ectonucleotidases e adenosina deaminase em ratos expostos a fumaça de cigarro

Activity of ectonuleotidases and adenosine deaminase in rats exposed to cigarette smoke

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informa
healthcare

RESEARCH ARTICLE

Activity of ectonucleotidases and adenosine deaminase in rats exposed to cigarette smoke

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Abstract

Cigarette smoke is a complex mixture of various toxic substances that are capable of initiating oxidative damage and promoting blood platelet alterations. In this study, we investigated the activities of the ectoenzymes NTPDase (ectonucleoside triphosphate diphosphohydrolase, CD39) and 5′-nucleotidase (CD73) in platelets as well as adenosine deaminase (ADA) in the plasma of rats exposed to aged and diluted sidestream smoke during 4 weeks. The rats were divided into two groups: I (control) and II (exposed to smoke). After the exposure period, blood was collected and the platelets and plasma were separated for enzymatic assay. The results demonstrated that NTPDase (with ATP as substrate) and 5′-nucleotidase (AMP as substrate) activities were significantly higher in group II ($p < 0.05$) as compared to group I, while no significant difference was observed for NTPDase with ADP as substrate. The ADA activity was significantly reduced in group II ($p < 0.05$) as compared with group I. Platelet aggregation was significantly increased in group II ($p < 0.05$) as compared with group I. We suggest that these alterations in the activity of enzymes from the purinergic system are associated with an increase in platelet aggregation. However, our study has demonstrated that the organism tries to compensate for this enhanced aggregation by increasing hydrolysis of AMP and reducing hydrolysis of adenosine, a potent inhibitor of aggregation and an important modulator of vascular tone.

Keywords: *Platelets; NTPDase; 5′-nucleotidase; adenosine deaminase; cigarette smoke; rat*

Introduction

Cigarette smoke is a complex mixture of over 4000 identified constituents including nicotine and reactive oxygen and nitrogen species. The reactive oxygen and nitrogen species are capable of initiating or promoting oxidative damage in biological systems (Rahman et al., 1996; Genbacev-Krtolica, 2005). Acute cigarette smoking significantly increases plasma epinephrine and induces platelet reactions, including the release of nucleotides (Mundal et al., 1998).

Extracellular adenine nucleotides such as adenosine triphosphate (ATP) and adenosine diphosphate (ADP) and the nucleoside adenosine are known to regulate the vascular response to endothelial damage by exerting a variety of effects on platelets (Birk et al., 2002b). The nucleotide ADP is the main promoter of platelet aggregation (Fusegawa et al., 2000; Braun et al., 2007), whereas adenosine is a potent inhibitor of this aggregation and an important modulator of vascular tone (Rozalski et al., 2005). Moreover, studies have demonstrated that ATP has a complex role in the regulation of platelet aggregation. High ATP concentrations inhibit aggregation induced by ADP, but low concentrations can contribute to enhanced collagen, thromboxane A2, and thrombin-induced aggregation (Birk et al., 2002b).

The most relevant ectoenzymes involved in adenine nucleotide extracellular hydrolysis are NTPDase (E.C. 3.6.1.5; ectonucleoside triphosphate diphosphohydrolase/CD39) and ecto-5′-nucleotidase (E.C. 3.1.3.5; CD73). The biological actions of these enzymes affect the extracellular levels

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of ATP, ADP, and adenosine monophosphate (AMP), which in turn affect purinoreceptor signaling (Lunkes et al., 2004). Both NTPDase and 5′-nucleotidase are present on the platelet membrane (Pilla et al., 1996; Kawashima et al., 2000) and play an important role in the maintenance of normal hemostasis and in the prevention of excessive platelet aggregation (Lunkes et al., 2004) by ADP hydrolysis. In fact, NTPDase is now accepted as a potent antithrombotic agent (Sévigny et al., 2002; Marcus et al., 2003). NTPDase hydrolyzes ATP and ADP to AMP, which is subsequently converted to adenosine by 5′-nucleotidase (Robson et al., 2006). Adenosine can be directly inactivated through the sequential action of adenosine deaminase (ADA; E.C. 3.5.4.4), which catalyzes the irreversible deamination of adenosine. This enzyme is involved in controlling adenosine levels, leading to inosine production (Spychala, 2000).

Platelets and the endothelium have been recognized for decades as key pathological components in processes of arterial thrombosis associated with vascular inflammation (Robson et al., 2001). In general, extracellular nucleotides tend to induce inflammation, whereas nucleosides largely result in cellular events that down-regulate such activation responses. Studies using selective pharmacological agents in mutant mice have reinforced these observations (Atkinson et al., 2006). Clinical models that interfere with platelet-mediated arterial thrombosis have resulted in successful strategies that target platelet receptors for extracellular nucleotides, in the inhibition of the platelet ADP receptor (Sharis et al., 1998; Hirsh & Bhatt, 2004; Savi & Herbert, 2005). Furthermore, platelet reactivity has been suggested to increase the incidence of coronary heart disease, and cigarette smoking has been reported to be an accelerator of platelet aggregation (Fusegawa & Handa, 2000).

In this context, considering that cigarette smoking presents alterations associated with blood platelets, it is relevant to study the activity of NTPDase and 5′-nucleotidase in platelets and ADA in plasma from rats exposed to aged and diluted sidestream smoke. In order to characterize this model of smoke exposure, we measured blood gas, pH, and carboxyhemoglobin and analyzed lung tissue, in addition to analyzing platelet aggregation.

Materials and methods

Chemicals

Nucleotides, HEPES, and Coomassie Brilliant Blue G were obtained from Sigma Chemical Co. (St. Louis, MO), and bovine serum albumin and K_2HPO_4 were obtained from Reagen (Quimibrás Indústrias Químicas S.A., Brazil). All the other chemicals used in this experiment were of the highest purity.

Animals

Adult male Wistar rats (200–300 g) were used in this experiment. The animals were kept on a 12-h light/12-h dark cycle, at a temperature of 22±2°C, with free access to food and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources (COBEA), and in accordance with international guidelines.

Cigarette composition

The brand of cigarette used in the experiment was manufactured by Philip Morris Brasil Indústria e Comércio Ltda from the city of Santa Cruz do Sul—Rio Grande do Sul, Brazil. Tobacco smoke contains thousands of different chemicals, including tar, nicotine, and carbon monoxide. The basic ingredients of cigarette smoke printed on cigarette packs of the brand used in the experiment are: sugar, paper, plant extracts, and flavoring agents, and the cigarette contains 10 mg tar, 0.8 mg nicotine, and 10 mg carbon monoxide. Moreover, ingredients in the 20-cigarette hard pack of the brand include burnt items (e.g. propylene glycol, glycerol, guar gum, side-seam adhesives, etc.) and unburnt items (e.g. filtration material, filter wraps, filter adhesives, etc.).

Cigarette smoke exposure

The animals were randomly divided into two groups (10 rats in each group): I (control) and II (exposed to smoke). Group II was exposed to the aged and diluted sidestream smoke of commercial cigarettes inside a whole-body smoke exposure chamber during 4 weeks. In the first, second, third, and fourth weeks, animals were concomitantly exposed to the smoke of one, two, three, and four cigarettes per day, respectively. The duration of each exposure was 15 minutes, 5 days each week. Control animals were placed in the same chamber for the same amounts of time, but without exposure to smoke. At no time were the control rats in the same environment during the exposure to cigarette smoke. The exposure chamber was washed with clean water and detergent and aired for 24 hours after use, ensuring that it was completely free of smoke when used with the control animals. At the end of the experimental period (28 days), rats were anesthetized with ketamine chlorhydrate and xylazine (5:1; 0.1 ml/100 g) and total blood was collected for the subsequent enzymatic assays. Immediately after collection the rats were killed by decapitation.

Smoke generation

The system of exposure to cigarette smoke was simple. After placing the rats inside the exposure chamber (size 564×385×371 mm; volume 53,100 ml/1795 oz; plastic material), between 1 and 4 cigarettes were lit (according to the protocol) and a stopwatch was turned on. Cigarettes were fixed in a metal holder, allowing them to be fully burned down within a period of 15 minutes. After lighting the cigarettes, the chamber was immediately closed, with only a small opening (371×40 mm) in the chamber for ventilation. A metal grille was placed on top of the cigarette holder to avoid direct contact with the cigarettes and thus prevent the rats from injuring themselves. The inhalation exposure of our study was to aged and diluted sidestream smoke, used as a surrogate of environmental tobacco smoke (ETS) that does not require smokers.

Platelet-rich plasma preparation

Platelet-rich plasma (PRP) was prepared by the method of Lunkes et al. (2004), with the following minor modifications.

Total blood was collected from cardiac puncture with 0.12 *M* sodium citrate as anticoagulant. The total blood–citrate system was centrifuged at 160 × *g* for 15 min. The PRP was centrifuged at 1400 × *g* for 30 min and washed twice by centrifugation at 1400 × *g* for 10 min with 3.5 m*M* HEPES isomolar buffer containing 142 m*M* NaCl, 2.5 m*M* KCl, and 5.5 m*M* glucose. The washed platelets were resuspended in HEPES isomolar buffer and adjusted to 0.4–0.6 mg of protein per milliliter.

NTPDase and 5′-nucleotidase assays

The NTPDase enzymatic assay was carried out in a reaction medium containing 5 m*M* $CaCl_2$, 100 m*M* NaCl, 5 m*M* KCl, 6 m*M* glucose, and 50 m*M* Tris-HCl buffer, pH 7.4, at a final volume of 200 μl as described by Pilla et al. (1996). Twenty microliters of the enzyme preparation (8–10 μg of protein) was added to the reaction mixture and the pre-incubation proceeded for 10 min at 37°C. The reaction was initiated by the addition of ATP or ADP at a final concentration of 1.0 m*M*, and the time of incubation was 60 min. For AMP hydrolysis, assay of 5′-nucleotidase activity was carried out as previously described, except that 5 m*M* $CaCl_2$ was replaced by 10 m*M* $MgCl_2$ and 2 m*M* AMP was added. Both reactions were stopped by the addition of 200 μl of 10% trichloroacetic acid (TCA) to provide a final concentration of 5%. After this, the inorganic phosphate released by ATP, ADP, and AMP hydrolysis was determined in triplicate by the method of Chan et al. (1986) using KH_2PO_4 as standard. The same process was carried out on control tubes to exclude non-enzymatic hydrolysis, by adding 20 microliters of protein to the reaction medium after TCA. The results are expressed as nmol inorganic phosphate released/minute/milligram of protein (nmol Pi released/min/mg protein).

Adenosine deaminase assay

Adenosine deaminase (ADA) in plasma was determined according to Giusti and Galanti (1984). Briefly, 50 μl of plasma was reacted with 21 m*M* adenosine, pH 6.5, and incubated at 37°C for 60 minutes. This method is based on the direct production of ammonia when ADA acts in an excess of adenosine. Results are expressed in units per liter (U/L). One unit (1 U) of ADA is defined as the amount of enzyme required to release 1 μmol of ammonia per minute from adenosine at standard assay conditions.

Protein determination

Protein was measured by the Coomassie Blue method according to Bradford (1976), using serum albumin as standard.

Blood gases, pH, and carboxyhemoglobin

To evaluate the reproducibility of this model of exposure to aged and diluted sidestream smoke, blood gas and pH, and carboxyhemoglobin (HbCo) were measured. Blood gas is expressed in terms of partial pressure of CO_2 ($PaCO_2$), partial pressure of oxygen (PaO_2), carbonate ion (HCO_3^-), total CO_2 (tCO_2), and saturation of oxygen (sO_2). The rats were previously anesthetized with ketamine chlorhydrate and xylazine (5:1; 0.1 ml/100 g), and the fur on the ventral aspect of the neck was clipped. The rats were then placed in the dorsal position, and antisepsis was carried out with alcohol–iodine–alcohol, after which the skin was cut, and the sternocleidomastoid muscle was pushed back with microsurgical instruments in order to identify the right common carotid artery by surgical microscope with a magnification of ×10; this was then isolated with mononylon 6.0. Then, arterial blood was collected by puncture with an insulin needle (0.45 × 13). The syringe was prepared in advance with a solution of sodium heparin, 5000 IU (100 μl/2 ml blood). Immediately after collection, the arterial blood was placed under ice until it was read in a Radiometer analyzer (model ABL-5; Copenhagen).

Platelet aggregation ex vivo

Blood samples were drawn, usually between 8:30 and 10:00 a.m., by cardiac puncture using sodium citrate 0.129 *M* as anticoagulant (1:9). The tests started within 3 hours after puncture and the samples were kept at room temperature (24–27°C). Platelet aggregation was measured using the method of Born and Cross (1963), by measuring turbidity with a Chrono-log optical aggregometer, with AGGRO/LINK® Model 810-CA software for Windows version 5.1. The preparation of platelet-rich plasma (PRP) was obtained by centrifugation of blood for 10 min at 500 rpm, and preparation of platelet-poor plasma (PPP) was obtained by centrifugation of the sample at 2400 rpm for 20 minutes. Aggregation was measured at 37°C and is expressed as the maximal percent change in light transmittance from baseline at 6 min after addition of the agonist, with platelet-poor plasma as a reference.

Anatomopathologic analysis of tissue lung

To confirm this model of exposure, an anatomopathologic analysis of lung tissue was carried out. Samples of lung tissue, ex vivo, were collected and fixed in 10% formalin solution, and then dehydrated and embedded in paraffin, followed by sectioning and histological staining with hematoxylin and eosin (H&E) (Tolosa & Rodrigues et al., 2003). The slides were observed in an optical microscope (×400) to check for possible changes in the lung tissue indicative of exposure to cigarette smoke.

Statistical analysis

Student's *t* test was used to evaluate differences between groups. A *p* value of <0.05 was considered statistically significant. Statistical analysis was performed with an SPSS software package (SPSS 10.0; Chicago, IL). All data are expressed as mean ± SEM.

Results

The results obtained for NTPDase activity are shown in Figure 1. As can be observed, platelet NTPDase activity with ATP as substrate was increased, and Student's *t* test revealed that ATP hydrolysis was significantly higher in group II (58.9%)

($p<0.05$) when compared to group I (Figure 1A). However, when ADP was used as substrate Student's *t* test revealed no significant difference between the groups (Figure 1B).

In addition, the results obtained for 5′-nucleotidase activity were similar to those found for NTPDase activity with ATP as substrate. As shown in Figure 1C, AMP hydrolysis was also altered, and Student's *t* test revealed that AMP hydrolysis was significantly increased in group II (141.7%) ($p<0.05$) when compared with group I.

The results obtained for ADA activity are shown in Figure 2. As can be seen, adenosine hydrolysis was altered, and Student's *t* test revealed that ADA activity in the plasma was significantly lower in group II (56%) ($p<0.05$) when compared to group I.

A significant increase in platelet aggregation was observed in group II ($p<0.05$) when compared with group I (Figure 3).

The analysis of blood gas, pH, and carboxyhemoglobin from control rats and those exposed to aged and diluted sidestream smoke are shown in Table 1. A significant increase in total CO_2 gas and carboxyhemoglobin ($p<0.05$) was observed in group II when compared to group I.

The pathological analysis of lung tissue revealed discrete architectural disorganization with small areas of pulmonary emphysema. In the wall of some bronchioles there was evidence of chronic inflammatory infiltrate with

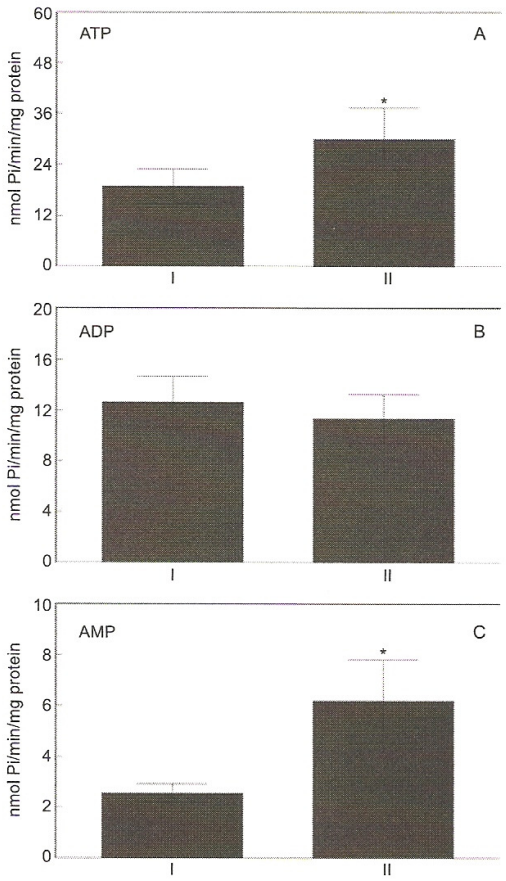

Figure 1. NTPDase activity in platelets of rats using ATP (A) and ADP (B) as substrate and 5′-nucleotidase activity in platelets of rats using AMP as substrate (C) after 4 weeks of exposure to cigarette smoke: group I (control) and group II (exposed to smoke). Enzyme activity is expressed in nmol inorganic phosphate (Pi) released/min/mg protein. Bars represent mean value ± SEM (*$p<0.05$; $n=10$).

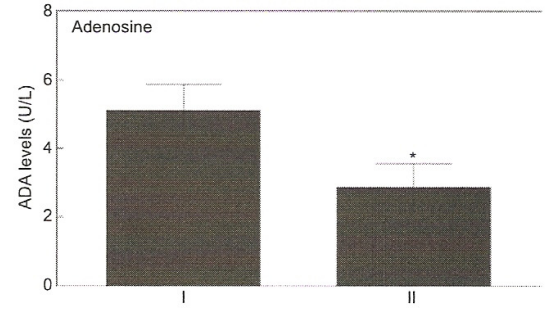

Figure 2. Adenosine deaminase (ADA) activity in plasma of rats using adenosine as substrate after 4 weeks of exposure to cigarette smoke: group I (control) and group II (exposed to smoke). This method is based on the direct production of ammonia when ADA acts in an excess of adenosine. Bars represent mean value ± SEM (*$p<0.05$; $n=10$).

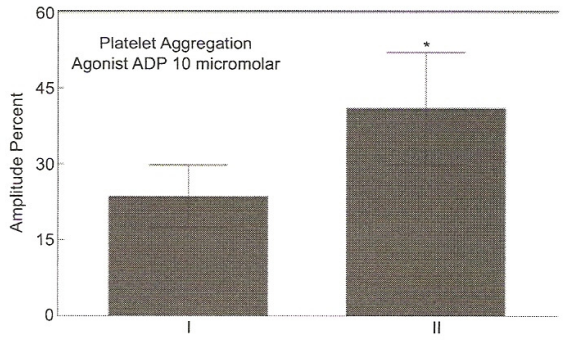

Figure 3. Aggregation of platelets in rats after 4 weeks of exposure to cigarette smoke: group I (control) and group II (exposed to smoke). Bars represent mean value ± SEM (*$p<0.05$; $n=10$).

Table 1. Measurement of gas and pH in arterial blood of rats after 4 weeks of aged and diluted sidestream smoke exposure: group I (control) and group II (exposed to smoke).

	Group I	Group II	*p* value
pH	7.31 ± 0.05	7.35 ± 0.03	0.0947
$PaCO_2$	50.50 ± 6.14	48.29 ± 5.77	0.4863
PaO_2	59.13 ± 11.84	64.86 ± 14.31	0.4110
HCO_3^-	24.13 ± 1.81	26.14 ± 2.85	0.1209
tCO_2	26.38 ± 2.56	29.72 ± 1.80*	0.0129
sO_2	85.88 ± 6.29	89.71 ± 5.82	0.2440
HbCo	0.56 ± 0.28	6.38 ± 0.81*	0.6116

Note. Partial pressures $PaCO_2$ and PaO_2 are expressed in mmHg; HCO_3^- and total carbon (tCO_2) are expressed in nmol/l; oxygen saturation (sO_2) is expressed in %; carboxyhemoglobin (HbCo) is expressed in mg/dl. Values represent mean ± SEM (*$p<0.05$; $n=10$).

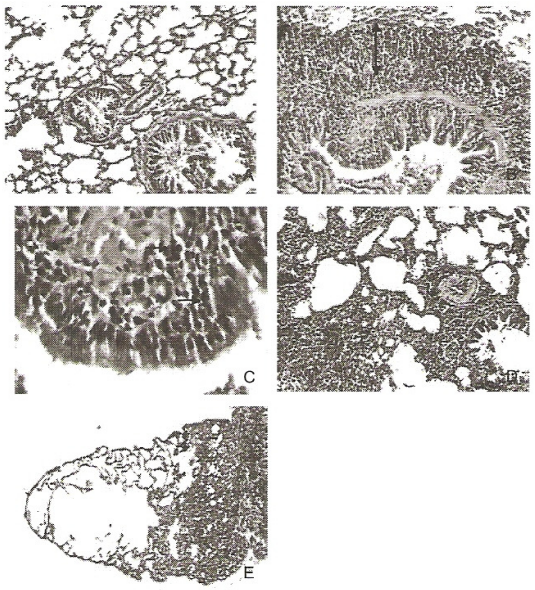

Figure 4. (A) Normal histological aspect of rat lung tissue, H&E (×100). (B) Image of rat lung tissue exposed to cigarette smoke, H&E (×100). The double-headed arrow indicates the extent of the bronchiole wall showing chronic inflammatory peribronchiolar infiltrate. (C) Higher magnification of (B), H&E (×400). (D) Chronic inflammatory peribronchiolar, perivascular, and septal infiltrate in rat lung tissue exposed to aged and diluted sidestream smoke. (E) An area of pulmonary emphysema, H&E (×100).

a predominance of lymphocytes and rare macrophages, which extended to the adjacent alveolar septa. In conclusion, histological sections of lung showed isolated areas of bronchiolitis with chronic inflammatory peribronchiolar (Figure 4B and C) as well as perivascular and septal (Figure 4D) infiltrate and some areas of pulmonary emphysema (Figure 4E). The normal lung tissue of controls is shown in Figure 4A.

Discussion and conclusions

Many studies have reported that acute and chronic effects of cigarette smoking induce proaggregatory conditions. However, there are no studies in the literature correlating the effect of subacute exposure to cigarette smoke on the activities of NTPDase and 5′-nucleotidase in platelets and adenosine deaminase in plasma of rats. These families of enzymes play an important role in the maintenance of adequate vascular hemostasis and thrombogenesis, mainly by regulating the platelet aggregation status (Kawashima et al., 2000; Marcus et al., 2003).

There is consistent evidence indicating that the damage caused by exposure to cigarette smoke may promote changes in the activity of NTPDase, 5′-nucleotidase, and adenosine deaminase. Our study shows an increase in NTPDase activity with ATP as substrate (Figure 1A). This may be due to the oxidative stress caused by cigarette smoke often occurring in extrapulmonary tissues (Bohmer et al., 2003; Ardite et al., 2005). This process enhances the level of ADP and causes the release of thromboxane A and serotonin, all three of which cause platelet aggregation.

Smokers with established macrovascular disease are reported to have platelet abnormalities (Mundal et al., 1998). In the present study, we did not find any change in the hydrolysis of ADP to AMP. Some studies suggest that ATP can be hydrolyzed directly to AMP in plasma without the intermediate formation of ADP (Goding, 2000; Birk et al., 2002a). The formation of ADP results in a proaggregatory action. In addition, AMP can be rapidly hydrolyzed by plasma 5′-nucleotidase to adenosine, a potent inhibitor of platelet aggregation (Birk et al., 2002b). Furthermore, cigarette smokers have more spontaneous platelet aggregates induced by epinephrine or ADP than non-smokers. Sensitivity to platelet aggregability may be enhanced by epinephrine or ADP (Fusegawa & Handa, 2000). Thus, we suggest that ATP may be hydrolyzed to AMP in platelets, which in turn is immediately catalyzed to adenosine. This might be a way of promoting hemostasis when there is high blood platelet aggregation caused by cigarette smoke. The analysis of our data showed an increase in platelet aggregation (agonist 10 μM ADP) in rats exposed to cigarette smoke (Figure 3).

Cigarette smoke consists of many chemicals, including nicotine, tar, with its many carcinogens, and gaseous compounds including carbon monoxide (CO) and carbon dioxide (CO_2). Nicotine has well known acute and chronic cardiovascular effects, mainly through sympathetic activation, although the contribution of nicotine per se in cardiovascular morbidity and mortality induced by cigarette smoking is uncertain (Benowitz & Gourlay, 1997).

One important result observed in this study was increased hydrolysis of AMP and a subsequent increase in the concentration of adenosine in platelets (Figure 1C). Adenosine helps to protect cells and tissues during stress conditions such as ischemia or anoxia (Downey & Forman, 1993), and the main mechanism by which carbon monoxide (CO) from smoke causes heart disease is the production of hypoxia (Zevin et al., 2001), which is implicated in the anti-inflammatory mechanisms (Cronstein, 1994). Following this line of thought, we believe that the increase in concentration of adenosine may contribute to compensate for the substantial activation of platelets and help to protect tissue damage by smoke. A possible role for adenosine during hypoxia may include vasodilatation. Thus, the increase in adenosine tissue concentrations during hypoxia may promote blood flow to hypoxic tissues, thereby providing an innate protective response to hypoxia (Colgan et al., 2006). In addition, a reduction in the hydrolysis of plasma adenosine, encouraging the accumulation of this nucleoside, was demonstrated in this study.

Thus, there is a strong association between cigarette smoking and cardiovascular disease (Whiss et al., 2000). Smoking can increase rates of cardiovascular mortality and morbidity associated with a higher prevalence of common

diseases such as atherosclerosis (Yanbaeva et al., 2007). This study has demonstrated the effects of aged and diluted sidestream smoke on platelet function, whereby exposure increased the stickiness of platelets. In general this can lead to myocardial infarction or might contribute to ischemic heart disease.

To evaluate the reproducibility of the model of exposure to aged and diluted sidestream smoke, blood gas, pH, and carboxyhemoglobin were measured and an anatomopathologic analysis of lung tissue was carried out. Histological sections of lung showed isolated areas of bronchiolitis with chronic inflammatory peribronchiolar, perivascular, and septal infiltrate and some areas of pulmonary emphysema (Figure 4). This process is associated with greater alveolar CO_2 retention, due to cigarette smoke, demonstrated by the high amount of carboxyhemoglobin biomarker in the blood (Table 1). This parameter of carboxyhemoglobin was used to confirm the efficiency of the animals' inhalation due to exposure to aged and diluted sidestream smoke. In addition, we expected a low pH due to $PaCO_2$. However, there was no alteration of pH. Blood gas ($PaCO_2$, PaO_2, HCO_3^-, and sO_2) also proved to be statistically unchanged. These parameters were unchanged due to the compensatory mechanism activated by the buffering system to maintain blood hemostasis (Smith & Taylor, 2007; Tan & Campbell, 2007). In addition, the fact that the protocol was subchronic, and thus may not have caused prominent injury in the lung, may explain the lack of alteration of these data. This buffering system acts as an immediate control of pH, but there are other compensatory mechanisms that also control pH, such as renal compensatory methods (Smith & Taylor, 2007). On the other hand, total carbon dioxide (tCO_2) from blood gas was increased in the group exposed to cigarette smoke, demonstrating that the animals had been exposed to the smoke (Table 1).

In conclusion, our study has demonstrated an alteration of ATP and AMP nucleotide hydrolysis in rat platelets and adenosine nucleoside hydrolysis in rat plasma, caused by aged and diluted sidestream smoke exposure. We suggest that these alterations in activity of enzymes from the purinergic system are associated with the increase in platelet aggregation observed in this study. However, our study also demonstrated that the organism attempts to compensate for this enhancement of aggregation by increasing AMP hydrolysis and reducing the hydrolysis of adenosine, a potent inhibitor of aggregation and an important modulator of vascular tone. Thus, we suggest that future studies be carried out in order to increase our understanding of the effects of cigarette smoke on platelet coagulant status.

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3.2 Artigo 2

Nicotina altera as atividades das ectonucleotidases em linfócitos: estudo *in vivo* e *in vitro*

Nicotine alter the ectonucleotidases activities in lymphocytes: *in vivo* and *in vitro* studies

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Nicotine alter the ectonucleotidases activities in lymphocytes: *in vitro* and *in vivo* studies

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Summary

The aim of the present study was to investigate the effects *in vivo* and *in vitro* of nicotine, an important immunosuppressive agent, on NTPDase and ADA activities in lymphocytes of adult rats. The following nicotine concentrations *in vivo* study were evaluated: 0, 0.25 and 1.0 mg/kg/day injected subcutaneously in rats for ten days. The activity of the enzymes were significantly decreased with nicotine 0.25 and 1 mg/kg which inhibited ATP (22%, 54%), ADP (44%, 30%) hydrolysis and adenosine (43%, 34%) deamination, respectively. The expression of the protein NTPDase in rat lymphocytes was decreased to nicotine 1mg/kg and the lymphocytes count was decreased in both nicotine concentrations studied. The purine levels measured in serum of the rats treated with nicotine 0.25mg/kg significantly increased to ATP (39%), ADP (39%) and adenosine (303%). The nicotine exposure marker was determinate by level of cotinine level which significantly increased in rats treated with nicotine 0.25 (39%) and 1 mg/kg (131%) when compared to rats that received only saline. The second set of study was *in vitro* assay which the ATP-ADP-adenosine hydrolysis were decreased by nicotine concentration 1 mM (0% - 0% - 16%, respectively), 5 mM (42% - 32% - 74%, respectively), 10 mM (80% - 27% - 80%, respectively) and 50 mM (96% - 49% - 98%, respectively) when compared with the control group. We suggest that alterations in the activities of these enzymes may contribute to the understanding of the mechanisms involved in the suppression of immune response caused by nicotine.

***Keywords**:* NTPDase, adenosine deaminase, nicotine, lymphocytes.

Abbreviations: NTPDase (ecto-nucleoside triphosphate diphosphohydrolase, CD39, E-NTPDase1); ADA (adenosine deaminase); ATP (adenosine triphosphate); ADP (adenosine diphosphate); AMP (adenosine monophosphate).

Introduction

Considerable effort has been expended in identifying the health effects of smoking, and a multitude of studies have suggested that exposure to cigarette smoke is ultimately detrimental to the immune system [1]. Chronic inhalation of cigarette smoke alters a wide range of immunological functions and many components of cigarette smoke such as nicotine might have immunomodulatory effects [1,2]. Nicotine is a major psychoactive compound and main immunosuppressive constituent of cigarette smoke, which inhibits both the innate and adaptive immune responses [3]. There is evidence that nicotine administration a dose-dependent and the mode of drug delivery decrease in the proliferative response of peripheral blood lymphocytes and induction of antibody-forming cells [4].

The regulation of immune responses also can be modulated by extracellular nucleotides (purines) which constitute a ubiquitous family of messengers that exert autocrine or paracrine actions [5]. Nucleotides, such as ATP, are known to modulate the immune and inflammatory responses [6] by exerting a variety of effects on lymphocytes [7]. Adenine nucleotides (ATP, ADP, and AMP) and their nucleoside derivative, adenosine, are important signaling molecules that mediate diverse biological and pathological processes [8,9]. Their release in fluids results from cell lysis, exocytosis of nucleotide-concentrating granules (synaptic vesicles, platelets-dense bodies), or efflux from cytoplasm through membrane transport proteins. They induce a wide spectrum of biological effects that are mediated by P2 purinergic receptors [10,11,12].

Nucleotides and nucleosides after exerting their function through purinoreceptors, are hydrolyzed in the extracellular medium by enzymes such as NTPDase (ecto-nucleoside triphosphate diphosphohydrolase, CD39, E-NTPDase1) and ADA (adenosine deaminase), respectively [13,14]. NTPDase hydrolyzes ATP and ADP to AMP, which is subsequently converted to adenosine by 5’-nucleotidase [15]. Adenosine can be directly inactivated through the sequential action of ADA, which catalyzes the irreversible deamination of adenosine. This enzyme is involved in controlling adenosine levels, leading to inosine production [16].

Thus, the duration and magnitude of purinergic signaling is governed by nucleotide-degrading ectoenzymes. In several tissue and cells, NTPDase operate jointly with other enzymes forming a “complex cell surface-located” nucleotide

hydrolysis and conversion mechanism [7]. These ectonucletidases form an enzymatic cascade which is physiologically important for signaling, as well as pathologically, situations that result in adenine nucleotide and nucleoside degradation in their microenvironment [16].

Considering that nicotine has some effects on the immune system and the NTPDase and ADA are important enzymes related to lymphocytes function, the aim of this study was to investigate the association of the immunologically suppression of NTPDase and ADA activities in lymphocytes of rats in an experimental model of immunosuppression using nicotine, which the data might help to comprehend this mechanisms in smokers.

Materials and Methods

Reagents

Nucleotides, Trizma Base, Percoll, HEPES and Comassie brilliant blue G were obtained from Sigma-Aldrich (St. Louis, MO, USA). Nicotine 98% was obtained from Aldrich (Germany). ENTPD1 rabbit polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, Inc). Laemmli sample buffer and polyvinylidine difluoride (PVDF) membrane were obtained from Bio-Rad Laboratories. All other chemicals used in this experiment were of the highest purity.

Animals

Male Wistar rats (90 days old) (30 animals) obtained from the General Animal House of the UFSM were transferred to our colony room and maintained in groups of 6 in opaque plastic cages, at room temperature (21-24 ºC). The animals were maintained on an inverted 12 h light/dark cycle, and they had free access to food and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources (COBEA) and are in accordance with international guidelines and, the project is registered in the Committee of Ethics of the Federal University of Santa Maria with number 23081.006211/2008-64.

In vitro and *in vivo* experiments with nicotine

For the *in vitro* studies were used 12 animals that were anesthetized with halothane and submitted to euthanasia. The whole blood was collected and the lymphocytes were separated. The lymphocytes were incubated with different concentrations of the nicotine (1, 5, 10 and 50 mM) and the biochemical parameters were determinates.
For the studies *in vivo* was used 18 animals that were randomly divided into three groups (n=6): I control (saline); II (nicotine 0.25 mg/kg/day); and III (nicotine 1.0 mg/kg/day). The nicotine was administered by subcutaneous (S.C.) injections in the dose of the 0.25 mg/kg/day in animals of the group II and in the dose of the 1.0 mg/kg/day in the animals of the group III, while that the animals the group I were treated with saline 0.9%. The period of the treatment was the 10 days and the administration of solutions was done in the light period of the cycle at the 8 a.m. After 10 days of treatment, the animals were anesthetized with halothane and submitted to euthanasia about 90 min after the last dose of nicotine or saline. The whole blood was collected and the lymphocytes were separated for biochemical assays.

Isolation of mononuclear cells from rat whole blood

Mononuclear leukocytes were isolated from rat blood collected with EDTA and separated on Ficoll-Hypaque density gradients as described by Böyum (1968) [17].

Cellular integrity

The activity of lactate dehydrogenase (LDH) was used as a marker of cell integrity. The measurement of LDH activity showed that most cells (approximately 85%) were intact after the isolation procedure (data not shown). The integrity of the cells after incubation was confirmed by microscopic observations in control samples and samples with different treatment doses (results not shown).

NTPDase assay for lymphocytes

After the isolation of mononuclear cells, NTPDase activity was determined by colorimetric assay in compliance with Leal et al. (2005) [18]. The reaction medium contained 0.5 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 6 mM glucose, and 50 mM Tris-HCl buffer, pH 8.0 at a final volume of 200 µL. Twenty microliters of intact

mononuclear cells suspended on saline solution were added to the reaction medium (2-4 mg protein) and preincubated for 10 min at 37 ºC. The reaction was started by the addition of substrate (ATP or ADP) at a final concentration of 2 mM and stopped with 200 µL 10% trichloroacetic acid (TCA) to provide a final concentration of 5%. The samples were chilled on ice for 10 min before assaying for the release of inorganic phosphate (Pi) as described for Chan et al. (1986) [19], using malachite green as a colorimetric reagent and H_2PO_4 *as standard. All samples were run in duplicate or triplicate and specific activity is reported as nmol Pi released/min/mg of protein.*

Adenosine Deaminase assay for lymphocytes

Lymphocytes ADA activities were estimated spectrophotometrically by the method of Giusti (1974) [20], which is based on the direct measurements of the formation of ammonia, produced when ADA acts in excess of adenosine. The samples of lymphocytes were added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 6.5). The samples were incubated at 37 ºC for 1 h and the reaction was started by addition of the substrate (adenosine). The reaction was stopped by adding the samples on a 106 mM/0.16 mM phenol-nitroprusside/mL. The reaction mixtures were immediately mixed to 125 mM/11 mM alkaline-hypochlorite (sodium hypochlorite) and vortexed. 75 mM ammonium sulphate was used as ammonium standard. All samples were run in duplicate and activity is reported as U/mg of protein.

Cotinine quantification

The cotinine quantification in plasma samples was realized by HPLC-UV, following the method of Cattaneoa et al. (2007) [21] after modifications as described following. The chromatographic equipment consisted of a chromatography system Agilents®, 1100 series, and injector with loop of 20 µL and UV-vis detector. The separation was a chieved using a reverse-phase column: Zorbax Eclipse column XDB-C8 (4.6 mm x 150 mm) Agilents® with 5 µm particle size. The mobile phase was a mixture of Milli-Q water: methanol: sodium acetate 0.1 M:acetonitrile (50:15:25:10, v/v), being added 1 mL of citric acid 0.034 mol/L and 5.0 mL of triethylamine for each liter of solution, pH

4.4, adjusted with acetic acid. The flow rate of 1 mL/min was maintained isocratically, the absorbance of the eluent was monitored at 260 nm and the total run time was 8 min. The internal standard was used 2-phenylimidazole. The detection limit of the method was 5 ng/mL and the limit of the quantification was 10 ng/mL. A volume of 500 µL of plasma was added to 25 µL of NaOH 10 M and 100 µL of internal standard (1 µg/mL). Then, the extraction with 2.0 mL of dicloromethane was carried out by vortex-mixed for 1 min and centrifuged at 3000 x g for 10 min. The organic phase was totally dried with nitrogen at ambient temperature. Following, was added 100 mL of the mobile phase and 20 µL was injected in to HPLC.

Protein determination

Protein was measured by the Coomassie blue method according to Bradford (1976) [22] using serum albumin as standard.

Western blot of protein NTPDase

Electrophoresis was performed using 12% polyacrylamide in a Bio-Rad Mini-Protean III apparatus. For Western blotting assays, peripheral blood lymphocytes were lysated inside microtubes containing an extraction buffer (50 mM Tris HCl, 1 mM EDTA, 1 mM PMSF, pH 7.5) in the presence of Triton ® X-100 (0.5%, v/v), glass pearls and vortexed for a minute, twice, on ice. Samples were centrifuged at 10000 x g for 20 min at 4^{o}C. The protein present in the supernatant, determined by colorimetric assay [22], was diluted (1:1, v:v) in the Bio-Rad Laemmli sample buffer (62.5 mM Tris HCl, pH 6.8; 25% glycerol, 2% SDS, 0,01% Bromophenol Blue) added 5% of mercaptoethanol and then loaded (10 µg) and size-separated in 15% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE, 100V). The running buffer used contained 25 mM Tris, 192 mM glycine, and 0,5% SDS. The proteins were blotted onto a polyvinylidine difluoride (PVDF) membrane for 1h (Bio-Rad) in blotting buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. Subsequently, the membrane was incubated with anti-rat ENTPD1 polyclonal antibody (primary antibody used at a dilution of 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1 h. The sensitivity and specificity of this antibody for rat antigen has been previously validated. The amount of protein was

corrected in order to load a fixed concentration of protein (10 ug) in 12% SDS-PAGE, and it was determined based on preliminary experiments by using different concentrations of proteins. To ensure equal protein loading, we used the Ponceau method to Western blot (Abercrombie et al., 1989). Membranes were developed using the substrate of alkaline phosphatase, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

Analysis of purines levels in serum by high performance liquid chromatography (HPLC)

The denaturation of sample proteins was performed using 0.6mol/L perchloric acid. All samples were then centrifuged (14000 × g for 10min) and the supernatants were neutralized with 4.0 N KOH and clarified with a second centrifugation (14000 × g for 15 min) [23]. High performance liquid chromatography (HPLC) was performed with a Shimadzu (Kyoto, Japan) equipment composed of a model LC-20AT reciprocating pumps, a model DGU-20A5 degasser, a diode array detector (DAD) model SPD-M20A, auto-sampler (SIL-20A) and model CBM-20A integrator, operated by software LC Solution 1.22 SP1. Separation was achieved with a Phenomenex Synergi 4µ Fusion RP-80A column (150 x 4.60 mm, 4 µm) with precolumn, using 0.04 M potassium dihydrogen orthophosphate (KH_2PO_4) and 0.06 M dipotassium hydrogen orthophosphate (K_2HPO_4) as mobile phase A and acetonitrile as mobile phase B. A gradient elution was used according to the specifications of Scherer et al. (2005) [24], at a flow rate of 0.7 mL/min. Mobile phases were filtered through a 0.45 µm Millipore filter prior to analysis, and all the reagent utilized were of HPLC grade. Purines in the samples (ATP, ADP, AMP and adenosine) were identified by their retention times and DAD spectrum (in the range 200-400 nm), and quantified by comparison of the peak's area with standards. The results are expressed by *p*mol of the different compounds per mL of serum.

Statistical analysis

Data were analyzed by analysis of variance (One-way ANOVA) followed by the Duncan multiple range test, and $P<0.05$ was considered to represent a significant difference in the analysis. All data were expressed as mean±S.E.M.

Results

In vivo assay

The results obtained in the present study show that both NTPDase and ADA activity and expression were altered in lymphocytes from nicotine-treated rats. One-way ANOVA, followed by Duncan's multiple range tests revealed that activity of NTPDase with ATP as substrates (Figure 1-A) was significantly decreased in the concentrations 0.25 mg/kg/day (22% inhibition) and 1.0 mg/kg/day (54% inhibition) of nicotine when compared with the control group ($P < 0.05$). The NTPDase activity using ADP as substrates (Figure 1-B) also was significantly decreased in the concentrations 0.25 mg/kg/day (44% inhibition) and 1.0 mg/kg/day (30% inhibition) of nicotine when compared with the control group ($P < 0.05$). In the lymphocytes the results obtained for ADA activity in vivo assay with adenosine as substrate significantly decreased in the concentration of 0.25 mg/kg/day (43% inhibition) and 1.0 mg/kg/day (34% inhibition) of nicotine when compared with the control group ($P < 0.05$) (Figure 1-C). Statistical analysis of the content of NTPDase-positive cells by Western blot quantification using labeled antibodies (ENTPD1) against NTPDase revealed that lymphocytes from rats treated with nicotine 1 mg/kg had a significant decreased in NTPDase expression when compared with control group ($P < 0.05$) (Figure 2-A,B). In addition, purines levels measurement in the serum of control animals and treated animals with nicotine were measured by HPLC. The levels of ATP, ADP and adenosine were significantly increased (39%, 39% and 303%, respectively) in animals treated with nicotine 0.25 mg/kg/day when compared to the control group ($P < 0.05$) (Table I). The peripheral blood lymphocyte counts in the animals treated with nicotine (0.25 and 1.0 mg/kg/day) and control group are showed in table II, where in the groups nicotine the number of lymphocytes were significantly decreased when compared to the control group ($P < 0.05$).

In vitro assay

In the lymphocytes the results obtained for NTPDase and ADA activities of rat in vitro assay are shown in table III. One-way ANOVA, followed by Duncan's multiple range tests revealed that activity of NTPDase with ATP as substrate significantly decreased

in the concentrations 5, 10 and 50 mM of nicotine (42%, 80% and 96%, respectively) ($P < 0.05$) when compared with the control group (nicotine 0 mM). When ADP was used as substrate the activity of NTPDase significantly decreased in the concentrations 5, 10 and 50 mM of nicotine (32%, 27% and 49%, respectively) ($P < 0.05$) when compared with the control group (nicotine 0 mM). In the lymphocytes the results obtained for ADA activity in vitro assay with adenosine as substrate significantly decreased in the concentration of 1, 5, 10 and 50 mM of nicotine (16%, 74%, 80%, 98%, respectively) when compared with the control group ($P < 0.05$).

Cotinine level

Table IV shows plasma cotinine level. Results demonstrated that rats treated for 10 days with nicotine (0.25 and 1.0 mg/kg/day) presented significantly increased level of cotinine (39.9±1.11 and 131.8±53.5, respectively) when compared to those treated with saline (control group) ($P < 0.05$).

Discussion and Conclusion

Alterations in the function of NTPDase have been reported in association with diseases in humans and animal models [25,18] including cigarette smoke which has a large amount of nicotine [9]. This ectonucleotidase (NTPDase) is also expressed on natural killer (NK) cells, monocytes, dendritic cells and subsets of activated T cells [26]. Regarding the some possible mechanisms that might mediate the effects of nicotine on the immune function we can cite the glucocorticoid hypersecretion produced by nicotine exposure, act directly on nicotine cholinergic receptors on lymphocytes in vitro, capacity of nicotine to stimuli the release of peripheral catecholamines, the action of nicotine at central and/or peripheral nicotinic-cholinergic receptors and, *inhibition of the expression of adhesion molecules [4,27]*. However, little attempt has been made to determine the potential contribution of nicotine to the effects on purinergic system in lymphocytes. The aim these study *in vivo* and *in vitro* was evaluate NTPDase and ADA activities in lymphocytes and the possible involvement of these enzymes in the regulation of immune function in nicotine-treated rats.

The immunosuppresion is observed after nicotine administration with a significant loss of antibody responses and T-cell proliferation and, the possible generation of homeostatic disorders [3]. Nicotine can suppression the immune system by many mechanisms such as glucocorticoid hypersecretion, act directly on lymphocytes by binding nicotinic acetylcholine receptors (nAChR) *in vitro*, release the peripheral catecholamines and nicotine also can intermediate its action at central and/or peripheral nicotinic-cholinergic receptors [4]. However, the modulation of immune system caused by nicotine and the involvement of the purinergic system has being little studied in lymphocytes.

To confirm the efficiency of the nicotine exposure the level of cotinine, exposure marker nicotine, was determined which in rats treated with nicotine was demonstrated significantly increased when compared to rats that received only saline (Table IV). This finding is to according with previous studies such as Cattaneoa et al. (2007) [21].

The results obtained of the *in vivo* study demonstrated that ATP, ADP and adenosine hydrolysis were significantly decreased in lymphocytes of rats treated for 10 days with nicotine in comparison to their respective control groups (Fig. 1). NTPDase plays an important role in lymphocytes as an activation marker essential for certain effectors functions, since extracellular nucleotides are mediators of immune and non-immune cell function [28,29]. Since ATP could accumulate in the extracellular milieu, and adenosine deamination is decreased by inhibition of adenosine deaminase activity, the immunodeficiency state may increase [30,31,32]. The cytolytic T-lymphocyte NTPDase activity has a protective effect against cell lysis caused by extracellular ATP. NTPDase activity is inhibited in nicotine treated animals, owing to its inability to remove extracellular ATP, the protective effect against cell lysis no was effective and the cytolytic mechanisms may act decreasing the number of lymphocytes showed in table II [32]. Moreover, Dombrowski et al. (1998) [28] reported that activated lymphocytes had NTPDase activity and, the decrease in NTPDase activity inhibited the effector functions of T, B and NK cells ie, the NTPDase is an activation marker that is essential for development of certain effector cell functions.

Our results demonstrate that the inhibition of the enzymes by nicotine increased the nucleotide ATP, ADP and nucleoside adenosine in serum of the rats treated with nicotine 0.25 mg/kg as demonstrated in table I. It is known that ATP

suppress the proliferation of peripheral lymphocytes and adenosine could incite apoptosis, contributing to the increase of the immunodeficiency state [33,34]. Besides, adenosine has anti-inflammatory and lymphotoxic effects, decreasing Class II MHC expression in macrophages and dendritic cells and lowering leukocyte adhesion [35]. However, ATP is considered pro-inflammatory agent, it induces an inflammatory response, and nicotine may be favorable regard to increase the ATP because nicotine inhibits the NTPDase. To the contrary the adenosine has immunosuppressive and anti-inflammatory effects then we may suggest that this decrease to ADA activity may be to defense mechanism of the organism against the effects of nicotine. In other words, greater amount of adenosine in the medium it could offset the pro-inflammatory effects of ATP.

Moreover, the enzyme NTPDase is expressed in numerous types of immune cells [36] and executes important role at control of lymphocytes function [37,29]. The observation that NTPDase expression is down-regulated in lymphocytes of the animals treated nicotine (1 mg/kg) (Fig. 2) also might contribute with immune response alterations [29]. Although the expression of NTPDase no change in nicotine 0.25 mg/kg, the inactivation of NTPDase produced by nicotine 1 mg/kg either by interaction in the active site or down-regulation in the expression.

Thus, we can speculate that the no change of purine (ATP, ADP and adenosine) levels in serum in the higher concentration of nicotine (1.0 mg/kg; Table I) *might be due to the hormetic effects (hormesis)* [38,39]*. This concept, which refers to the willful exposure to toxins in an attempt to develop immunity against them* [40]*, might be happening to hydrolysis of ADP, ADP and adenosine* in lymphocytes. Where, high doses of nicotine (1.0 mg/kg) no change purine level and we may suggest that a compensatory mechanism occur in order to maintain the appropriate level of purine [34].

In the last set of experiments, *in vitro* study, we evaluated the role of the nicotine on NTPDase and ADA activities in lymphocytes from rats. An important data observed in our *in vitro* study is that the nicotine per se promoted a decrease in adenine nucleotides hydrolysis in lymphocytes of rats (Table III). Our results demonstrated a decrease in the activity of NTPDase and ADA in both *in vivo* and *in vitro* assays, with nicotine 0.25 and 1.0 mg/kg/day (Figure 1) and 1, 5, 10 and 50 mM (Table III), respectively. All the concentration of nicotine tested decreased the ADA activity in vitro assay and, the highest concentration of nicotine (50 mM) promoted

the highest inhibition of the enzyme ($P < 0.05$) (Table III). These results suggest that the inhibition of enzymes by nicotine might be due to the allosteric interaction in the active site of the enzyme increasing the levels of purines. It is believed that both in vivo and in vitro study are in accordance, which nicotine promoted an increase in ATP and adenosine production which favorable effects immunosuppressant in biologic system. Thus we can suggest that one of the mechanisms by which nicotine exerts its degenerative effects may be through the modulation of the ectonucleotidase pathway in the lymphocytes.

In conclusion, the results obtained *in vivo* and *in vitro* studies demonstrate alterations in NTPDase and ADA activities in lymphocytes from rats, indicating that purinergic system is altered with nicotine. Our data propose that during treatment with nicotine there is an increase of ATP and adenosine in the extracellular medium. The hydrolysis of these molecules by enzymes such as NTPDase and ADA is very important for inactivation of its toxic effects on the cells and save them from cytolytic events. These findings may help to understand the mechanisms involved in the suppression of immune response caused by nicotine and become useful to smokers tobacco. However, more study is necessary and our next step will be the determination of kinetic parameters which appears to be involved with immunosuppressive agent, nicotine.

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LEGENDS OF FIGURES AND TABLES

Figure 1

(A) ATP hydrolysis in lymphocytes of rats *in vivo* treated during period of 10 days with nicotine in the concentrations of 0.25 and 1.0 mg/kg/day. (B) ADP hydrolysis in lymphocytes of rats *in vivo* treated during period of 10 days with nicotine in the concentrations of 0.25 and 1.0 mg/kg/day. Enzymes specific activities are reported as nmol Pi released/min/mg of protein. (C) Adenosine deaminação in lymphocytes of rats *in vivo* treated during period of 10 days with nicotine in the concentrations of 0.25 and 1.0 mg/kg/day. Enzyme activities are reported as U/mg of protein. Bars represent mean±S.E.M. *Significant difference from control activity by one-way ANOVA (P<0.05) (n=6).

Figure 2

(A) Expression of the protein NTPDase in rat lymphocytes. Samples were loaded on an SDS-gel, and after electrophoresis, the proteins were blotted onto a polyvinilidine difluoride membrane and incubated with a polyclonal antibody against protein NTPDase. (B) Densitometric analysis (arbitrary units, A.U.) of the protein NTPDase. Results are representative of three individual experiments (n=3). Data are represented as the mean±S.E.M *P<0.05.

Table I. Purine level in serum of rats (n=6) treated with nicotine 0.25 and 1.0 mg/kg/day for 10 days measured by HPLC. The levels of ATP, ADP and adenosine were significantly increased in nicotine 0.25mg/kg/day when compared to the control group (P < 0.05). The results are presented as mean±S.E.M. The levels of purines are reported as *p*mol/mL.*Significant difference from control level by one-way ANOVA (P<0.05).

Table II. Peripheral blood lymphocyte counts in the rats treated for 10 days with nicotine (0.25 and 1.0 mg/kg/day) and control group. The results are presented as mean±S.E.M. The numbers of lymphocytes are reported as unid/µl of blood. *Significant difference from control activity by one-way ANOVA (P < 0.05).

Table III. ATP and ADP hydrolysis in lymphocytes of rat *in vitro* assay in the final nicotine concentrations 1, 5, 10 and 50 mM and enzymes specific activities are reported as nmol Pi released/min/mg of protein. Adenosine deaminação in lymphocytes of rat *in vitro* assay in the final nicotine concentrations 1, 5, 10 and 50 mM and enzyme activities are reported as U/mg of protein. The results are presented as mean±S.E.M. *Significant difference from control activity by one-way ANOVA (P<0.05) (n=6).

Table IV. Plasma cotinine level of rats (n=6) treated with saline or nicotine (0.25, 0.5, 1.0 and 2.0 mg/kg/day) during period of 10 days. The results are presented as mean±S.E.M., ND = not detectable (detection limit = 5 ng/mL). *Significant difference from control activity by one-way ANOVA (P < 0.05).

Figure 1.

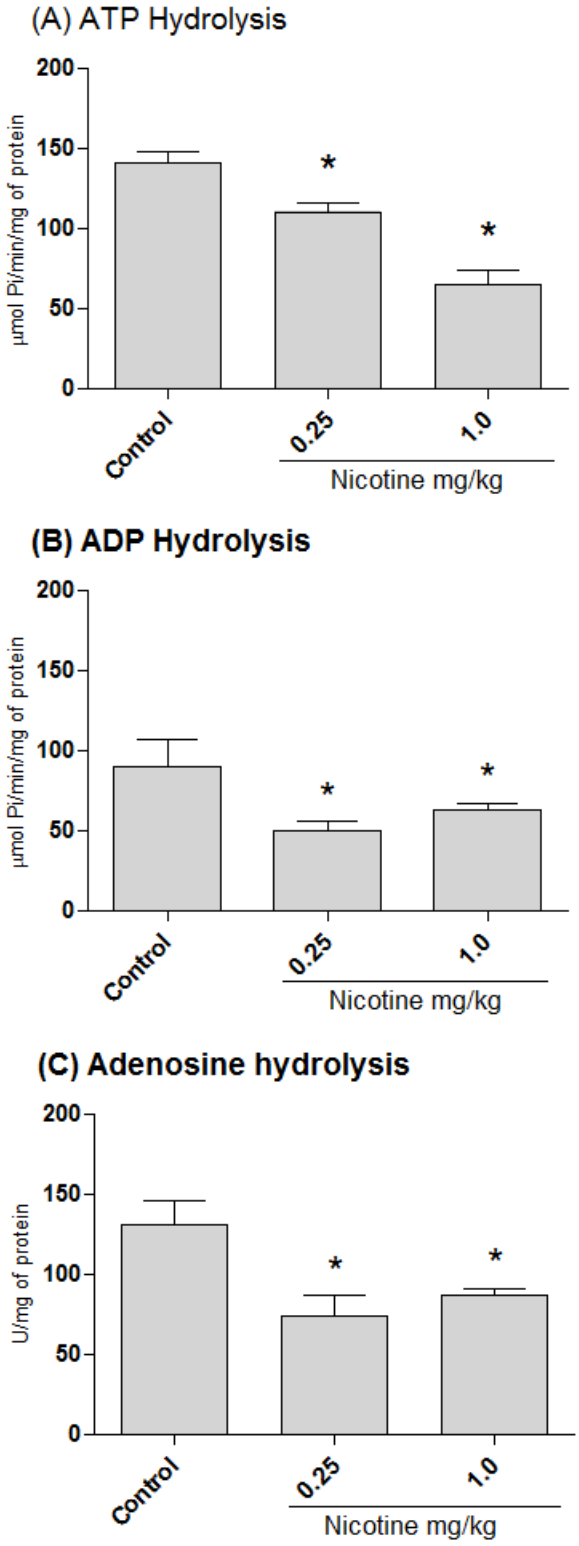
(A) ATP Hydrolysis
µmol Pi/min/mg of protein
200
150
100
50
0
*
*
Control
0.25
1.0
Nicotine mg/kg
(B) ADP Hydrolysis
µmol Pi/min/mg of protein
200
150
100
50
0
*
*
Control
0.25
1.0
Nicotine mg/kg
(C) Adenosine hydrolysis
U/mg of protein
200
150
100
50
0
*
*
Control
0.25
1.0
Nicotine mg/kg

Figure 2.

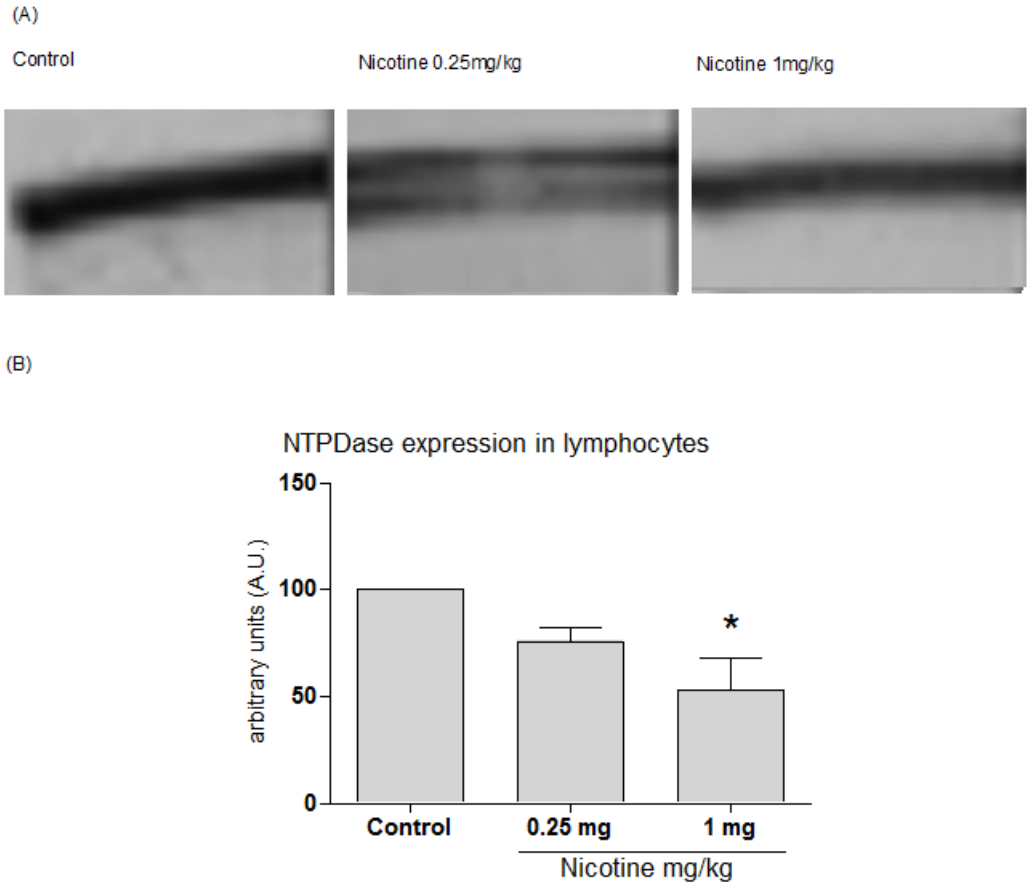
(A)
Control
Nicotine 0.25mg/kg
Nicotine 1mg/kg
(B)
NTPDase expression in lymphocytes
arbitrary units (A.U.)
150
100
50
0
*
Control
0.25 mg
1 mg
Nicotine mg/kg

Table I. Purines levels in serum.

Treatment (mg/kg)	**ATP** (*p*mol/mL)	**ADP** (*p*mol/mL)	**AMP** (*p*mol/mL)	**Adenosine** (*p*mol/mL)
Control (saline)	227.8±12.69	148.4±9.14	4.50±0.50	2.30±0.81
Nicotine 0.25	316.3±20.10*	204.1±6.53*	3.54±0.95	9.29±0.86*
Nicotine 1.0	261.5±13.80	146.9±9.90	4.00±0.83	2.84±1.64

Table II. Lymphocyte count.

Treatment (mg/kg)	Lymphocytes (unid/µL)
Control (saline)	4497.5±343
Nicotine 0.25	2910.0±416*
Nicotine 1.0	3242±381*

Table III. ATP, ADP and adenosine hydrolysis in lymphocytes of rat in vitro assay.

Treatment (mM)	ATP	ADP	Adenosine
Control (saline)	268.0±24.72	237.8±16.07	23.4±1.46
Nicotine 1	269.6±16.96	237.2±7.84	19.5±1.55*
Nicotine 5	155.7±7.81*	160.4±6.89*	5.9±2.54*
Nicotine 10	52.0±6.64*	173.4±22.01*	4.5±1.06*
Nicotine 50	9.9±0.79*	121.0±17.21*	0.3±0.08*

Table IV. Plasma cotinine level.

Treatment (mg/kg)	Cotinine (ng/mL)
Control (saline)	ND
Nicotine 0.25	39.9±11.1*
Nicotine 1.0	131.8±53.5*

3.3 Artigo 3

Vitamina E diminui a atividade da acetilcolinesterase e nível de peroxidação lipídica no encéfalo de ratos expostos à fumaça de cigarro diluída e envelhecida inalada por via secundária

Vitamin E decreased the activity of acetylcholinesterase and level of lipid peroxidation in brain of rats exposed to agend and diluted sidestream smoke

Gustavo Roberto Thomé, Rosélia Maria Spanevello, Alexandre Mazzanti, Amanda Maino Fiorenza, Marta Maria Medeiros Frescura Duarte, Sônia Cristina Almeida da Luz, Maria Ester Pereira, Vera Maria Morsch, Maria Rosa Chitolina Schetinger, Cinthia Melazzo Mazzanti.

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Original Investigation

Vitamin E Decreased the Activity of Acetylcholinesterase and Level of Lipid Peroxidation in Brain of Rats Exposed to Aged and Diluted Sidestream Smoke

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Abstract

Introduction: The biological systems of both smoker and passive smoking suffer changes caused by toxic compounds from cigarette smoke such as inflammation, lipid peroxidation, and deficiency of vitamin E. The aim of the present study was to evaluate the effect of vitamin E on acetylcholinesterase (AChE) activity and the lipid peroxidation level in the brain of rats in the model of exposure to aged and diluted sidestream smoke (ADSS).

Methods: Adult male Wistar rats (200–300 g) were exposed to ADSS for 4 weeks and treated with vitamin E (50 mg/kg/day) loaded by gavage. In the first, second, third, and fourth weeks, animals were concomitantly exposed to the smoke of 1, 2, 3, and 4 cigarettes/day, respectively. The duration of each exposure was 15 min, daily.

Results: For rats exposed to ADSS, the AChE activity and lipid peroxidation level increased in the striatum, cerebral cortex, and cerebellum. In contrast, the activity of AChE and the level of lipid peroxidation decreased in the smoke group treated with vitamin E.

Conclusions: The results suggest that the rats exposed to ADSS and treated with vitamin E significantly reduced the raised activity of AChE and level lipid peroxidation from the brain structures studied. The study, therefore, concludes that vitamin E could be considered as a therapeutic agent in this type of exposure.

Introduction

The sidestream of smoke contains not only the gas phase of exhaled smoke but also the products of combustion on the top of a cigarette. Therefore, persons exposed to environmental tobacco smoke (ETS) are exposed to up to 50 times higher concentration of some chemicals than smokers themselves (Domagala-Kulawik, 2008). The toxic associations of cigarette smoke observed with active smoking also appear to hold for passive smoking (Swan & Lessov-Schlaggar, 2007; Witschi, Joad, & Pinkerton, 1997). In animal models and the human condition, several studies have demonstrated that both mainstream and sidestream cigarette smoke exposure are associated with neurotoxic effects (Durazzo, Cardenas, Studholme, Weiner, & Meyerhoff, 2007; Swan & Lessov-Schlaggar, 2007). Exposure of rat brain to cigarette smoke results in an increase of reactive oxygen species and nitric oxide synthase leading to lipid peroxidation and protein oxidation (Swan & Lessov-Schlaggar, 2007).

Moreover, tobacco contributes to the aggravation of many diseases in various tissues such as damaging effects on the healing of wounds (Ejaz & Lim, 2006), neuropsychiatric disorders (Dome, Lazary, Kalapos, & Rihmer, 2010), repair process of DNA damage in lymphocytes (Jianlin et al., 2009), perinatal deaths and, later in life, higher rates of learning disabilities, behavioral problems, attention deficit, and hyperactivity disorder (Bell & Lau, 1995; DiFranza & Lew, 1995; Naeye, 1992). In terms of developmental liability, nicotine is one of the key components of cigarette smoke. The nicotine itself is a neuroteratogen with adverse effects on brain cell number, synaptic development, and neurobehavioral function (Slotkin, 1998, 2004). Nicotine may activate nicotinic receptors on neurons resulting in a release of acetylcholine changing cholinergic system in central nervous system (CNS; Rowell & Winkler, 1984).

Some common features in the pathogenesis of various smoking-associated diseases are inflammation (Anto, Mukhopadhyay,

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Shishodia, Gairola, & Aggarwal, 2002), lipid peroxidation (Anbarasi, Sabitha, & Devi, 2005, Anbarasi, Vani, Balakrishna, & Devi, 2005; Baskaran, Lakshmi, & Prasad, 1999; Delibas, Ozcankaya, Altuntas, & Sutcu, 2003; Pryor & Stone, 1993), and deficiency of vitamin E (Reiter, Jiang, & Christen, 2007). An increase in pro-inflammatory markers in all areas of the brain was also observed (Manna et al., 2006). Yet, the mechanism underlying the role of smoking in these diseases is presently unknown or poorly understood (Anto et al., 2002).

In the brain, the cholinergic systems provide diffuse innervations to practically all brain (Woolf, 1991), and the various subunit compositions of nicotine acetylcholine receptors (nAChRs) are widely distributed in the regions of striatum, cerebral cortex, cerebellum, and hipoccampus. The broad cholinergic innervations acting via nAChRs have been found to influence arousal, attention, sleep, fatigue, anxiety, central processing of pain, and a number of cognitive functions (Dani, 2001).

Vitamin E is the main and the most important lipid-soluble nonenzymatic antioxidant in biological membranes (Singh & Jialal, 2004; M. L. Thakur & Srivastava, 1996). Because of its structure and the localization on the surface of the lipoproteins and biological membranes, vitamin E may react with lipid peroxy free radicals as well as with free water-phase alkoxyl radicals, which frequently initiate the chain reaction of peroxidation. Several studies indicate that vitamin E is a molecule that exhibits potential anti-inflammatory activity (Grammas et al., 2004; S. N. Meydani, Han, & Wu, 2005; Reiter et al., 2007; Singh, Devaraj, & Jialal, 2005; Singh & Jialal, 2004) and neuroprotective properties (Onem et al., 2006). In fact, some studies have suggested a protective role for vitamins and antioxidants in modifying the major diseases related to cigarette smoking (Tiwari, 2004). However, the effects of vitamin E on the exposure aged and diluted sidestream smoke (ADSS) model have been little studied.

Considering that vitamin E is a potent antioxidant and neuroprotector, the main aim of this study was to evaluate the effects of vitamin E on acetylcholinesterase (AChE) activity and lipid peroxidation level in tissue of brain in the model of exposure by inhalation of cigarette smoke that was ADSS termed as a surrogate of ETS that does not require smokers.

Methods

Animals

Adult male Wistar rats (200–300 g) were used in this experiment. The 24 animals were kept on a 12-h light and 12-h dark cycle, at a temperature of 22 ± 2 °C, with free access to food and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources (COBEA) and are in accordance with international guidelines. The research project was approved by the Ethics Committee of the Federal University of Santa Maria, Brazil, by the number 23081.006211/2008-64.

Cigarette Smoke Exposure

The animals were randomly divided into four groups (six rats in each group): C (control, not exposed to ADSS), CV (control vitamin E, only was given vitamin E orally and not exposed to ADSS), S (smoke, exposed to ADSS), and SV (exposed to ADSS and treated with vitamin E orally). The smoke group was exposed to ADSS of commercial cigarettes via inhalation through the nose only inside an exposure chamber (size 564 × 385 × 371 mm; volume 53,100 ml/1,795 oz; plastic material) during 4 weeks. After lighting the cigarettes, the chamber was immediately closed, with only a small opening (371 × 40 mm) in the chamber for ventilation. In the first, second, third, and fourth weeks, animals were concomitantly exposed to the smoke of 1, 2, 3, and 4 cigarettes/day, respectively. The duration of each exposure was 15 min, daily. Control animals were placed in another clean chamber for the same amounts of time and without exposure to ADSS.

Smoke Generation

The system of exposure to ADSS was determined according to our research group (Thomé et al., 2009). Briefly, after placing the rats inside the chamber of exposure, between one and four cigarettes were lit. Cigarettes were fixed in a metal holder allowing them to be fully burned down within the period of 15 min. A metal grille was placed on top of the cigarette holder for the rats not to injure themselves and to avoid direct contact with the cigarette. The inhalation was ADSS termed as a surrogate of ETS that does not require smokers. Sidestream smoke is emitted from the tip of a smoldering cigarette, and mainstream smoke is emitted at the mouthpiece of a cigarette and exhaled by the smoker. ETS is composed of exhaled mainstream smoke (11%) and sidestream smoke (85%), contaminants emitted into the air during the puff, and contaminants that diffuse through the cigarette paper and mouth end between puffs (Pieraccini et al., 2008).

Treatment With Vitamin E

Thirty minutes before of the exposure to ADSS, the animals were treated with vitamin E. The animals from vitamin group were given 50 mg/kg/day of vitamin E diluted in canola oil (1 ml/kg) orally for 28 days.

Cigarette Composition

The brand of cigarette used in the experiment was manufactured by Philip Morris Brazil Industry and Company LTDA from the city of Santa Cruz do Sul, Rio Grande do Sul, Brazil. The basic ingredients of cigarettes as printed on cigarette packs of the brand used in the experiment are: sugar, paper, plant extracts, and agents of flavor, and each cigarette contains 10 mg tar, 0.8 mg nicotine, and 10 mg carbon monoxide. Moreover, the ingredients in cigarette brand hard pack 20 cigarettes contain burnt items (such as propylene glycol, glycerol, guar gum, side seam adhesives among others) and unburnt items (such as filtration material, filter wraps, filter adhesives among others).

Anatomopathologic Analysis of Lung Tissue

To confirm this model of exposure ADSS, an anatomopathologic analysis of lung tissue was carried out. The samples of lung tissue, ex vivo, were collected and fixed in formalin solution of 10% and then dehydrated and embedded in paraffin, followed by sectioning and histological staining with hematoxylin and eosin (H&E) determined by the method described by Tolosa

and Rodrigues (2003). The slides were observed in an optical microscope to check for possible changes in the lung tissue indicative of exposure to ADSS.

Blood Gases, pH, and Carboxyhemoglobin

To evaluate the reproducibility of this model of exposure ADSS, blood gas, pH, and carboxyhemoglobin (HbCo) were measured. Blood gas consists of partial pressure of CO_2 (pCO_2), partial pressure of oxygen (pO_2), carbonate ion (HCO_3^-), total CO_2 (tCO_2), and saturation of oxygen (sO_2). The rats were previously anesthetized with ketamine chlorhydrate and xylazine (5:1; 0.1 ml/100 g), and from right common carotid artery, the blood was collected by puncture with an insulin needle (0.45 × 13). The syringe was prepared in advance with a solution of sodium heparin 5,000 IU (100 µl/2 ml blood). Immediately after collection, the arterial blood was placed under ice for up to 3 hr until it was read in the Radiometer analyzer (model ABL-5, Copenhagen).

Cotinine Quantification

The cotinine quantification in plasma samples was realized by high-performance liquid chromatography ultraviolet, following the method of Cattaneoa et al. (2007) after modifications as described in the following subsections.

Instrumentation and Chromatographic Conditions

The chromatographic equipment consisted of a chromatography system Agilents, 1100 series, and injector with loop of 20 µL and UV–vis detector. The separation was achieved using a reverse-phase column: Zorbax Eclipse column XDB-C8 (4.6 × 150 mm) Agilents with 5-µm particle size. The mobile phase was a mixture of Milli-Q water: methanol: sodium acetate 0.1 M: acetonitrile (50:15:25:10, vol/vol), being added 1 ml of citric acid 0.034 mol/L and 5.0 ml of triethylamine for each liter of solution, pH 4.4, adjusted with acetic acid. The flow rate of 1 ml/min was maintained isocratically, the absorbance of the eluent was monitored at 260 nm, and the total run time was 8 min. As internal standard, we used 2-phenylimidazole. The detection limit of method was 5 ng/ml, and the limit of quantification was 10 ng/ml.

Sample Preparation

A volume of 500 µl of plasma was added to 25 µl of NaOH 10 M and 100 µl of internal standard (1 µg/ml). Then, extraction with 2.0 ml of dichloromethane was carried out by vortex-mixed for 1 min and centrifuged at 3,000 × g for 10 min. The organic phase was totally dried with nitrogen. Then 100 ml of the mobile phase was added and 20 ml was injected into HPLC.

AChE Activity Assay

The cranium was opened and the structures were gently removed and separated into striatum, cerebral cortex, cerebellum, and hippocampus. The brain regions were then homogenized in a glass potter in the same solution of 320 mM sucrose, 0.1 mM EDTA, and 5 mM Tris–HCl (pH 7.5), at a proportion of 1:10 (g/vol). AChE activity was determined according to Ellman, Courtney, Andres, and Featherstone (1961), modified by Rocha, Emanuelli, and Pereira (1993). The reaction mixture (2 ml final volume) was composed of 100 mM phosphate buffer (pH 7.5) and 1 mM 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB). The method is based on the formation of a yellow anion, 4,4′-dithio-bis-acid nitrobenzoic measured by absorbance at 412 nm during 3-min incubation at 25 °C. The enzyme was preincubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide. The enzyme activity was expressed in µmole AcSCh/h/mg of protein.

Thiobarbituric Acid Reactive Substances Measurement

Brain thiobarbituric acid reactive substances (TBARS) levels were determined by the method described previously by Ohkawa, Ohishi, and Yagi (1979). In short, the reaction mixture contained 200 µl of brain homogenates or standard (0.03 mM MDA-malondialdehyde), 200 µl of 8.1% sodium dodecyl sulfate (SDS), 500 µl of acetic acid solution (2.5 M HCl, pH 3.5), and 500 µl of 0.8% thiobarbituric acid (TBA). The absorbance was measured at 532 nm. TBARS tissue levels were expressed as nmol MDA/mg of protein. The effect of TBARS in structure brain on AChE activity in striatum, cerebral cortex, cerebellum, and hipoccampus was analyzed by linear regression of normalized variables.

Chemicals

Acetylthiocholine iodide, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), SDS, malondialdehyde (MDA), thiobarbituric acid, and trizma base were purchased from Sigma Chemical Co. (St. Louis, MO). Vitamin E acetate (C31H52O3; *dl*α-tocopheryl acetate) was obtained from Pharma Nostra (São Paulo, Brazil). All other reagents used in the experiments were of the highest purity available.

Protein Determination

Protein was measured by the Coomassie blue method according to Bradford (1976) using bovine serum albumin as standard. For AChE activity, the protein was determined previously and adjusted for each structure: striatum (0.4 mg/ml), cerebral cortex (0.6–0.8 mg/ml), cerebellum (0.6 mg/ml), and hippocampus (0.8 mg/ml).

Statistical Analysis

Data were analyzed by one-way analysis of variance followed by the Duncan multiple range test, and $p < .05$ was considered to represent a significant difference in the analysis. All data were expressed as mean ± *SEM*.

Results

The anatomopathologic analysis of lung tissue is presented in Figure 1. Representative lung sections from control rats (not exposed to ADSS) with normal alveolar and bronchiolar structures were maintained and showed no inflammatory cell infiltration (H&E, ×40; Figure 1A). The anatomopathologic analysis of lung tissue by microscopy of rats exposed to ADSS revealed the presence of interalveolar septa with an intense diffuse chronic interstitial infiltrate represented by lymphocytes (Figure 1B, Arrow 1) and foamy macrophages (Figure 1B, Arrow 2, ×40; Figure 1C, ×400; H&E). The lung section shows cellular interstitial infiltrate composed of lymphocytes (Figure 1D, Arrows 2), plasma cells, and bronchiole, which presents mild

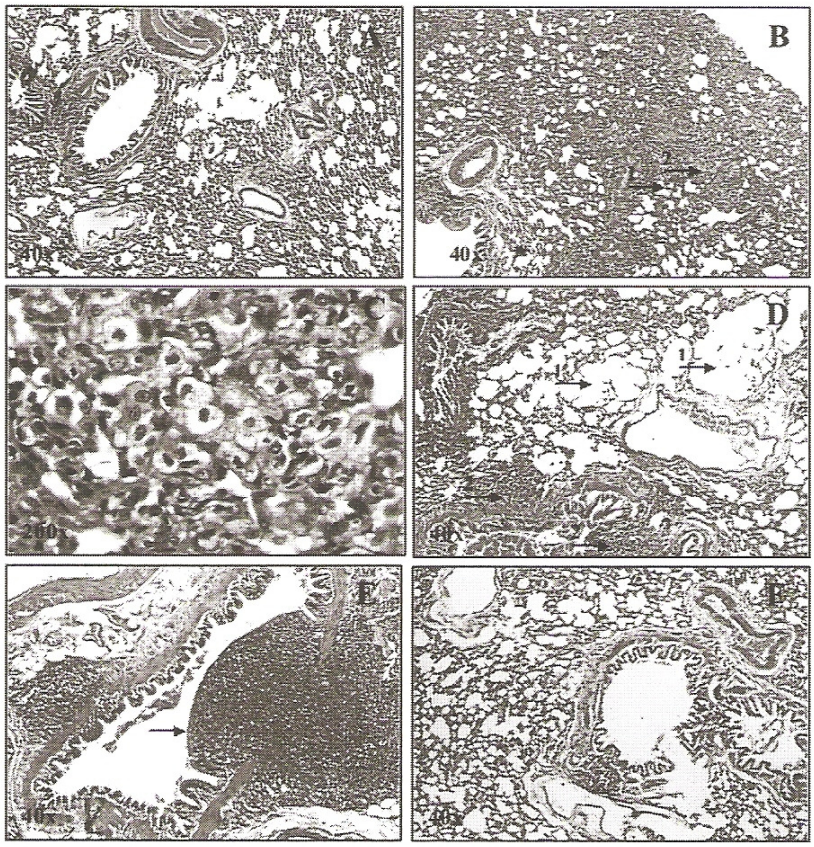

Figure 1. (A) Normal histological aspect of rat lung tissue, hematoxylin and eosin (H&E; ×40). (B) Image of rat lung tissue exposed to ADSS with thickened interalveolar septa with an intense diffuse chronic interstitial infiltrate represented by lymphocytes (Arrow 1) and foamy macrophages (Arrow 2), H&E (×40). (C) Foamy macrophages showed in higher magnification, H&E (×200). (D) Focal emphysema (Arrows 1) of parenchyma around the affected airways and infiltrate composed of lymphocytes (Arrows 2), H&E (×40). (E) Lymphoid follicle with germinal center in the wall of bronchiole, H&E (×40). (F) Representative lung sections of rats exposed to ADSS and treated with vitamin E with normal appearance of rat lung with absence of interstitial infiltrate and absence of emphysema, H&E (×40).

chronic inflammation in the wall (chronic bronchiolitis; Figure 1D). In addition, prominent lymphoid tissue presents germinal center along airways (Figure 1D) and focal emphysema of parenchyma around the affected airways (Figure 1D, Arrows 1, ×40, H&E). In the smoke group, lymphoid follicle with germinal center in the wall of bronchiole was also found (Figure 1E, ×40, H&E). Figure 1F shows representative lung sections of rats exposed to ADSS and treated with vitamin E. The lung sections showed normal appearance, absence of interstitial infiltrate and emphysema, as well as occasional lymphoid aggregates (×40, H&E).

The analysis of blood gas, pH, carboxyhemoglobin, and cotinine from control rats and those exposed to ADSS are shown in Table 1. A significant increase in pCO_2, tCO_2, and carboxyhemoglobin (HbCo) was observed in the smoke group ($p < .05$) when compared with the control group. The exposure of ADSS did not alter the cotinine level parameter when compared with the control group.

TBARS production was increased in the striatum (Figure 2A), cerebral cortex (Figure 2B), and cerebellum (Figure 2C) of rats from the ADSS group when compared with the control rats ($p < .05$). Post hoc comparisons by Duncan's test revealed that the increase in TBARS levels induced by the ADSS exposure was decreased by vitamin E administration ($p < .05$). TBARS levels in the structures of brain concentration were positively correlated ($p < .05$) with AChE activity in cerebral cortex ($R = .638$) and cerebellum ($R = .657$); hippocampus ($R = -.210$) and striatum ($R = .154$) had no correlation. In the hippocampus, there was no difference in the TBARS levels among the groups (Figure 2D). In all brain structures studied where TBARS level were determined, no significant changes were observed when vitamin E group was compared with the control group.

The AChE activity was modified by ADSS exposure in the striatum ($p < .05$), and post hoc comparisons by Duncan's test revealed that the enzyme activity was significantly higher in the ADSS group when compared with the control group (Figure 3A). Besides, vitamin E administration was effective in decreasing the AChE activity increased by the ADSS exposure (Figure 3A). We also observed a decrease in the AChE activity of ADSS vitamin E group ($p < .05$) when compared with the control group (Figure 3A). There were similar results for the AChE activity in

Table 1. Measurement of Gas, pH, Carboxyhemoglobin, and Cotinine in Arterial Blood of Rats After 4 Weeks of ADSS Exposure: Control Group and Exposed to ADSS Group

	Control group	Smoke group (ADSS)
pH	7.27 ± 0.04	7.32 ± 0.11
pCO_2	45.25 ± 5.51	**55.62 ± 2.29***
pO_2	59.02 ± 13.10	76.2 ± 29.80
(HCO_3^-)	17.00 ± 2.82	16.12 ± 0.25
tCO_2	18.50 ± 3.50	**28.75 ± 0.50***
sO_2	77.32 ± 17.00	87.35 ± 11.60
HbCo	0.32 ± 0.09	**7.62 ± 0.27***
Cotinine	0.00 ± 0.00	Low 5 ng/ml

Note: Partial pressure level of carbon dioxide (pCO_2) was significantly increased in group ADSS when compared with the control group ($p<0.05$). Total carbon level (tCO_2) was significantly increased in group ADSS when compared with the control group ($p<0.05$). Carboxyhemoglobin (HbCo) level was significantly increased in group ADSS when compared with the control group ($p<0.05$). Partial pressures of pCO2 and pO2 are expressed in mmHg; carbonate ion (HCO^-_3) and total carbon (tCO_2) are expressed in nmol/L; oxygen saturation (sO_2) is expressed in %; carboxyhemoglobin (HbCo) is expressed in mg/dl. Cotinine is expressed in ng/ml of plasma. Data are reported as mean ± SEM of six animals per group. ADSS=aged and diluted sidestream smoke.

*Denotes $p < .05$ as compared with the control group (one-way analysis of variance/Duncan)

the cerebral cortex where post hoc comparisons by Duncan's test revealed that the enzyme activity was significantly higher ($p < .05$) in the ADSS group when compared with the control group, and vitamin E administration decreased the AChE activity increased by the ADSS exposure (Figure 3B). In the cerebellum, post hoc comparisons by Duncan's test revealed that the enzyme activity was significantly higher ($p < .05$) in the ADSS group when compared with the control group, however, vitamin E administration decreased the AChE activity increased by the ADSS exposure (Figure 3C). In the hippocampus, the AChE activity was not changed in the presence of ADSS exposure when compared with control, vitamin E, and ADSS vitamin E groups (Figure 3D).

Discussion

The main objective of this work was to investigate the effects of vitamin E on the AChE activity as well as to determine lipid peroxidation levels in the brain of rats using the model of ADSS exposure termed as a surrogate of ETS that does not require smokers.

To evaluate the reproducibility of the model of exposure to ADSS, blood gas, pH, carboxyhemoglobin, and cotinine were measured and an anatomopathologic analysis of the lung tissue was carried out. The histological sections of lungs of rats exposed to ADSS showed the presence of ticked interalveolar septa with an intense diffuse chronic interstitial infiltrate represented by lymphocytes, foamy macrophages, plasma cells, and chronic bronchiolitis (Laniado-Laborín, 2009) with sparse areas characteristic of focal emphysema of parenchyma around the affected airways. Moreover, there was also lymphoid follicle with germinal center in the wall of bronchiole caused by ADSS exposure. This process is associated with greater alveolar CO_2 restraint due to ADSS demonstrated by the high amount of carboxyhemoglobin. Both carboxyhemoglobin and total carbon dioxide (tCO_2) are biomarkers of cigarette smoke exposure in blood (Burtiz & Ashwood, 1994; Smith & Taylor, 2007). Here, we demonstrated that these parameters were increased in the group exposed to ADSS confirming the efficiency of the inhalation of ADSS. However, the cotinine level in the sample plasma of rats subjected to the model of exposure to ADSS was not detected because the technique of determining the cotinine was not sensitive enough to detect this level of exposure to nicotine (detection limit of the method is 5 ng/ml). According to the assumption of Vizi et al. (2004), the relatively low concentration of nicotine in the brain during cigarette smoking may induce its neurobiological effects.

In order to know the effect of vitamin E on the cognitive deficit of ADSS exposure in rats and whether it is involved with the cholinergic enzyme alternations in brain, we analyzed the activity of AChE. The animals exposed to ADSS had increased AChE activity in striatum (Figure 3A), cerebral cortex (Figure 3B), and cerebellum (Figure 3C). It is suggested that a decrease in the level of acetylcholine (ACh) occurs, which may be related to cognitive impairment observed in smokers, such as disruption of attention and memory (Jacobsen et al., 2005). In the striatum, however, the cholinergic neurons are interneurons that provide very dense local innervations. The cholinergic interneurons provide an ongoing ACh signal by firing action potentials tonically at about 5 Hz (Zhou, Wilson, & Dani, 2002). Thus, the function in the striatum may underline the cholinergic participation in sensorimotor planning, learning, and memory (Zhou et al., 2002). Cholinergic neurons make broad projections mainly throughout the cortex and hippocampus. Evidence indicates that excitatory hippocampal afferents gate information flow from the prefrontal cortex to the nucleus accumbens (Goto & O'Donnell, 2001; Grace, 2000). The nucleus accumbens projects to the basal forebrain cholinergic neurons, providing another pathway for the nucleus accumbens to affect cortical arousal, attention, and cognitive function (Sarter & Bruno, 2000). Since the cerebellum has been implicated in different types of procedural memory (the learning of motor skills and habits), such as eye-blink classical conditioning and motor-skill learning (Krakauer & Shadmehr, 2006), we also analyzed AChE activity in this structure.

The present study demonstrated that the treatment with vitamin E improved the neurotoxicity in brain of rats after exposure to ADSS. Vitamin E decreased the AChE activity to the normal level in the striatum in rats ADSS (Figure 3A), cerebral cortex (Figure 3B), and cerebellum (Figure 3C). However, the results were not homogeneous for the different cerebral structures studied, and no changes were observed in the AChE activity in the ADSS group in the hippocampus (Figure 3D). It should also be considered that vitamin E might have a neuroprotective effect on these structures in the cholinergic system by decreases of the AChE activity in animals ADSS. Thus, it has been suggested that vitamin E may prevent the lowering of ACh

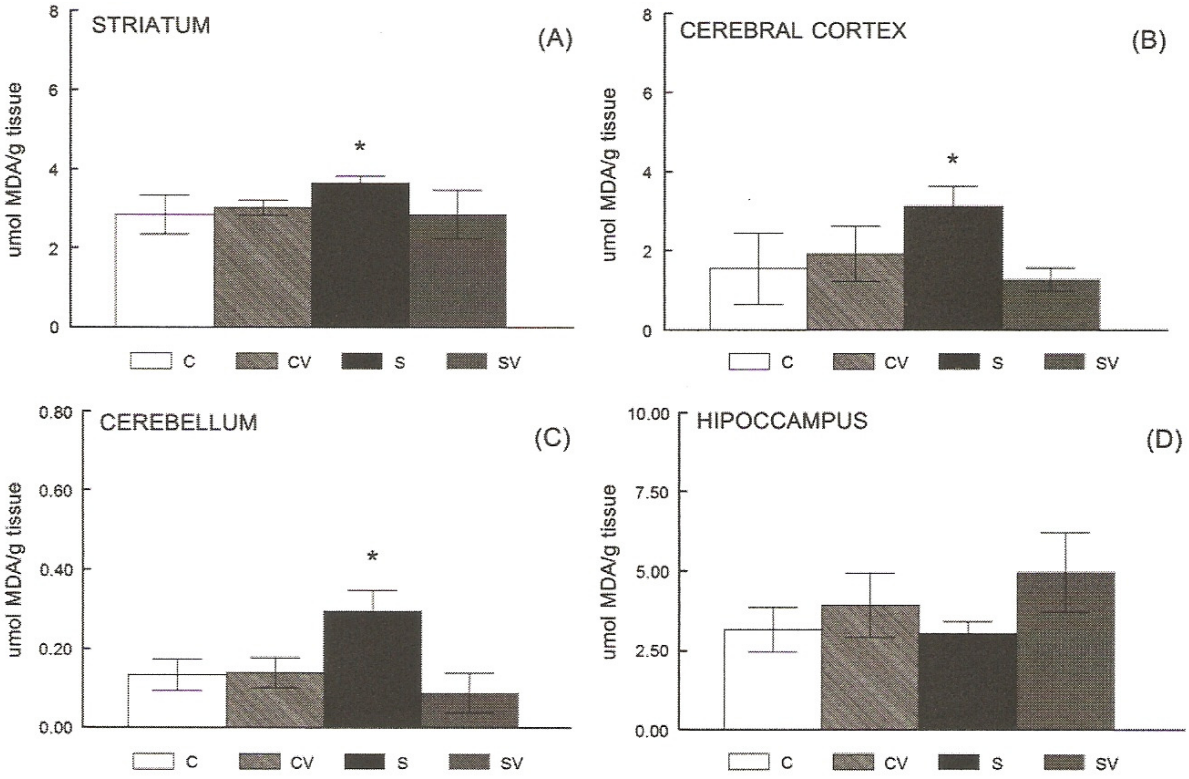

Figure 2. Effect of exposure of ADSS and oral vitamin E on thiobarbituric acid reactive substances (TBARS) production in the striatum (A), cerebral cortex (B), cerebellum (C), and hippocampus (D). C, control group; CV, control vitamin E; S, smoke, exposed to ADSS; and SV, exposed to ADSS and treated with vitamin E orally. The TBARS product was expressed as μmol MDA/g of tissue. Data are reported as mean ± *SEM* of six animals per group. *Denotes $p < 0.05$ as compared with the control group (one-way analysis of variance/Duncan).

levels in the brain structures studied, whose results are in accordance with Eidi, Eidi, Mahmoodi, and Oryan (2006). A number of studies have demonstrated that nicotine exposure induces increased numbers of nAChRs and ACh release (Rowell & Winkler, 1984) in the CNS in animals and human smokers (Sabbagh, Lukas, Sparks, & Reid, 2002). Perhaps the different results showing both potentiating and no change of exposure ADSS in the cholinergic system in CNS are simply due to the distribution of nAChRs throughout the brain (Brody et al., 2004) by increased number of nAChRs and increased ACh release in these structures.

In addition to direct stimulation of nAChRs, nicotine might provide cascading effects through stimulation of the release of a variety of transmitters (M. K. Thakur, 2000) including acetylcholine (ACh; McGehee & Role, 1995) or direct stimulation via dopamine release or monoamine oxidase (MAO) inhibition or a combination of these factors (Swan & Lessov-Schlaggar, 2007). It should be considered that vitamin E might have a neuroprotective effect in the striatum, cerebral cortex, and cerebellum cholinergic system. Thus, it has been suggested that vitamin E may prevent the lowering of ACh level in some parts of the brains studied, whose results are in accordance with Eidi et al. (2006).

An important aspect is that the enhanced activity of AChE may be related to the increase of ligands, such as nicotine by direct stimulation of nAChRs. Several studies have reported the effects of nicotine by its activation with high selectivity to nAChRs (Léna et al., 1999; Shao & Feldman, 2001). Interference with the normal function of these nAChRs may be the basis of the detrimental side effects of nicotine present in cigarette smoke. Still, it is speculated that not only nicotine but hundreds of other compounds derived from cigarette smoke may also contribute to structural brain abnormalities in smokers (Dome et al., 2010), and this might promote changes in the AChE activity, leading to a change in the levels of ACh, and consequently changing cholinergic neurotransmission.

Membrane lipids in the brain contain high levels of polyunsaturated fatty acids and are therefore particularly sensitive to oxidation (Vajragupta et al., 2000). Lipid peroxidation is the oxidative deterioration of the polyunsaturated lipids through the formation of hydroperoxides into short-chain aldehydes, ketones, and other oxygenated compounds considered to be responsible for the development of diseases in human and animals (Baskaran et al., 1999; Butterfield, Lange, & Sultana, 2010). Lipid peroxidation is an important cause of neuronal damage, as in ischemic injuries, neurotrauma, neural disorders (Coyle & Puttfarken, 1993), and cigarette smoke exposure (Anbarasi, Sabitha, et al., 2005; Anbarasi, Vani, et al., 2005; Baskaran et al., 1999; Delibas et al., 2003; Pryor & Stone, 1993). The major degradation product of lipid hydroperoxides is the malondialdehyde (MDA), and it has attracted much attention as a marker

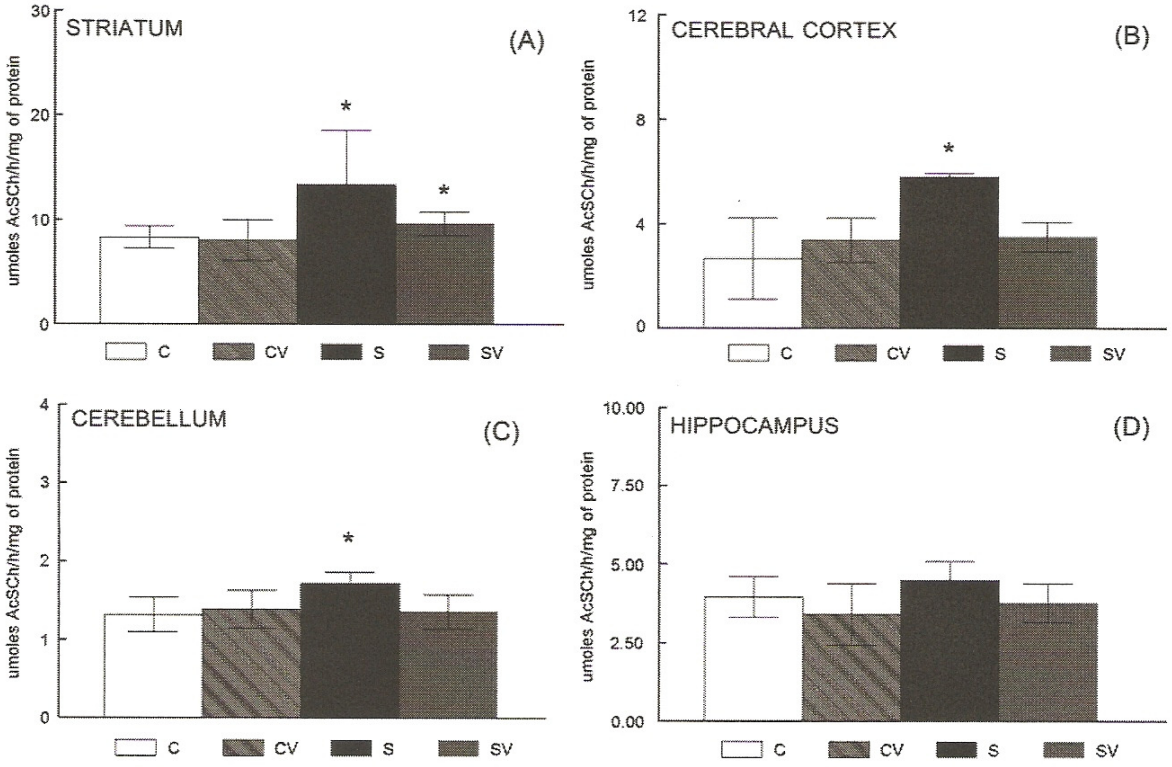

Figure 3. Effect of exposure of ADSS and oral vitamin E on AChE activity in the striatum (A), cerebral cortex (B), cerebellum (C), and hippocampus (D). C, control group; CV, control vitamin E; S, smoke, exposed to ADSS; and SV, exposed to ADSS and treated with vitamin E orally. The AChE activity was expressed as µmol AcSCh/h/mg of protein. Data are reported as mean ± *SEM* of six animals per group. *Denotes $p < 0.05$ as compared with the control group (one-way analysis of variance/Duncan).

for assessing the extent of lipid peroxidation (Raharjo & Sofos, 1993). The most common method for measuring MDA in biological samples seems to be the TBARS test, which is based on spectrophotometric quantitation of the pink complex formed after reaction of MDA with two molecules of thiobarbituric acid (TBA; Ohkawa et al., 1979). The present data of the study demonstrate that the exposure of ADSS enhanced lipid peroxides in striatum, cerebral cortex, and cerebellum. No significant increases in lipid peroxidation levels in the ADSS-exposed group suggest that ADSS does not cause free radical–mediated tissue damage in the hippocampus. The present study showed that ADSS did not induce lipid peroxidation in the hippocampus, which is in accordance with the result of Baskaran et al. (1999), Delibas et al. (2003), and Gumustekin et al. (2003), who reported that the nicotine did not increase TBARS levels in hippocampus.

In our study, ADSS increased the lipid peroxidation levels and AChE activity in the brain, and these parameters were decreased by vitamin E. This finding is in accordance with the studies that reported a decrease of the susceptibility in cerebral structures to hydrogen peroxide–induced lipid peroxidation (Escames et al., 1997; M. Meydani, Macauley, & Blumberg, 2006) in smokers (Brown, Morrice, Arthur, & Duthie, 1996; Brown, Morrice, & Duthie, 1997) and the prevention of the increase in lipid peroxidation (Meydani et al., 2006) in rats tissues exposed to ADSS by vitamin E supplemented (Gumustekin et al., 2003; Hoshino et al., 1990).

Conclusions

In conclusion, alterations in the brain of rats in the model of exposure of cigarette smoke studied demonstrated that ADSS can affect the functionality of the cholinergic system and increase the lipid peroxidation levels, which were turned into normal levels in animals treated with vitamin E. This may suggest that vitamin E could be considered a potential therapeutic agent in this type of exposure.

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Declaration of Interests

None declared.

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4 DISCUSSÃO

A fumaça de cigarro é uma complexa mistura de centenas de diferentes compostos químicos com capacidade de promover dano ao tecido biológico (WITSCHI et al., 1997; SWAN & LESSOV-SCHLAGGAR, 2007). As ações promovidas por este tipo de exposição alteram a agregabilidade das plaquetas, as defesas antioxidantes e deprime o sistema imunológico. (HOFFMANN & HOFFMANN, 1997; HOOKER et al., 2007). No entanto, não foi encontrado na literatura estudos que relacionassem o efeito da fumaça de cigarro e nicotina na atividade das ectonucleotidases.

Em relação aos resultados obtidos do estudo em plaquetas foi demonstrado um aumento significativo na hidrólise do ATP e AMP, e nenhuma mudança para a hidrólise do ADP. Também foi observada uma redução significativa na desaminação da adenosina no soro, em ratos expostos à fumaça de cigarro. Estes achados sugerem um aumento na atividade da NTPDase e 5´-nucleotidase, uma vez que essas enzimas são responsáveis pela hidrólise dos nucleotídeos ATP e AMP (MARCUS et al., 2003). Um aumento na atividade da NTPDase (substrato ATP) pode elevar o nível de ADP o qual não demonstrou alteração significativa de hidrólise para formação de AMP. Isso resulta numa ação pró-agregante e deste modo pode promover doenças macrovasculares como demonstrado em estudos com fumantes (MUNDAL et al., 1998) devido a anormalidades em plaquetas.

Em fumantes de cigarro há uma maior espontaneidade na agregação de plaquetas induzido por epinefrina ou ADP do que não fumantes (FUSEGAWA & HANDA, 2000). Corroborando com estes achados as análises dos dados deste estudo demonstraram um aumento significativo na agregação de plaquetas com o agonista ADP em ratos expostos à fumaça de cigarro. A fumaça de cigarro induz condições pró-agregantes (MUNDAL et al., 1998), o que predispoe a doenças cardiovasculares (WHISS et al., 2000) e aumento da mortalidade e morbidade cardiovascular associada com alta prevalência de doenças comuns como a aterosclerose (WAGNER & BURGER, 2003; YANBAEVA et al., 2007).

Em relação a hidrólise de AMP e adenosina houve um aumento na atividade da 5´-nucleotidase e um decréscimo na atividade da ADA. Estas alterações podem

sugerir um estado compensatório do organismo com o objetivo de aumentar os níveis de adenosina. A adenosina é uma importante molécula que atua na regulação do tônus vascular e da função plaquetária (PILLA et al., 1996). A adenosina ajuda a proteger as células e os tecidos durante as condições de estresse tais como a isquemia e a hipóxia (DOWNEY & FORMAN, 1993), sendo que o principal mecanismo pelo qual o monóxido de carbono da fumaça de cigarro causa doenças do coração é a produção de hipóxia (ZEVIN et al., 2001). Assim, pode-se sugerir que o aumento na concentração de adenosina pode contribuir para modular a aumentada ativação de plaquetas e ajudar a proteger o tecido danificado pela fumaça de cigarro. Em adição, o aumento na concentração de adenosina no tecido durante a hipóxia pode promover melhora do fluxo sanguíneo e resposta de proteção inata a hipóxia (COLGAN et al., 2006).

Um importante dado observado na análise anatomopatológica foi a presença de áreas isoladas com bronquiolite e processo inflamatório crônico peribronquiolar, perivascular e infiltrado septal. Também foi observado em algumas áreas do tecido pulmonar a presença de enfisema pulmonar das seções do tecido de pulmão. Estes achados estão associados com a grande retenção de dióxido de carbono, devido à fumaça de cigarro, demonstrado pela grande quantidade de carboxihemoglobina observada neste estudo (SMITH & TAYLOR, 2007). A carboxihemoglobina é um biomacador do sangue de exposição à fumaça e juntamene com o aumento do dióxido de carbono demonstraram que os ratos foram eficientemente expostos à fumaça de cigarro confirmando a boa reprodutibilidade deste modelo de exposição.

Em relação ao tratamento com a nicotina em linfócitos também foi observado uma alteração na atividade das ectonucleotidases. Houve um decréscimo da hidrólise de nucleotídeos (ATP e ADP) e o nucleosídeo (adenosina) em ambos os estudos *in vivo* e *in vitro*. A nicotina é o composto com maior efeito psicoativo e principal constituinte imunossupressor que inibe a resposta imune inata e adaptativa (SOPORI, 2002). Há evidências que a administração de nicotina diminui a resposta proliferativa de linfócitos do sangue periférico e indução de células formadoras de anticorpos (MCALLISTER-SISTILLI et al., 1998). No estudo *in vivo* as concentrações de nicotina utilizadas foram de 0,25 e 1,0 mg/kg/dia administrado por via subcutânea em ratos por um período de dez dias. Após este período de exposição foi observado um aumento (39% e 131%, respectivamente) no nível de cotinina. A cotinina é o marcador biológico da nicotina e seu nível aumentado demonstra que exposição foi

eficiente durante todo o período de tratamento (CATTANEOA et al., 2007). Os resultados obtidos neste estudo demonstraram um decréscimo significativo para a hidrólise de ATP, ADP e desaminação da adenosina. Este achado sugere uma diminuição na atividade das enzimas NTPDase e ADA, uma vez que estas enzimas modulam a sinalização dos nucleotídeos de adenina e seu derivado adenosina, os quais medeiam diversos processos fisiológicos e patológicos via receptores purinérgicos P1-2 (SCHETINGER et al., 2007).

A atividade da NTPDase em linfócitos T citolíticos tem um efeito protetor contra a lise celular causada pelo ATP. Sugere-se que a inibição da NTPDase em ratos tratados com nicotina causa uma inabilidade de remover o ATP extracelular (MIDDLEBROOK et al., 2002). O efeito protetor contra a lise celular pode não ser efetivo e o mecanismo citolítico age diminuindo o número de linfócitos como foi observado neste estudo.

Embora os resultados deste estudo tenham indicado que ambas as doses de nicotina (0,25 e 1 mg/kg) inibem a atividade da NTPDase e ADA, somente na dose de 0,25 mg/kg foi detectado os níveis aumentados de ATP, ADP e de adenosina no soro. Este achado pode ser atribuído ao efeito hormético da nicotina. Este efeito refere-se à relação dose-resposta, ou seja, uma resposta fisiológica de um agente físico ou químico com uma dose mais baixa pode ser completamente oposta quando uma dose maior é administrada (FURST, 1987). A maior dose de nicotina (1,0 mg/kg) utilizada neste estudo não alterou os níveis de ATP, ADP e adenosina. Deste modo, sugere-se que um mecanismo compensatório pode estar ocorrendo com objetivo de manter o apropriado nível de ATP, ADP e adenosina (RESTA & THOMPSON, 1997).

Neste estudo, foi observado um decréscimo na expressão da NTPDase, um achado importante para as alterações na resposta imune em animais tratados com nicotina. A expressão diminuída da enzima pode afetar a hidrólise de seus substratos e comprometer a cascata enzimática com menor quebra de nucleotídeos e isso pode aumentar tanto os níveis de ATP, ADP quanto de adenosina. O ATP faz supressão da proliferação de linfócitos periféricos e da adenosina e isto pode iniciar o processo de apoptose, o que contribui para o aumento do estado de imunodeficiência (FISHMAN et al., 1980; RESTA & THOMPSON, 1997). Em adição, a adenosina tem efeito antiinflamatório e linfotóxico diminuindo a expressão do complexo de histocompatibilidade (MHC) de classe II em macrófagos, células dendríticas e decréscimo da adesão de leucócitos (HASKÓ & CRONSTEIN, 2004).

Em relação aos resultados do estudo *in vitro* a nicotina *per se* diminuiu a hidrólise de nucleotídeos (ATP e ADP) e na desaminação da adenosina em linfócitos de ratos. A hidrólise de ATP-ADP-adenosina foram diminuídas pelas concentrações de nicotina 1mM (0%-0%-16%, respectivamente), 5mM (42%-32%-74%, respectivamente), 10mM (80%-27%-80%, respectivamente) e 50mM (96%-49%-98%, respectivamente). Apesar de poucos estudos na literatura sobre a estrutura anatômica da família das NTPDases (ZIMMERMANN, 2001), estes resultados sugerem que a inibição na atividade das ectonucleotidases pela nicotina pode ser devido a sua interação com o sitio ativo desta enzima, e assim pode aumentar os níveis de nucleotídeos.

Em ambos os estudos *in vivo* e *in vitro* em linfócitos estão de acordo os quais a nicotina aumentou o nível de ATP e adenosina favorecendo os efeitos imunossupressores em sistemas biológicos. Pode-se sugerir que um dos mecanismos pelo qual a nicotina exerce seu efeito degenerativo pode ser através da modulação da via das ectonucleotidases em linfócitos. Neste contexto, pode-se sugerir que estudos futuros possam ser conduzidos na ordem de investigar a cinética destas enzimas, visto que o efeito inibitório causado pela nicotina pode estar relacionado com uma interação com a NTPDase e a ADA.

Outro estudo foi conduzido com objetivo de investigar os efeitos da vitamina E (50 mg/Kg) sobre a atividade da AChE e a determinação do nível de peroxidação lipídica no encéfalo de ratos submetidos a exposição à fumaça de cigarro. Nesse estudo, foi observado após quatro semanas de exposição um aumento na atividade da AChE no estriado, no córtex cerebral e no cerebelo. Estes achados sugerem que há um decréscimo no nível de ACh o qual pode estar associado com o prejuízo cognitivo observado em fumantes, tais como distúrbios de atenção e memória (JACOBSEN et al., 2005). As estruturas encefálicas estudadas possuem inervações de neurônios colinérgicos as quais podem ter participação no planejamento sensório-motor, aprendizado, memória, excitação e atenção (GRACE, 2000; SARTER & BRUNO, 1999; GOTO & O´DONNELL, 2001; ZHOU et al., 2002; KRAKAUER & SHADMEHR, 2006), visto que a AChE possui papel fundamental na modulação colinérgica do SNC.

O presente estudo demonstrou que o tratamento com a vitamina E diminuiu a neurotoxicidade no encéfalo de ratos depois da exposição com fumaça de cigarro. A vitamina E diminuiu a atividade da AChE no estriado, no córtex cerebral e no

cerebelo de ratos expostos à fumaça de cigarro. Entretanto, os resultados não foram homogêneos para as diferentes estruturas encefálicas estudadas, e nenhuma mudança foi observada na atividade da AChE no hipocampo tanto para animais expostos à fumaça de cigarro quanto tratados com vitamina E. Tem sido demonstrado por MANEESUB et al. (1993) que a vitamina E pode restaurar parcialmente a hipofunção do sistema colinérgico no envelhecimento e também pode ativar este sistema na retenção da memória (EIDI et al., 2006). Embora estes estudos tenham demonstrado que a vitamina E possa melhorar a função cognitiva pouco é conhecido sobre seu mecanismo de ação. Pode-se sugerir que um dos mecanismos de ação deste composto seja através da diminuição na atividade da AChE como observado neste estudo.

Em relação à peroxidação lipídica no encéfalo, os resultados demonstraram um aumento no estriado, no córtex cerebral e no cerebelo de ratos expostos à fumaça de cigarro, focalizando o envolvimento deste tipo de exposição com a formação de EROs. O acúmulo do malondialdeído, o produto final da peroxidação lipídica, é considerado um dos principais mecanismos de toxicidade associados ao tabagismo (PRYOR & STONE, 1993; BASKARAN et al., 1999; DELIBAS et al., 2003; ANBARASI et al., 2005a; ANBARASI et al., 2005b; SWAN & LESSOV-SCHLAGGAR, 2007). Além disso, os resultados demonstraram que a exposição à fumaça de cigarro não causa aumento da peroxidação lipídica no hipocampo. Corroborando com esses resultados foi verificado por Bascaran et al. (1999), Delibas et al. (2003) e Gumustekin et al. (2003) que o hipocampo não é alvo de peroxidação lipídica em animais expostos à nicotina.

Um importante resultado obtido neste estudo é que a vitamina E reduziu a aumentada peroxidação lipídica no estriado, no córtex cerebral e no cerebelo de ratos expostos à fumaça de cigarro. A vitamina E é um antioxidante com alto potencial redutor e tem sido relatada como importante defesa antioxidante não enzimática devido as suas propriedades de neutralizar diretamente as EROs e proteger as membranas da peroxidação lipídica (MAYNE, 2003). Um das características comuns da patogênese de várias doenças associadas ao tabagismo é a deficiência de vitamina E (REITER et al., 2007). Em exposições como à fumaça de cigarro a qual produz estresse oxidativo, a diminuição dos níveis desta vitamina pode estar ocorrendo devido ao aumento da utilização deste antioxidante (REITER et al., 2007). Logo, a suplementação pode fortalecer as defesas do organismo contra

esse tipo de exposição, principalmente para as membranas de lipídios no encéfalo que contém altos níveis de ácidos graxos poliinsaturados que são particularmente sensíveis a oxidação (VAJRAGUPTA et al., 2000).

Neste trabalho, um modelo experimental de exposição à fumaça de cigarro tóxica foi utilizado para avaliar a atividade das ectonucleotidases em plaquetas e ADA em plasma e pelos resultados compilados, pode-se sugerir que existe uma disfunção purinérgica, a qual é causada pela exposição passiva à fumaça de cigarro. Também foram investigados os efeitos da nicotina per se no sistema purinérgico em linfócitos, demonstrando que este alcalóide interfere com a atividade das ectonucleotidases, enzimas importantes para modulação do sistema imune. Em relação ao tratamento utilizado neste estudo foi investigado o efeito da vitamina E sobre a atividade da AChE e a peroxidação lipídica no encéfalo. Os resultados demonstraram uma interação deste composto com o sistema colinérgico por diminuir a atividade da AChE, além de diminuir o dano oxidativo, em animais exposto à fumaça de cigarro. Estes resultados podem ajudar a compreender os mecanismos envolvidos na exposição passiva à fumaça de cigarro e da nicotina e futuras investigações poderão ser conduzidas com o intuito de encontrar novas terapias para beneficiar dependentes de tabaco.

5 CONCLUSÕES

- O aumento na atividade da NTPDase em plaquetas de ratos expostos à fumaça de cigarro sugere uma condição que propicia o aumento da formação de ADP que pode resultar numa ação pró-agregante.

- O aumento na atividade da 5´-nucleotidase e a diminuição na atividade da ADA em plaquetas de ratos expostos à fumaça de cigarro sugerem uma resposta orgânica compensatória, com o objetivo de manter os níveis de adenosina, um potente inibidor da agregação e modulador do tônus vascular.

- A diminuição na expressão da NTPDase e da atividade da ADA em linfócitos de ratos expostos à nicotina e o aumento nos níveis de ATP, ADP e adenosina contribuem para aumento do estado de imunodeficiência.

- A inibição da hidrólise de nucleotídeos e desaminação da adenosina pela nicotina *per se* em linfócitos de ratos *in vitro* sugere que a inibição pode ser devido à interação no sitio ativo da enzima.

- O aumento na atividade da AChE e do nível de peroxidação lipídica no encéfalo de ratos expostos à fumaça de cigarro sugere que este tipo de exposição pode atingir o SNC.

- A diminuição na atividade da AChE e do nível de peroxidação lipídica em ratos expostos à fumaça de cigarro e tratados com vitamina E sugere que este composto antioxidante possui um importante papel como agente terapêutico restaurador neste tipo de exposição.

5.1 Importância do trabalho e perspectivas

Este foi o primeiro trabalho que avaliou a atividade das ectonucleotidases na exposição à fumaça de cigarro e de nicotina, sendo observada uma alteração na sinalização purinérgica. Outro resultado interessante foi que a vitamina E, demonstrou uma interação com a acetilcolinesterase, além de promover uma diminuição na peroxidação lipídica em animais expostos à fumaça de cigarro, sugerindo que esta molécula antioxidante pode representar uma importante opção terapêutica para dependentes de tabaco. Adicionalmente, a nicotina possui um efeito supressor no sistema imune, visto que, o resultado mais relevante deste estudo foi o aumento nos nucleotídeos e nucleosídeos os quais podem contribuir para a compreensão dos mecanismos envolvidos na imunossupressão por este alcalóide. Em ambos os sistemas, vascular e sistema nervoso central, a nicotina apresenta um enorme efeito biológico, e sua dependência em modelos animais aparece em 21 dias de tratamento. É importante ressaltar que a nicotina induz a estimulação do prazer e reduz a ansiedade e quando a pessoa para de fumar aparece os sintomas de abstinência a este alcalóide, os quais servem de estímulo para a pessoa voltar a fumar. Deste modo, poderíamos aprofundar ainda mais este estudo a partir da concretização dos seguintes objetivos:

- ✓ Avaliar a atividade das enzimas NTPDase, 5'-nucleotidase e adenosina desaminase no sistema nervoso central de ratos submetidos ao modelo de síndrome de abstinência à nicotina.

- ✓ Identificar através de métodos histoquímicos a expressão da NTPDase, 5'-nucleotidase e adenosina desaminase no sistema nervoso central de ratos submetidos ao modelo de síndrome de abstinência à nicotina.

- ✓ Realizar os testes comportamentais em ratos submetidos ao modelo de síndrome de abstinência à nicotina.

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