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A CURCUMINA PREVINE OS EFEITOS DA EXPOSIÇÃO À FUMAÇA DO CIGARRO - SISTEMA PURINÉRGICO, SISTEMA COLINÉRGICO E MEMÓRIA

TESE DE DOUTORADO

Jeandre Augusto dos Santos Jaques

Santa Maria, RS, Brasil

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A CURCUMINA PREVINE OS EFEITOS DA EXPOSIÇÃO À FUMAÇA DO CIGARRO – SISTEMA PURINÉRGICO, SISTEMA COLINÉRGICO E MEMÓRIA

Jeandre Augusto dos Santos Jaques

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

Orientadora: Daniela Bitencourt Rosa Leal
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Centro de Ciências Naturais e Exatas
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A CURCUMINA PREVINE OS EFEITOS DA EXPOSIÇÃO À FUMAÇA DO CIGARRO – SISTEMA PURINÉRGICO, SISTEMA COLINÉRGICO E MEMÓRIA

elaborada por
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como requisito parcial para obtenção do grau de
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RESUMO

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

A CURCUMINA PREVINE OS EFEITOS DA EXPOSIÇÃO À FUMAÇA DO CIGARRO – SISTEMA PURINÉRGICO, SISTEMA COLINÉRGICO E MEMÓRIA

Autor: Jeandre Augusto dos Santos Jaques
Orientadora: Daniela Bitencourt Rosa Leal
Co-orientadora: Maria Rosa Chitolina Schetinger
Data e Local da Defesa: Santa Maria, 09 de maio de 2013.

A exposição à fumaça do cigarro é um fator de risco para o desenvolvimento de doenças cardiovasculares, déficits neurocognitivos e neurobiológicos. Atualmente, o emprego de fitoterápicos é uma das alternativas para o tratamento de diversas doenças. A curcumina, um polifenol obtido a partir de rizomas de *Curcuma longa* e amplamente utilizado na culinária e na medicina tradicional oriental, possui diversas propriedades farmacológicas como antioxidante, anti-agregante e neuroprotetora. Em virtude de seu amplo espectro de propriedades farmacológicas a curcumina possui potencial para a prevenção dos efeitos causados pela exposição à fumaça do cigarro. Neste contexto, o objetivo deste estudo foi avaliar o efeito da curcumina sobre a memória e parâmetros envolvidos na homeostase do sistema nervoso central (SNC) em ratos expostos de forma passiva à fumaça do cigarro. Os experimentos foram realizados em duas etapas, sendo a primeira delas dividida em duas fases. Na primeira fase, os animais foram divididos aleatoreamente em quatro grupos, denominados: veículo; curcumina 12,5 mg/kg; curcumina 25 mg/kg; e curcumina 50 mg/kg. Na segunda fase, os animais foram divididos aleatoreamente em cinco grupos, denominados: veículo; cigarro; cigarro + curcumina 12,5 mg/kg; cigarro + curcumina 25 mg/kg; e cigarro + curcumina 50 mg/kg. Na segunda etapa experimental, os animais foram divididos aleatoreamente em dez grupos, denominados: veículo; curcumina 12,5 mg/kg; curcumina 25 mg/kg; curcumina 50 mg/kg; curcumina nanoencapsulada 4 mg/kg; cigarro; cigarro + curcumina 12,5 mg/kg; cigarro + curcumina 25 mg/kg; cigarro + curcumina 50 mg/kg; cigarro + curcumina nanoencapsulada 4 mg/kg. O tratamento com a curcumina e com a fumaça do cigarro foi realizada uma vez por dia, cinco dias por semana, durante trinta dias. A curcumina foi administrada de forma oral e, após aproximadamente dez minutos, os grupos fumantes eram expostos à fumaça de quatro cigarros comerciais (0,9 mg de nicotina, 10 mg de alcatrão cada) dentro de uma câmara de exposição. Após trinta dias, os animais foram eutanasiados, o sangue coletado e o encéfalo dissecado em córtex cerebral, hipocampo, hipotálamo, estriado e cerebelo. O grupo de ratos expostos à fumaça do cigarro apresentou um aumento na atividade das enzimas E-NTPDase (ATP como substrato) e E-5'-NT, e uma redução na atividade da enzima E-NTPDase (ADP como substrato) em plaquetas; um aumento nas atividades das enzimas E-NTPDase, E-5'-NT e AChE em sinaptossomas de córtex cerebral; um aumento na atividade da enzima AChE em cerebelo, córtex cerebral, hipocampo, estriado, hipotálamo e sangue periférico; uma redução nas atividades das enzimas Na^+,K^+-ATPase e Ca^{2+}-ATPase e um desequilíbrio no balanço redox. Além disso, neste mesmo grupo de animais observou-se um déficit cognitivo avaliado através dos testes da esquiva inibitória e do reconhecimento de objetos. Concluimos que o uso de ambas as formulações de curcumina livre e nanoestruturada previne os efeitos observados nas atividades das enzimas do sistema purinérgico, colinérgico, nas enzimas envolvidas na formação do gradiente iônico e nos parâmetros de estresse oxidativo. Por fim, os resultados obtidos neste estudo indicam que a administração da curcumina através de nanocápsulas de núcleo lipídico possa ser uma alternativa para o aumento de sua eficácia, provavelmente pelo aumento da biodisponibilidade da curcumina administrada de forma oral.

Palavras-chave: Curcumina. *Curcuma longa.* Fumaça do cigarro. Sistema purinérgico. Sistema colinérgico. Memória. Estresse oxidativo. Nanocápsulas.

ABSTRACT

Thesis of Doctor's Degree
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Federal University of Santa Maria, RS, Brazil

CURCUMIN PREVENTS AGAINST THE EFFECTS OF CIGARETTE SMOKE EXPOSURE – PURINERGIC SYSTEM, CHOLINERGIC SYSTEM AND MEMORY

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Place and Date: Santa Maria, may 09th, 2013.

Cigarette smoke exposure is a major risk factor to the development of cardiovascular diseases, neurocognitive and neurobiological deficits. Nowadays, phytotherapy is widely employed in the treatment of many illnesses. Curcumin, a polyphenol obtained from the rhizomes of *Curcuma longa* and commonly used in the oriental culinary and traditional medicine, has several pharmacological properties such as antioxidant, antiaggregant and neuroprotective. Despite its wide-ranging spectrum of pharmacological properties, curcumin possess potential to prevent the noxious effects caused by cigarette smoke exposure. In this context, the purpose of this study was to evaluate the effect of curcumin on memory and parameters involved in the homeostasis of central nervous system (CNS) in rats passively exposed to cigarette smoke. The experiments were performed in two different stages, being the first divided in two sets. In the first set, animals were randomly assigned into four groups: vehicle; curcumin 12.5 mg/kg; curcumin 25 mg/kg; and curcumin 50 mg/kg. In the second set, animals were randomly assigned into five groups: vehicle, cigarette smoke; curcumin 12.5 mg/kg along with cigarette smoke; curcumin 25 mg/kg along with cigarette smoke; and curcumin 50 mg/kg along with cigarette smoke. In the second experimental stage, animals were randomly divided into ten groups: vehicle; curcumin 12.5 mg/kg; curcumin 25 mg/kg; curcumin 50 mg/kg; nanoencapsulated curcumin 4 mg/kg; cigarette smoke; curcumin 12.5 mg/kg along with cigarette smoke; curcumin 25 mg/kg along with cigarette smoke; curcumin 50 mg/kg along with cigarette smoke; and nanoencapsulated curcumin 4 mg/kg along with cigarette smoke. The treatment with curcumin and cigarette smoke was carried out once a day, 5 days each week, during 30 days. Curcumin was administered orally and, approximately 10 minutes later, the smoking groups were exposed to the sidestream smoke of four commercial cigarettes (nicotine 0.9 mg, tar 10 mg each) inside a whole-body smoke exposure chamber. After thirty days, the animals were euthanized, the blood collected and the brain dissected in cerebral cortex, hippocampus, hypothalamus, striatum and cerebellum. The group of rats exposed to cigarette smoke showed an increase in the activity of the enzymes E-NTPDase (ATP as substrate) and E-5'-NT, and a reduction in the activity of the enzyme E-NTPDase (ADP as substrate) in platelets; an increase in the activities of the enzymes E-NTPDase, E-5'-NT and AChE in synaptosomes from the cerebral cortex; an increase in the activity of AChE in cerebellum, cerebral cortex, hippocampus, striatum, hypothalamus and peripheral blood; a decrease in the activities of the enzymes Na^+,K^+-ATPase and Ca^{2+}-ATPase and a redox imbalance. Furthermore, in the same group of animals, it was observed a cognitive impairment evaluated through the inhibitory avoidance test and the object recognition test. We conclude that the use of both formulations of curcumin, free and nanostructured, prevents the effects observed in the purinergic and cholinergic system, in the enzymes involved in the ion homeostasis and in the oxidative stress parameters. Finally, the results obtained in this study indicate that curcumin administration as lipid-core nanocapsules may be an alternative to increase its efficacy, probably by the increase of its bioavailability when administered orally.

Keywords: Curcumin. *Curcuma longa.* Cigarette smoke. Purinergic system. Cholinergic system. Memory. Oxidative stress. Nanocapsules.

LISTA DE ILUSTRAÇÕES

INTRODUÇÃO

Figura 1 – Separação da fumaça do cigarro em fase gasosa e fase particulada . 15
Figura 2 – Dano oxidativo induzido por EROs …… 16
Figura 3 – Função plaquetária na homeostase …….. 18
Figura 4 – Componentes da sinalização autócrina purinérgica 20
Figura 5 – Árvore filogenética hipotética derivada de 22 membros selecionados da família das NTPDases (NTPDase1 a NTPDase8) de ratos, seres humanos e camundongos, após o emparelhamento da sequência de aminoácidos 21
Figura 6 – Esquema da sinapse colinérgica …… 23
Figura 7 – Esquema do ciclo funcional da Na^+,K^+-ATPase 25
Figura 8 – Esquema do ciclo funcional da SERCA 1a 26
Figura 9 – Isolamento, extração e estrutura química da curcumina 27

ARTIGO 1

Figure 1 – Ectonucleoside triphosphate diphosphohydrolase activity in the platelets from cigarette smoke-exposed rats treated with curcumin … 34
Figure 2 – 5'-Nucleotidase activity in platelets from cigarette smoke-exposed rats treated with curcumin ……... 35
Figure 3 – Adenosine deaminase activity in the platelets from cigarette smoke-exposed rats treated with curcumin …….. 35

ARTIGO 2

Figure 1 – NTPDase and 5'-Nucleotidase activities in cerebral cortex synaptosomes from cigarette smoke-exposed rats treated with curcumin …… 41
Figure 2 – Acetylcholinesterase activity in cerebral cortex synaptosomes from cigarette smoke-exposed rats treated with curcumin ……. 42

ARTIGO 3

Figure 1 – Effect of oral administration of vehicle (corn oil) or curcumin in cigarette smoke-exposed rats on the inhibitory avoidance task performance of adult rats measured as the test step-down latencies.. 47
Figure 2 – AChE activity in the cerebellum, cerebral cortex and hippocampus of cigarette smoke-exposed rats and those treated with curcumin plus smoke……... 48
Figure 3 – AChE activity in striatum of cigarette smoke-exposed rats and those treated with curcumin plus smoke …….. 48
Figure 4 – AChE activity in the hypothalamus of cigarette smoke-exposed rats and those treated with curcumin plus smoke ……... 49
Figure 5 – AChE activity in whole blood of cigarette smoke-exposed rats and those treated with curcumin plus smoke ……... 49

ARTIGO 4

Figure 1 – Recognition index on test trial (memory test) in object recognition memory task of cigarette smoke-exposed rats and those treated with curcumin free and nanoencapsulated along with smoke …… 56
Figure 2 – Na^+,K^+-ATPase activity in cerebral cortex homogenates of cigarette smoke-exposed rats and those treated with curcumin free and nanoencapsulated along with smoke …… 56
Figure 3 – Ca^+-ATPase activity in cerebral cortex homogenates of cigarette smoke-exposed rats and those treated with curcumin free and nanoencapsulated along with smoke …… 57
Figure 4 – Thiobarbituric acid reactive substances levels in cerebral cortex homogenates of cigarette smoke-exposed rats and those treated with curcumin free and nanoencapsulated along with smoke ……..……. 57
Figure 5 – Nitrites and nitrates levels in cerebral cortex homogenates of cigarette smoke-exposed rats and those treated with curcumin free and nanoencapsulated along with smoke …… 58
Figure 6 – Non-protein sulfhydryl groups levels in cerebral cortex homogenates of cigarette smoke-exposed rats and those treated with curcumin free and nanoencapsulated along with smoke …… 58
Figure 7 – Superoxide dismutase activity in cerebral cortex homogenates of cigarette smoke-exposed rats and those treated with curcumin free and nanoencapsulated along with smoke …… 58

LISTA DE TABELAS

ARTIGO 3

Table 1 – Effect of oral administration of vehicle or curcumin 5 days each week, during 30 days on the latency to training on the inhibitory avoidance apparatus and on the behavior of rats in the open-field immediately after the inhibitory avoidance testing session …… 47

Table 2 – Effect of oral administration of vehicle, smoke, and smoke plus curcumin 5 days each week, during 30 days on the latency to training on the inhibitory avoidance apparatus and on the behavior of rats in the open-field immediately after the inhibitory avoidance test session ……... 47

Table 3 – Effect of oral administration of vehicle and curcumin 5 days each week, during 30 days on AChE activity in the five brain structures and whole blood of rats ……... 48

ARTIGO 4

Table 1 – Physicochemical characteristics of C-LNC …….. 55

Table 2 – Effect of oral administration of curcumin free and nanoencapsulated along with smoke on the training trial (sample phase) recognition index and on behavior of rats (number of crossing and rearing responses) in the open-field immediately after the object recognition testing session …….. 55

Table 3 – Effect of oral administration of curcumin free and nanoencapsulated *per se* on the training and test recognition indexes, on crossing and rearing responses in the open field immediately after the object recognition test session, on the activities of Na^{+},K^{+}-ATPase and Ca^{2+}-ATPase, and on parameters of oxidative stress (TBARS, NOx, NPSH and SOD)…… 56

LISTA DE ABREVIATURAS E SIGLAS

AA	ácido araquidônico
Acetil-CoA	acetil-coenzima A
ACh	acetilcolina
AChE	acetilcolinesterase
ADO	adenosina
ADP	nucleosídeo difosfato de adenosina
$AlCl_3$	cloreto de alumínio
AMP	nucleosídeo monofosfato de adenosina
As	arsênio
ATP	nucleosídeo trifosfato de adenosina
BHE	barreira hematoencefálica
CaM	calmodulina
Ca^{2+}	cálcio
Ca^{2+}-ATPase	bomba de cálcio
cAMP	AMP cíclico
CD	*cluster of differentiation*, grupamento de diferenciação
ChAT	*choline acetyltransferase*, colina acetil-transferase
C-LNC	*curcumin-loaded nanocapsules*, nanocápsulas carregadas com a curcumina
CXCL4	fator plaquetário 4
DAG	diacilglicerol
DDT	dicloro-difenil-tricloroetano
DNA	*deoxyribonucleic acid,* ácido desoxirribonucléico
DPOC	doença pulmonar obstrutiva crônica
E-ADA	ecto-adenosina desaminase
E-NTPDases	ecto-nucleosídeo trifosfato difosfohidrolases
ERNs	espécies reativas de nitrogênio
EROs	espécies reativas de oxigênio
E-5'-NT	ecto-5'-nucleotidase
FAP	fator de agregação plaquetário
Fe^{2+}	íon ferroso

GPI	glicosil-fosfatidilinositol
HIV	vírus da imunodeficiência humana
$HO^{\bullet}$	radical hidroxila
H_2O_2	peróxido de hidrogênio
IBGE	Instituto Brasileiro de Geografia e Estatística
i.p.	injeções intraperitoneais
i.v.	administração intravenosa
K^+	potássio
K_m	constante de Michaelis
LTM	*long term memory*, memória de longa duração
mAChRs	receptores colinérgicos muscarínicos
MRT	*mean residence time*, tempo médio de residência
nAChRs	receptores colinérgicos nicotínicos
Na^+	sódio
Na^+,K^+-ATPase	bomba de sódio-potássio
$NO^{\bullet}$	óxido nítrico
NO_2	dióxido de nitrogênio
NTPDase	nucleosídeo trifosfato difosfohidrolase
$ONOO^-$	peroxinitrito
$O_2^{\bullet-}$	ânion radical superóxido
O_2NOO^-	peroxinitrato
P-ATPases	ATPases do tipo P
PKA	proteína cinase A
PKC	proteína cinase C
PLGA	poliéster de ácido láctico-co-glicólico
PMCA	*plasma membrane Ca^{2+}-ATPase*, Ca^{2+}-ATPase da membrana plasmática
P1 / P2Y	receptores purinérgicos metabotrópicos
P2X	receptores purinérgicos ionotrópicos
$Q^{\bullet-}$	radical semiquinona
RNA	*ribonucleic acid,* ácido ribonucléico
STZ	estreptozotocina
SERCA	*sarco-endoplasmic reticulum Ca^{2+}-ATPase*, Ca^{2+}-ATPase do retículo sarco-endoplasmático

SNC	sistema nervoso central
TXB_2	tromboxano B_2
$t_{1/2}$	meia vida
vAChT	*vesicular ACh transporter*, transportador vesicular de ACh
VGCC	*voltage-gated calcium channels,* canais de cálcio dependentes de voltagem
12-HETE	12-hidroxieicosatetraenóico
12-HHT	12-hidroxiheptadecatrienóico
12-HPETE	ácidos monohidroperóxidoeicosateraenoicos

SUMÁRIO

INTRODUÇÃO 15
Objetivos 30
Objetivo geral 30
Objetivos específicos 30
ARTIGO 1 - EFFECTS OF CURCUMIN ON THE ACTIVITIES OF THE ENZYMES THAT HYDROLYSE ADENINE NUCLEOTIDES IN PLATELETS FROM CIGARETTE SMOKE-EXPOSED RATS..... 31
Abstract 32
Introduction 32
Materials and methods 33
Results 34
Discussion 35
References 36
ARTIGO 2 – THE EFFECT OF CURCUMIN IN THE ECTONUCLEOTIDASES AND ACETYLCHOLINESTERASE ACTIVITIES IN SYNAPTOSOMES FROM THE CEREBRAL CORTEX OF CIGARETTE SMOKE-EXPOSED RATS..... 38
Abstract 39
Introduction 39
Materials and methods 40
Results 42
Discussion 42
References 43
ARTIGO 3 – CURCUMIN PROTECTS AGAINST CIGARETTE SMOKE-INDUCED COGNITIVE IMPAIRMENT AND INCREASED ACETYLCHOLINESTERASE ACTIVITY IN RATS..... 44
Abstract 45
Introduction 45
Material and methods 46
Results 47
Discussion 48
Conclusions 50
References 50
ARTIGO 4 – FREE AND NANOENCAPSULATED CURCUMIN PREVENTS CIGARETTE SMOKE-INDUCED COGNITIVE IMPAIRMENT AND REDOX IMBALANCE..... 51
Abstract 52
Introduction 52
Materials and methods 53
Results 55
Discussion 58
Conclusions 59
References 60
DISCUSSÃO 62
CONCLUSÃO 76
REFERÊNCIAS..... 77

APRESENTAÇÃO

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos. As seções Materiais e Métodos, Resultados, Discussão e Referências encontram-se nos próprios artigos e representam a íntegra deste estudo.

Os itens Discussão e Conclusões, encontrados no final desta tese, apresentam interpretações e comentários gerais sobre os artigos contidos neste trabalho.

As referências referem-se somente às citações que aparecem nos itens Introdução e Discussão desta tese.

Os artigos estão estruturados de acordo com as normas das revistas científicas nas quais encontram-se publicados:

Artigo 1: Cell Biochemistry and Function

Artigo 2: Cell Biochemistry and Function

Artigo 3: Physiology & Behavior

Artigo 4: Neurobiology of Learning and Memory

INTRODUÇÃO

Hoje, aproximadamente 20% da população mundial (± 1,4 bilhão de pessoas) utiliza produtos derivados do tabaco, a maioria na forma de cigarros e, em consequência disso, cerca de seis milhões de pessoas morreram somente em 2011 (ERIKSEN et al., 2012). De acordo com o Instituto Brasileiro de Geografia e Estatística (IBGE, 2012) o Brasil possui uma população estimada de 190 milhões de pessoas. Uma pesquisa realizada pelo Ministério da Saúde revelou que a frequência de fumantes na população brasileira é de 15,1% (BRASIL, 2011), aproximadamente 28,6 milhões de fumantes.

Mesmo os não fumantes se expõem diariamente à fumaça do cigarro, a qual possui mais de 4.000 substâncias tóxicas (GENBACEV-KRTOLICA, 2005). Dentre elas, encontram-se: a acetona, o acetileno, o arsênico, o benzeno, o butano, o cádmio, o monóxido de carbono, o dicloro-difenil-tricloroetano (DDT), o formaldeído, o chumbo, o cianeto de hidrogênio, o metanol, a nicotina, o fenol, o polônio 210, o tolueno, o cloreto de vinila e diversas outras dezenas de substâncias carcinogênicas (ERIKSEN et al., 2012). Além destes compostos tóxicos, o cigarro também é fonte de espécies reativas de oxigênio (EROs) como o ânion radical superóxido ($O_2^{\bullet-}$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($HO^{\bullet}$); e espécies reativas de nitrogênio (ERNs) como o óxido nítrico ($NO^{\bullet}$), o peroxinitrito ($ONOO^-$) e o peroxinitrato (O_2NOO^-) (Figura 1) (PRYOR; STONE, 1993).

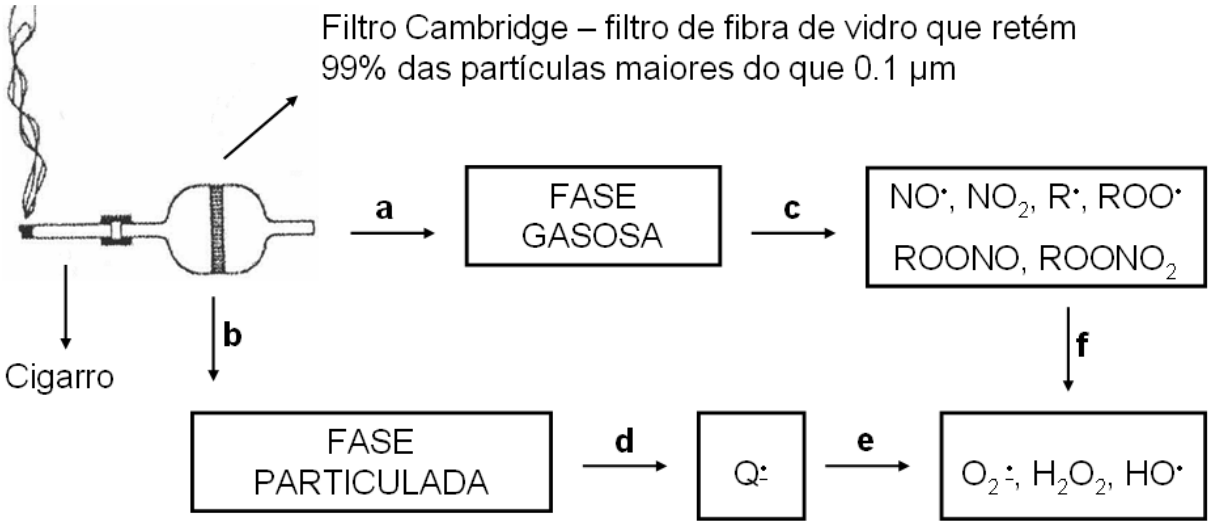

Figura 1 – Separação da fumaça do cigarro em (a) fase gasosa e (b) fase particulada. (c) A fase gasosa do cigarro contém radicais que são produzidos a partir da reação de $NO^{\bullet}/NO_2$ com os compostos presentes na fumaça do cigarro. (d) A fase particulada contém o radical semiquinona ($Q^{\bullet-}$), que por sua vez pode gerar (e) $O_2^{\bullet-}$, H_2O_2 e, na presença de Fe^{2+}, $HO^{\bullet}$. (f) Além disso, a fase gasosa também é fonte das EROs. Adaptado de (PRYOR & STONE, 1993).

Estudos demonstram que as espécies reativas geradas pela exposição a estes compostos e pela combustão do cigarro podem causar dano oxidativo em macromoléculas biológicas como a peroxidação de ácidos graxos poliinsaturados de membrana, oxidação protéica, quebra da dupla fita de ácido desoxirribonucléico (*deoxyribonucleic acid*, DNA) e oxidação de ácido ribonucléico (*ribonucleic acid*, RNA) desencadeando a despolarização da membrana mitocondrial, a apoptose (CHEN et al., 2004; DEMARINI, 2004; KONG; LIN, 2010; PRYOR et al., 1998; RAIJ et al., 2001; STONE et al., 1995; TSUCHIYA et al., 2002) e uma cascata inflamatória que pode levar ao desenvolvimento e/ou facilitar a progressão de diversas doenças que envolvem o sistema nervoso central (SNC) (Figura 2) (ALMEIDA et al., 2008; DEANE; ZLOKOVIC, 2007; MCQUAID et al., 2009; SUNDSTROM et al., 2008).

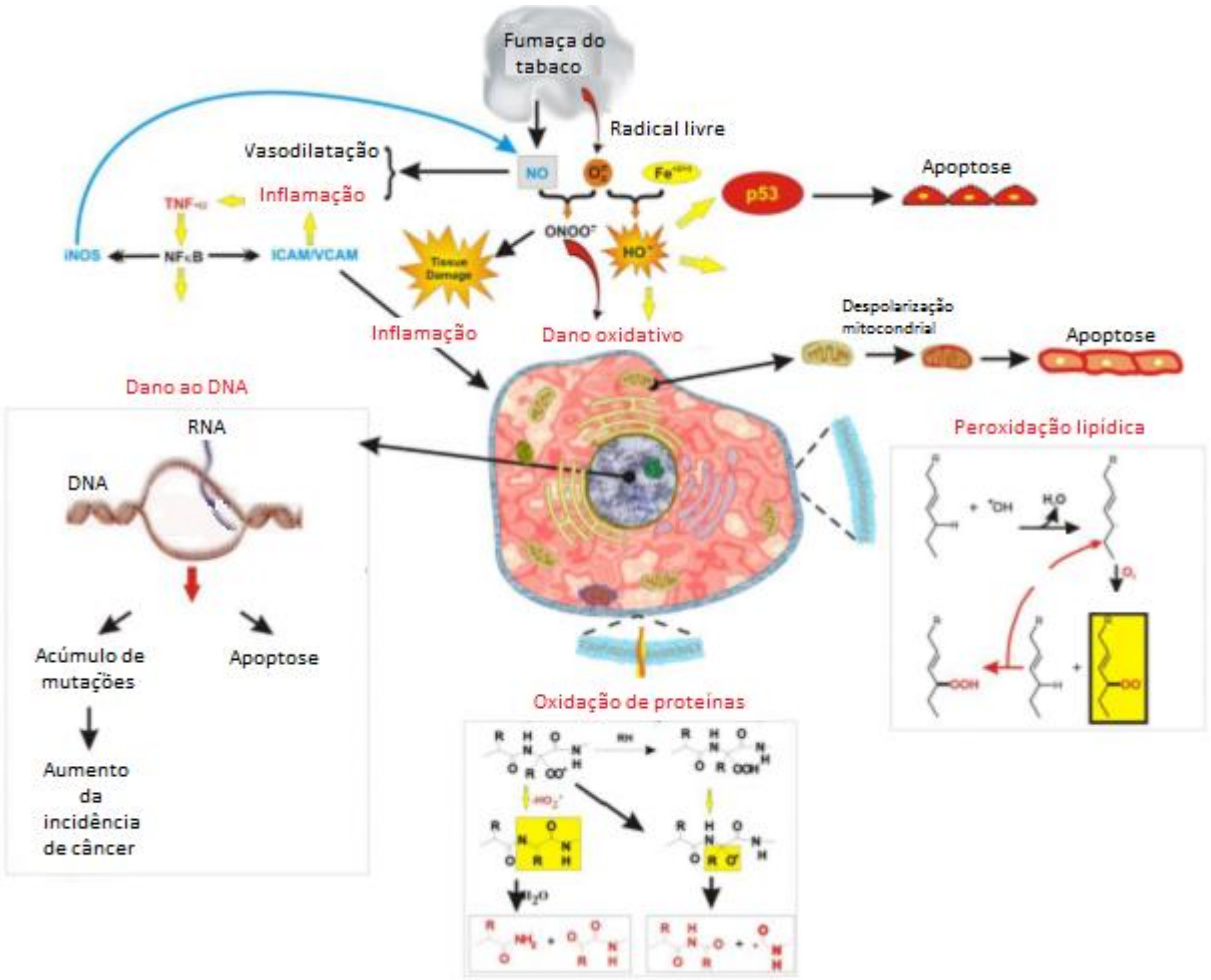

Figura 2 – Dano oxidativo induzido por EROs. Representação esquemática das diversas vias pelas quais as EROs originadas da combustão do tabaco podem causar dano celular e inflamação. Adaptado de (MAZZONE et al., 2010).

O dano oxidativo que ocorre em consequência da exposição à fumaça do cigarro também está associado com a disfunção endotelial caracterizada pelo déficit no relaxamento vascular dependente do endotélio (ARGACHA et al., 2008; OTA et al., 1997; RAIJ et al., 2001). A exposição ao tabaco é um dos fatores fortemente associados com doenças cardiovasculares como a doença arterial coronariana e a aterosclerose (CASTELLI et al., 1981; GLANTZ; PARMLEY, 1991; GUARINO et al., 2011; STEENLAND et al., 1996). O dano ao tecido endotelial também está associado à ativação dos trombócitos ou plaquetas em consequência da ligação ao colágeno, à outras proteínas expostas da matriz extracelular e da subsequente liberação de mediadores solúveis armazenados em vesículas (Figura 3) (SEMPLE et al., 2011). Estudos indicam que a agregabilidade plaquetária é mais acentuada em indivíduos fumantes e que estes apresentam níveis séricos de proteínas secretadas por plaquetas ativadas como o fator plaquetário 4 (CXCL4), a β-tromboglobulina, o fator de agregação plaquetário (FAP) e a P-selectina superiores aos basais, e também aumento da expressão de P-selectina na superfície das plaquetas (DOTEVALL et al., 1987; FITZGERALD et al., 1983; FUSEGAWA; HANDA, 2000; IMAIZUMI, 1991; MIYAURA et al., 1992; PERNERSTORFER et al., 1998).

Durante cerca de um século e meio as plaquetas foram exclusivamente associadas à trombogênese (BIZZOZERO, 1882). Atualmente, embora sejam primariamente associadas à hemostase e à trombogênese, existe uma gradual compreensão de que as plaquetas desempenham uma importante função na modulação da resposta imune inata e adaptativa (ELZEY et al., 2003; HENN et al., 1998; KISSEL et al., 2006; SEMPLE et al., 2011; SPRAGUE et al., 2008; VON HUNDELSHAUSEN; WEBER, 2007). Além disso, estudos demonstram que plaquetas ativadas estão envolvidas na etiologia de diversas doenças que acometem o SNC por possuírem uma grande variedade de receptores e produtos de secreção, além daqueles envolvidos com as funções clássicas de hemostase e trombogênese, os quais estão ativos em processos imunes, inflamatórios e de reparo tecidual (CIABATTONI et al., 2007; HORSTMAN et al., 2010; PUTNAM, 1935; SHEREMATA et al., 2008).

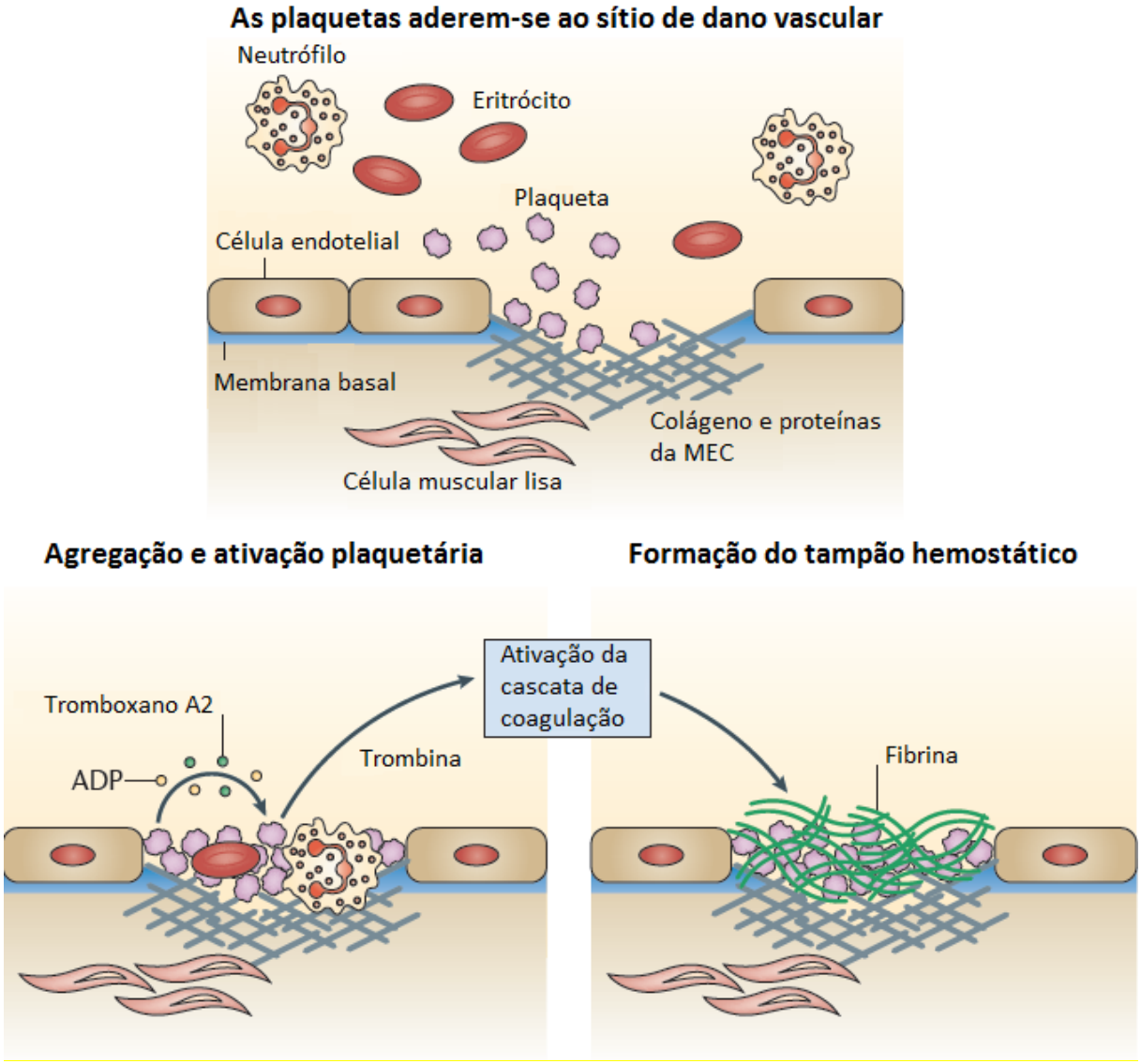

Figura 3 – Função plaquetária na hemostase. Acredita-se que a principal função fisiológica das plaquetas é a hemostase. No primeiro passo deste processo, um dano vascular expõe o colágeno e proteínas da membrana basal que permitem a adesão das plaquetas ao substrato. As plaquetas aderidas formam um agregado e iniciam a liberação de mediadores da ativação plaquetária como o ADP e o tromboxano A2. Em seguida, as plaquetas produzem trombina, a qual catalisa o início da cascata de coagulação e a deposição de fibrina. ECM, matriz extracelular. Adaptado de (SEMPLE et al., 2011).

Alguns autores propõem (HORSTMAN et al., 2010) que as plaquetas juntamente com os linfócitos estariam envolvidas em doenças que acometem o SNC por aumentar a permeabilidade da barreira hematoencefálica (BHE). Especificamente, o FAP, um dos principais agonistas liberados pelas plaquetas e um potente mediador pró-inflamatório, seria secretado pela cooperação entre plaquetas e leucócitos e facilitaria o rompimento da BHE, uma vez que uma das funções reconhecidas e mais proeminentes do FAP é a ruptura das junções endoteliais

(ADAMSON et al., 2008; BRKOVIC; SIROIS, 2007; JIANG et al., 2008; KNEZEVIC et al., 2009). O envolvimento do FAP com o dano encefálico já foi proposto anteriormente (LINDSBERG et al., 1991) e acredita-se que ele seja responsável pela ruptura da BHE no início do desenvolvimento da esclerose múltipla (CALLEA et al., 1999; LOCK et al., 2002). Sabe-se que a exposição à fumaça do cigarro está associada com o aumento na liberação do FAP (IMAIZUMI, 1991), o que provavelmente contribui para as alterações neurobiológicas e neurocognitivas observadas em indivíduos fumantes e expostos de forma passiva ao tabaco. Além disso, a fumaça do cigarro também contém níveis elevados de $NO^{\bullet}$, que pode afetar a função da BHE diretamente ou via alteração do tônus vascular, ativação plaquetária e pela adesão de leucócitos ao endotélio (BORLAND; HIGENBOTTAM, 1987). A migração de leucócitos através da BHE é uma das principais características de diversas desordens neuroimunes (MAZZONE et al., 2010).

Diversos grupos de pesquisa que investigam as consequências neurocognitivas da exposição crônica à fumaça do cigarro apontam um declínio na precisão da memória de trabalho (JACOBSEN et al., 2005), na memória áudio-verbal (FRIED et al., 2006), na velocidade de processamento de informações (SPILICH et al., 1992), na inteligência geral (WEISER et al., 2010), na velocidade psicomotora e na flexibilidade cognitiva (KALMIJN et al., 2002) e em habilidades intelectuais gerais (DEARY et al., 2003). Estes efeitos observados no desempenho cognitivo de fumantes, tais como o declínio na velocidade psicomotora e flexibilidade cognitiva sugerem alguma alteração no córtex cerebral (substância branca) (DE GROOT et al., 2000; YLIKOSKI et al., 1993), que pode ser resultante de lesões em pequenos vasos, uma vez que já foram descritas alterações vasculares e no fluxo sanguíneo cerebral de fumantes (ROGERS et al., 1984) e estas alterações estão diretamente relacionadas com o desempenho cognitivo (MEYER et al., 1988).

O sistema purinérgico, que é constituído por enzimas, nucleotídeos, nucleosídeos, e receptores purinérgicos (Figura 4) (JUNGER, 2011), participa de forma notável em processos que envolvem o SNC como a neurotransmissão (BURNSTOCK, 1976; BURNSTOCK, 2007), a neuromodulação (BURNSTOCK, 2007) e a plasticidade sináptica (WIERASZKO; EHRLICH, 1994) além de processos como a tromborregulação e hemostase (ZIMMERMANN, 1999).

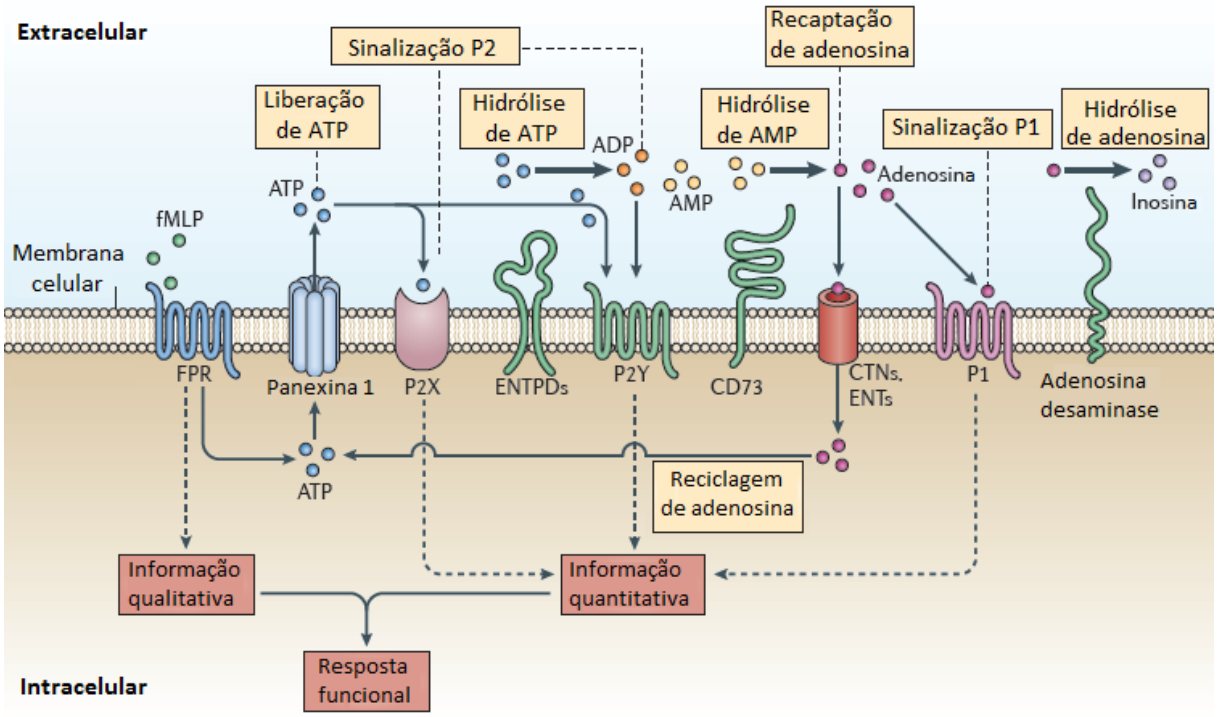

Figura 4 – Componentes da sinalização autócrina purinérgica. Esquema dos elementos chave envolvidos na sinalização purinérgica. A ativação de receptores do tipo formil peptídeo (FPRs) pelo *N*-formil-metionil-leucil-fenilalanina (fMLP) leva a abertura de semicanais de panexina 1 e liberação de ATP pela célula. O ATP liberado promove a ativação autócrina de receptores do tipo P2. As enzimas ectonucleotidases, como a ectonucleosídeo trifosfato difosfohidrolase (E-NTPDase) e a 5'-nucleotidase promovem a hidrólise do ATP e a formação de adenosina, a qual ativa receptores do tipo P1. A adenosina é "neutralizada" pela ecto-adenosina desaminase, a qual converte a adenosina em inosina, ou reciclada via transportadores de nucleosídeos (CNTs). Adaptado de (JUNGER, 2011).

Dentre as enzimas do sistema purinérgico, as ecto-nucleotidases atuam na regulação dos níveis extracelulares dos nucleotídeos e nucleosídeo de adenina, que são moléculas sinalizadoras. Dentre elas destacam-se as famílias ecto-nucleosídeo trifosfato difosfohidrolase (E-NTPDase, EC 3.6.1.5) e ecto-5'-nucleotidase (E-5'-NT, EC 3.1.3.5) por representarem as principais enzimas envolvidas na hidrólise de nucleotídeos que participam da sinalização purinérgica (ZIMMERMANN et al., 2012). As E-NTPDases possuem esta nomenclatura por possuírem a capacidade de hidrolisarem bases púricas e pirimídicas (VITIELLO et al., 2012). Até então, oito membros parálogos desta família foram identificados e diferem quanto à especificidade de substratos, distribuição tecidual e localização celular (Figura 5); destes, 4 (NTPDAse1, NTPDase2, NTPDase3 e NTPDase8) são tipicamente expressos na superfície celular; e 4 (NTPDase 4-7) estão presentes no meio intracelular em organelas citoplasmáticas (BIGONNESSE et al., 2004; ROBSON et al., 2006; SHI et al., 2001; ZIMMERMANN, 2001; ZIMMERMANN et al., 2012). A E-

NTPDase1 (CD39, ATP difosfohidrolase, apirase) catalisa a desfosforilação sequencial do nucleosídeo trifosfato de adenosina (ATP) em nucleosídeo difosfato (ADP) até a formação do nucleosídeo monofosfato (AMP) e é encontrada em diversas células como em plaquetas, células endoteliais, células musculares lisas dos vasos, neurônios e células da glia (BRAUN et al., 2000; ZIMMERMANN, 1999; ZIMMERMANN, 2006a). A E-5'-NT é uma enzima ancorada à membrana plasmática por glicosil-fosfatidilinositol (GPI) e possui forma estrutural homodimérica com pontes dissulfeto entre as cadeias. Esta enzima possui um importante papel na cascata purinérgica, pois hidrolisa preferencialmente moléculas de AMP em ADO com valores

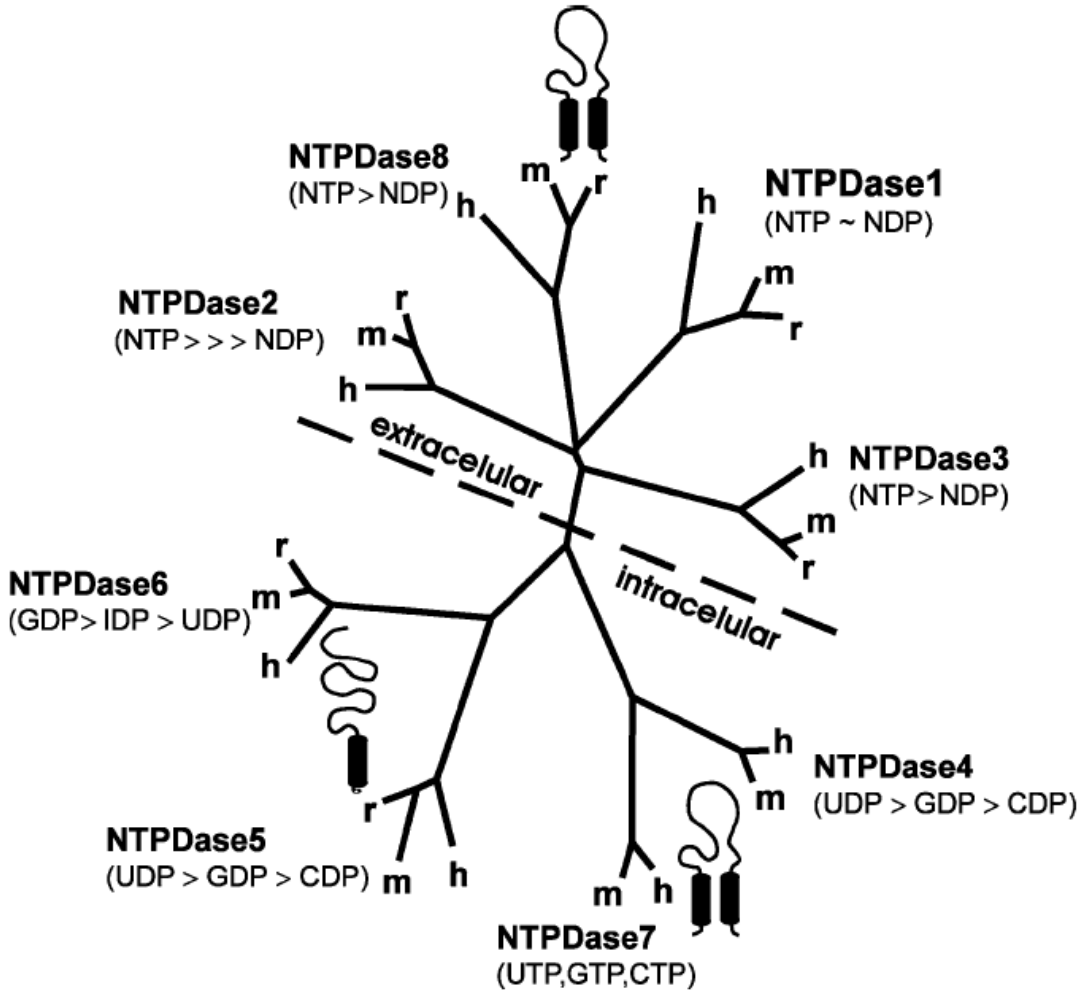

Figura 5 – Árvore filogenética hipotética derivada de 22 membros selecionados da família das NTPDases (NTPDase1 a NTPDase8) de ratos (r), seres humanos (h) e camundongos (m), após o emparelhamento da sequência de aminoácidos. O comprimento das linhas indica a diferença existente entre as sequências de aminoácidos. Pode observar-se a clara separação entre as enzimas ancoradas à membrana (acima da linha pontilhada) e às formas intracelulares. Além disso, são indicadas as principais afinidades por substratos para cada membro e a sua respectiva topografia (um ou 2 domínios transmembrana). Adaptado de (ROBSON et al., 2006).

da constante de Michaelis (K_m) na faixa de micromolar (ZIMMERMANN, 1992; ZIMMERMANN, 1996; ZIMMERMANN, 2001; ZIMMERMANN et al., 2012). Quanto à sua distribuição, a E-5'-NT também é expressa em plaquetas, células endoteliais, neurônios e células da glia (ZIMMERMANN, 1996; ZIMMERMANN et al., 1998). Além das ecto-nucleotidases a enzima ecto-adenosina desaminase (E-ADA, EC 3.5.4.4) também participa da regulação dos níveis extracelulares de adenosina (ADO), através da desaminação da ADO em inosina (COLGAN et al., 2006; YEGUTKIN, 2008; ZIMMERMANN, 2001).

Os nucleotídeos e o nucleosídeo de adenina, que têm suas concentrações extracelulares reguladas pelas atividades dessas enzimas, são agonistas de receptores purinérgicos metabotrópicos (P1, P2Y) e ionotrópicos (P2X). Através da ligação com estes receptores os nucleotídeos e a adenosina participam de respostas fisiológicas diversas como, por exemplo: no desenvolvimento sináptico (BRAUN et al., 1995; SCHOEN; KREUTZBERG, 1994); na neurogênese (ZIMMERMANN, 2006b); no pré-condicionamento isquêmico e adaptação à hipoxia (SCHETINGER et al., 1998a; SCHETINGER et al., 1998b); na tromborregulação e hemostase e na função plaquetária (ZIMMERMANN, 1999). Um ponto que merece destaque é que, assim como outros neurotransmissores, o ATP é armazenado em vesículas sinápticas, liberados de maneira dependente da concentração de Ca^{2+}, atua em receptores metabotrópicos ou ionotrópicos e é hidrolisado extracelularmente (ZIMMERMANN, 2008).

O sistema colinérgico, por sua vez, desempenha um papel fundamental em várias funções vitais tais como o aprendizado e a memória (MESULAM et al., 2002; WINKLER et al., 1995). A acetilcolina (ACh), a primeira molécula a ser identificada como um neurotransmissor, liga-se a duas classes de receptores: os receptores colinérgicos nicotínicos (nAChRs), de ação ionotrópica, e os receptores colinérgicos muscarínicos (mAChRs), de ação metabotrópica (DESCARRIES et al., 1997; KANDEL et al., 2000). A sua síntese é realizada nos terminais nervosos pré-sinápticos pela colina-acetiltransferase (ChAT, EC 2.3.1.6) a partir de dois precursores: a colina e a acetil-coenzima A (acetil-CoA). A ACh é armazenada em vesículas que liberam seu conteúdo por exocitose, após influxo de cálcio no terminal nervoso. Ao ser liberada, a ACh interage com receptores específicos causando despolarização e propagação do potencial de ação na célula pós-sináptica. A enzima acetilcolinesterase (AChE, EC 3.1.1.7), localizada na fenda sináptica, realiza

a terminação da transmissão sináptica colinérgica através da hidrólise da ACh em colina e acetato (Figura 6) (SILVA, 1998; SOREQ; SEIDMAN, 2001; TAYLOR; BROWN, 1999).

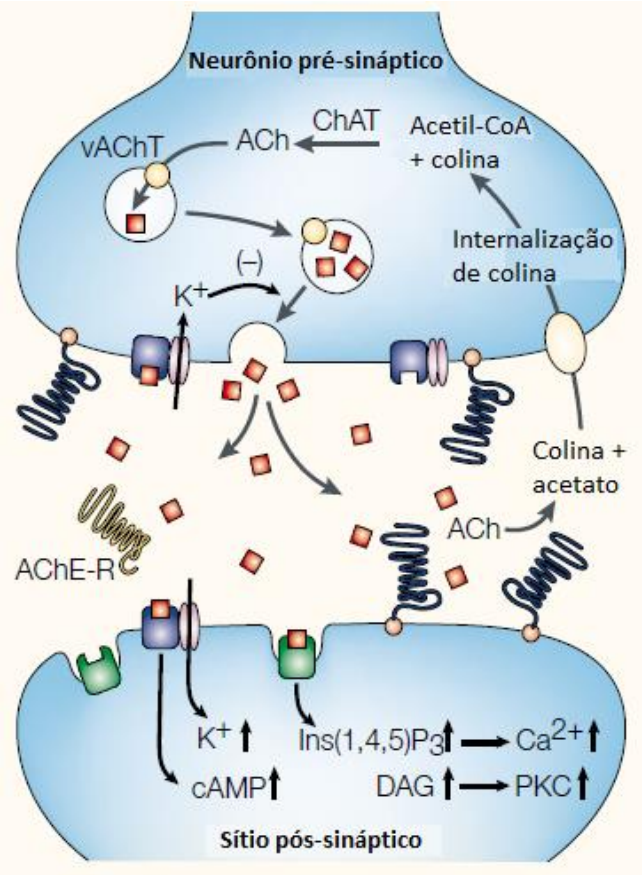

Figura 6 – Esquema da sinapse colinérgica. A acetilcolina (ACh) que está contida na vesícula sináptica do terminal nervoso é liberada na fenda sináptica por exocitose. Liga-se no neurônio pós-sináptico através de receptores específicos e é hidrolizada por colinesterases específicas, que a degradam em colina e acetil-CoA. O transportador de colina recolhe a colina resultante da reação que está livre na fenda sináptica e leva novamente para o neurônio pré-sináptico para a sua reutilização. Colina acetil-transferase (ChAT); transportador vesicular de ACh (vAChT); AMP cíclico (cAMP); diacilglicerol (DAG); proteína cinase C (PKC). Adaptado de (SOREQ; SEIDMAN, 2001).

O envolvimento do sistema colinérgico em processos cognitivos pode ser caracterizado pela participação da modulação colinérgica muscarínica em algumas das características patológicas presentes na doença de Alzheimer, como, por exemplo, a degeneração de neurônios colinérgicos que leva a uma redução significativa na liberação de ACh na amígdala, hipocampo e neocórtex e uma consequente diminuição da modulação dos mAChRs sobre as funções neuronais nessas estruturas (COYLE et al., 1983; LEVEY, 1996; THATHIAH; DE STROOPER, 2009; WILKINSON et al., 2004). Além disso, autores sugerem o envolvimento do sistema colinérgico na modulação da memória através de diversos sistemas, uma vez que antagonistas colinérgicos atenuam a influência no armazenamento da

memória pela atividade opióide (INTROINI; BARATTI, 1984), GABAérgica (CASTELLANO; MCGAUGH, 1991) e β-adrenérgica (INTROINI-COLLISON et al., 1996).

Outro aspecto importante para a regulação da função encefálica é a capacidade de manter um gradiente iônico através da membrana plasmática neuronal, ou seja, a capacidade de manter um desequilíbrio nas concentrações intra e extracelulares de íons como sódio (Na^+), potássio (K^+) e cálcio (Ca^{2+}) que são necessários para a geração, processamento e transmissão de impulsos nervosos (ERECINSKA; SILVER, 1994). A bomba de sódio-potássio (Na^+,K^+-ATPase; EC 3.6.3.9) e a bomba de cálcio (Ca^{2+}-ATPAse; EC 3.6.3.8) são membros da família das ATPases do tipo P (P-ATPAses) de bombas de cátions que utilizam a energia livre proveniente da hidrólise do ATP para transportar ativamente cátions contra os seus gradientes eletroquímicos (JORGENSEN et al., 2003).

A Na^+,K^+-ATPase realiza o transporte ativo de Na^+ para o meio extracelular e de K^+ para o meio intracelular, contribuindo para regulação do potencial de membrana, necessário para a comunicação entre as redes neurais (Figura 7) (HORISBERGER, 2004). A Na^+,K^+-ATPase é uma proteína de membrana e consiste de uma subunidade catalítica α com 10 segmentos transmembrana, uma única subunidade β glicosilada transmembrana, necessária para a estabilização estrutural assim como para propriedades funcionais como a afinidade catiônica, e também uma subunidade γ expressa especialmente nos rins e envolvida na regulação do canal iônico (COLONNA et al., 1997; FAMBROUGH et al., 1994; GEERING, 2001; KUSTER et al., 2000; THERIEN et al., 2001). A Na^+,K^+-ATPase é composta por 4 isoformas α (α1-4), sendo 3 destas expressas no encéfalo de ratos e camundongos: a isoforma α1 é encontrada em diversos tipos celulares, a isoforma α2 predomina em astrócitos, e a isoforma α3 é localizada essencialmente em neurônios (LINGREL et al., 2007; MOSELEY et al., 2007). Foi demonstrado que a haploinsuficiência (ausência de um gene funcional) para as isoformas α2 e α3 da Na^+,K^+-ATPAse resultam em alterações comportamentais em camundongos (LINGREL et al., 2007). Também já foi demonstrado que camundongos haploinsuficientes para a isoforma α2 da Na^+,K^+-ATPase apresentam um comportamento similar à ansiedade e que camundongos haploinsuficientes para a isoforma α3 da Na^+,K^+-ATPase desenvolveram déficits cognitivos espaciais (MOSELEY et al., 2007). Além disso, a

redução na atividade desta enzima em córtex cerebral de ratos foi associada à disfunção cognitiva induzida pelo dano encefálico traumático (LIMA et al., 2008).

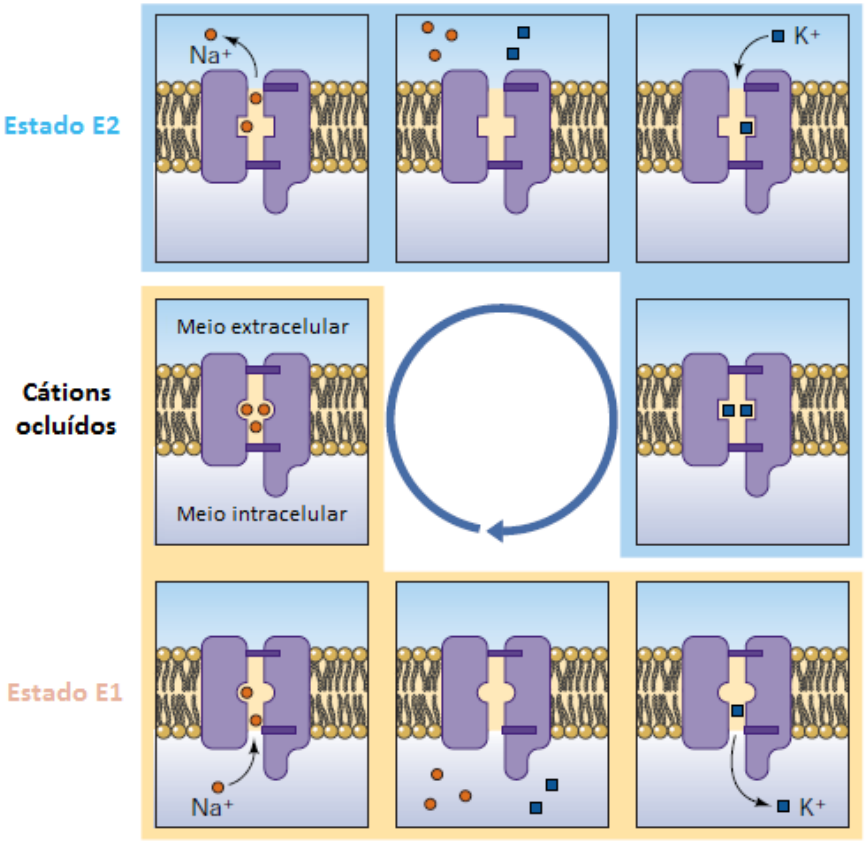

Figura 7 – Esquema do ciclo funcional da Na^+,K^+-ATPase. A proteína é ancorada à membrana celular (com o seu lado extracelular representado para cima e o seu lado intracelular para baixo). Os sítios de ligação para os cátions Na^+ e K^+ estão localizados na região transmembrana da proteína e são acessíveis alternativamente do lado citoplasmático (conformação E1) e do lado extracelular (conformação E2) da membrana. O acesso aos sítios de ligação é regulado por dois "portões" (*gates*) ilustrados pelas barras horizontais escuras, sendo uma para o meio extracelular e outra para o meio intracelular. Adaptado de (HORISBERGER, 2004).

A Ca^{2+}-ATPase, por sua vez, transporta dois íons de Ca^{2+} através da membrana plasmática (para o meio extracelular) ou do retículo endoplasmático (para dentro da vesícula) contra o seu gradiente de concentração, obtendo energia através da hidrólise de uma molécula de ATP durante cada ciclo de transporte (Figura 8) (MOLLER et al., 2010). A baixa concentração intracelular de Ca^{2+} é mantida principalmente pela atividade da Ca^{2+}-ATPase (ativada pela calmodulina, CaM) localizada na membrana plasmática (PMCA, *plasma membrane* Ca^{2+}*-ATPase*) (CARAFOLI, 1991; CARAFOLI, 1992; INESI; KIRTLEY, 1992) e no retículo endoplasmático (SERCA, *sarco-endoplasmic reticulum* Ca^{2+}*-ATPase*) (IKEMOTO, 1982). A função destas enzimas é fundamental, uma vez que a sua inibição

(RICHARDS et al., 1988; WANG et al., 1994) leva à alteração dos gradientes iônicos, acúmulo de Ca^{2+} e à morte celular (KEHRER, 1993; ROHN et al., 1996).

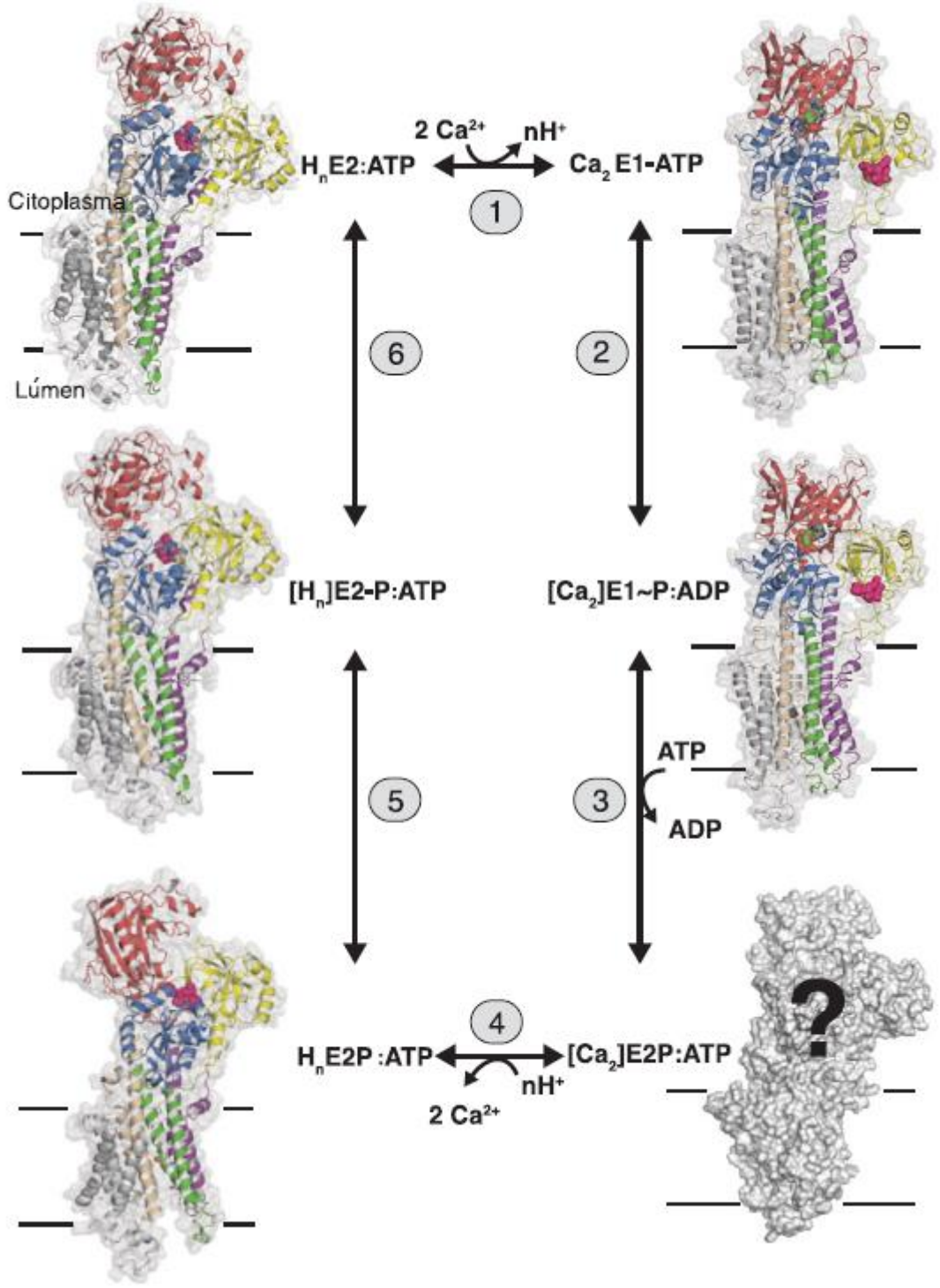

Figura 8 – Esquema do ciclo funcional da SERCA 1a. Principais estados do ciclo de transporte: 1) Troca de n prótons (n=2-3) por 2 íons Ca^{2+}; 2) Reação de fosforilação com o ATP com a formação do intermediário $[Ca_2]$E1~P:ADP; 3) Conversão do estado $[Ca_2]$E1~P:ADP para $[Ca_2]$E1~P:ATP após a troca de ADP/ATP (*estrutura ainda desconhecida*); 4) Estado basal após a abertura luminal e a troca dos íons Ca^{2+} por íons H^+. 5) Formação do estado fechado [Hn]E2~P:ATP; 6) Desfosforilação do estado de transição [Hn]E2~P:ATP em HnE2:ATP. [ ] representa o estado fechado da enzima. Adaptado de (MOLLER et al., 2010).

Dentre as alternativas para o tratamento de diversas doenças destaca-se o emprego de compostos naturais extraídos das plantas, denominados fitoterápicos. Desde os tempos pré-históricos os fitoterápicos são empregados na medicina popular.

A curcumina (diferuloilmetano) é um dos principais constituintes do açafrão da Índia ou cúrcuma (GOEL et al., 2008), a especiaria gastronômica de cor amarelada extraída dos rizomas da planta conhecida como *turmeric* (*Curcuma longa*). Esta planta é uma herbácea perene que pertence à família Zingiberaceae e possui distribuição em zonas tropicais e subtropicais; seu cultivo é muito difundido em países asiáticos, principalmente na Índia e na China (ARAUJO; LEON, 2001). A curcumina é um polifenol empregado na medicina hindu (AMMON; WAHL, 1991) e na medicina tradicional Indiana e Chinesa para tratamento de diversas doenças (AMMON et al., 1992; PATWARDHAN et al., 2005).

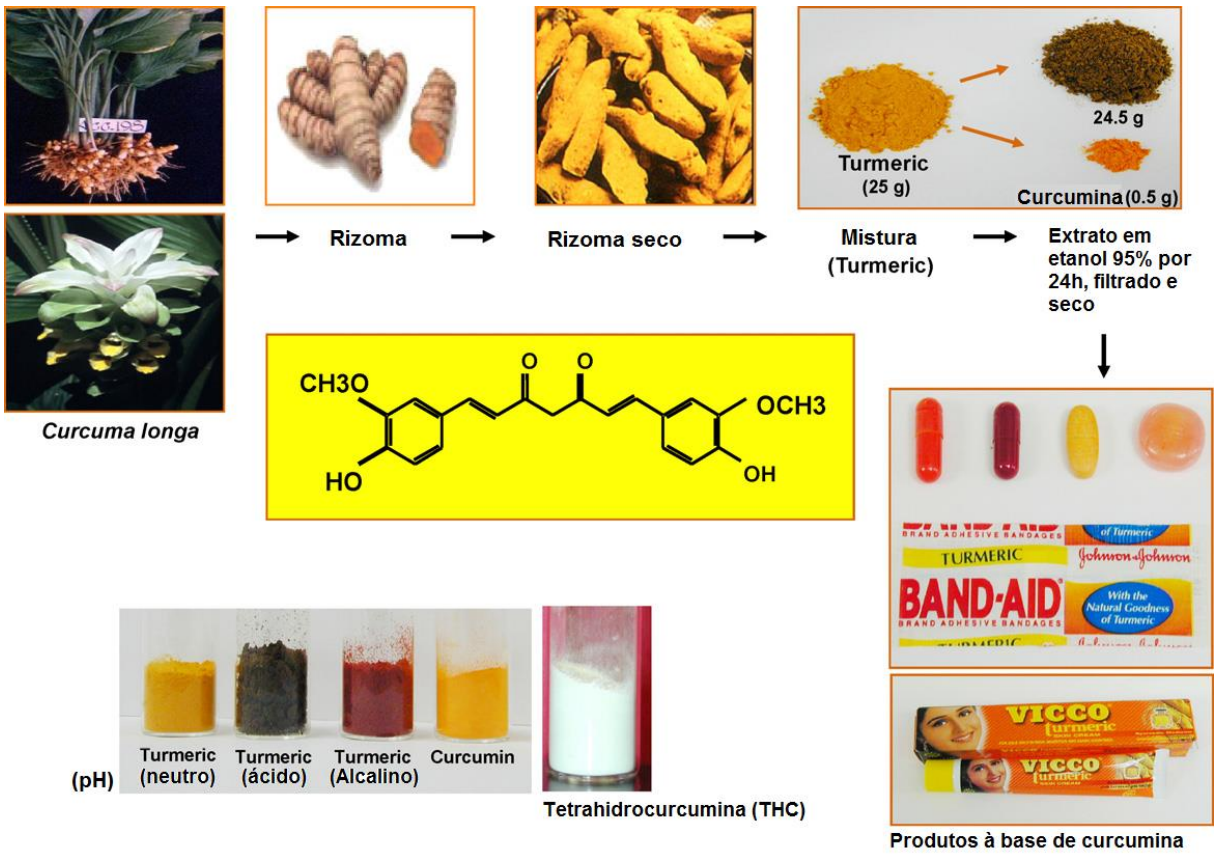

Figura 9 – Isolamento, extração e estrutura química da curcumina. São apresentados alguns produtos à base de curcumina comumente comercializados em países orientais como comprimidos, cápsulas, pílulas, fitas curativas e cremes para o tratamento de ferimentos e para barbear. As alterações na colocação do extrato bruto (turmeric) em condições neutras, acídidas ou alcalinastambém podem ser observadas. Adaptado de (GOEL et al., 2008).

A curcumina é uma molécula lipofílica e dentre as suas propriedades farmacológicas destaca-se o seu forte potencial antioxidante (ILBEY et al., 2009; KAMAT et al., 2008; KOWLURU; KANWAR, 2007), atribuído à sua capacidade de atenuar o efeito de EROs e ERNs e de se ligar à íons metálicos associada com características estruturais tais como a presença do grupo funcional metoxilo no anel fenólico e das ligações 1,3-dicetona em sua estrutura (AGGARWAL; SUNG, 2009; KAPOOR; PRIYADARSINI, 2001).

Pesquisas também demonstram que este composto possui atividade anti-agregante. A curcumina inibe a agregação plaquetária induzida pelo ácido araquidônico (AA), adrenalina, colágeno e FAP; altera o metabolismo de eicosanóides reduzindo a liberação de AA pela deacilação de fosfolipídios com a concomitante redução da síntese de tromboxano B_2 (TXB_2), ácido 12-hidroxiheptadecatrienóico (12-HHT) e ácido 12-hidroxieicosatetraenóico (12-HETE); além de inibir a mobilização de Ca^{2+} das plaquetas (SHAH et al., 1999; SRIVASTAVA et al., 1995).

Estudos experimentais indicam que a curcumina é capaz de atravessar a BHE (GARCIA-ALLOZA et al., 2007; YANG et al., 2005), o que fortalece os resultados de diversos estudos que demonstram o seu potencial neuroprotetor na prevenção da disfunção cognitiva (PAN et al., 2008; REETA et al., 2009; TANG et al., 2009), neurotoxicidade (SETHI et al., 2009), neuroinflamação e doenças de Alzheimer e Parkinson (BEGUM et al., 2008), assim como na promoção de neuroplasticidade e neurogênese (BEGUM et al., 2008; KIM et al., 2008). Acredita-se que os efeitos neuroprotetores providos pela curcumina sejam parcialmente relacionados à regulação de importantes enzimas e moléculas envolvidas na inflamação como a cicloxigenase-2 (COX-2), lipoxigenase, fator nuclear kappa-B (FN-κB) e citocinas (GOEL et al., 2008). Alguns estudos também inferem que a curcumina é capaz de modular vias de sinalização intracelulares pela regulação da atividade das proteínas cinases A, B e C (PKA, PKB e PKC) dependentes de Ca^{2+} e pela expressão do receptor do inositol 1,4,5-trifosfato (GOEL et al., 2008). Além disso, destaca-se sua propriedade citoprotetora na prevenção do dano oxidativo à células neuronais (SCAPAGNINI et al., 2006).

Adicionalmente, algumas pesquisas apontam que embora a curcumina não exerça um efeito *per se* sobre o sistema colinérgico no SNC, ela é capaz de normalizar o aumento na atividade da enzima AChE em córtex cerebral promovido pela estreptozotocina (STZ) em modelo de demência (AGRAWAL et al., 2010; RINWA et al., 2010) e em modelo de diabetes (KUHAD; CHOPRA, 2007), e também atenua a redução da atividade enzimática causada pela exposição ao arsênio (As) nesta estrutura encefálica (YADAV et al., 2011). A curcumina também não exerce efeito *per se* sobre o sistema purinérgico avaliado através da atividade da enzima E-NTPDase em linfócitos periféricos e pulmonares, contudo foi eficaz em prevenir a redução promovida pela exposição passiva à fumaça do cigarro (JAQUES et al., 2011).

Ao mesmo tempo em que a curcumina possui diversas propriedades promissoras para a terapia de muitas condições clínicas, sua atividade biológica é bastante limitada em virtude de suas propriedades farmacocinéticas, especialmente a sua restrita biodisponibilidade (KELLOFF et al., 1996). Frente à este problema, diversos grupos de pesquisa têm empregado o uso da nanotecnologia com o intuito de aumentar a biodisponibilidade da curcumina administrada por via oral e as formulações nanoestruturadas têm proporcionado resultados promissores (RAY et al., 2011; SHAIKH et al., 2009; THANGAPAZHAM et al., 2008).

Como apresentado anteriormente, a fumaça do cigarro possui diversos efeitos nocivos conhecidos como o dano oxidativo ao sistema vascular cerebral, a acentuação da agregabilidade plaquetária, as disfunções neurocognitivas e de memória, dentre outros. Desta forma, é de interesse clínico investigar a ação terapêutica de compostos com propriedades antioxidantes, antiagregantes e neuroprotetoras como a curcumina sobre o perfil redox e sobre a atividade de enzimas que desempenham funções vitais no SNC como a E-NTPDase, a E-5'-NT, a E-ADA, a AChE, a Na^+,K^+-ATPase e a Ca^{2+}-ATPase em um modelo animal de exposição passiva à fumaça do cigarro.

OBJETIVOS

Objetivo Geral

Avaliar o efeito da curcumina sobre a memória e parâmetros envolvidos na homeostase do sistema nervoso central em ratos expostos de forma passiva à fumaça do cigarro.

Objetivos específicos

→ Determinar *ex vivo* em ratos expostos de forma passiva à fumaça do cigarro e tratados com diferentes doses de curcumina:

- A atividade das enzimas E-NTPDase, E-5'-nucleotidase e E-ADA em plaquetas de sangue periférico;
- A atividade das enzimas E-NTPDase, E-5'-nucleotidase e AChE em sinaptossomas de córtex cerebral;
- A atividade da enzima AChE em diferentes estruturas encefálicas e em sangue total;

→ Avaliar a memória dos animais pela tarefa da esquiva inibitória;

→ Determinar *ex vivo* em ratos expostos de forma passiva à fumaça do cigarro e tratados com diferentes doses de curcumina livre e nanoestruturada:

- A atividade das enzimas Na^+,K^+-ATPase e Ca^{2+}-ATPase em homogenatos de córtex cerebral;
- Os níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS), nitritos e nitratos (NOx), tióis não-protéicos (NPSH) e a atividade da enzima superóxido dismutase (SOD) em homogenatos de córtex cerebral;

→ Avaliar a memória dos animais pela tarefa de reconhecimento de objetos.

ARTIGO 1 – EFFECTS OF CURCUMIN ON THE ACTIVITIES OF THE ENZYMES THAT HYDROLYZE ADENINE NUCLEOTIDES IN PLATELETS FROM CIGARETTE SMOKE-EXPOSED RATS

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Effects of curcumin on the activities of the enzymes that hydrolyse adenine nucleotides in platelets from cigarette smoke-exposed rats

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The aim of the present study was to investigate the effect of curcumin (Cur) on the activity of ectonucleoside triphosphate diphosphohydrolase (CD39), 5'-nucleotidase (CD73) and adenosine deaminase in platelets of cigarette smoke-exposed rats. For that purpose, we subjected male Wistar rats to a treatment with Cur and cigarette smoke, once a day, 5 days each week, for 30 days. The rats were treated by gavage with Cur or corn oil and then exposed to cigarette smoke. The experimental procedures were divided into two sets of experiments. In the first, the animals were divided into four groups: vehicle (corn oil) or Cur 12·5, 25 or 50 $mg \cdot kg^{-1}$. In the second, the animals were divided into five groups: vehicle (corn oil), smoke, or smoke and Cur 12·5, 25 or 50 $mg \cdot kg^{-1}$. The results showed that treatment with Cur significantly prevented the increased adenosine triphosphate (ATP) (121%) and adenosine monophosphate (AMP) (159%) and the decreased adenosine diphosphate (ADP) (51%) hydrolysis observed in the cigarette smoke-exposed rats Our results suggest that those purinergic enzyme alterations observed in the cigarette smoke-exposed rats could be related to an excessive platelet aggregation and point toward the potential of Cur to modulate purinergic signalling and, consequently, regulate the thrombus formation. Copyright © 2011 John Wiley & Sons, Ltd.

KEY WORDS—curcumin; cigarette smoke; platelets; purinergic signalling

INTRODUCTION

Cigarette smoking is a risk factor for the development of many disorders such as chronic obstructive pulmonary disease, lung cancer,[1] brain deficits,[2] coronary artery disease[3] and atherosclerosis.[4] This widely spread behaviour is associated with increased mortality from cardiovascular disease, and its effects over the platelets have been investigated. *In vitro* studies have shown that smokers presented higher small (spontaneous aggregation), medium and large aggregates (induced by 1 or 5 $\mu mol \cdot l^{-1}$ of epinephrine) than nonsmokers, which confirmed that platelet aggregability is enhanced in smokers.[5] Some studies also demonstrated that smokers show elevated platelet specific proteins, which are released from platelet α-granules into circulating blood plasma when platelets are activated such as plasma levels of platelet factor 4[6] and β-thromboglobulin,[7] higher P-selectin expression on the surface of platelets and a higher plasma soluble P-selectin level.[8] Furthermore, Takajo and colleagues[9] demonstrated that the bioactivity of platelet-derived nitric oxide (NO) is impaired in chronic smokers, reinforcing the knowledge about their prothrombotic state.

Platelets or thrombocytes are tiny, anucleated and regularly shaped cell fragments produced by large, multinucleated cells in the bone marrow, the megakaryocytes. They play an important role in the blood hemostasis, being source of growth factors and involved in the formation of blood clots.[10] The enzymes ectonucleoside triphosphate diphosphohydrolase (NTPDase; EC 3.6.1.5; CD39), 5'-nucleotidase (EC 3.1.3.5; CD73) and adenosine deaminase (ADA; EC 3.5.4.4) are present in the platelet membrane and play an important role in regulating the levels of extracellular nucleotides such as adenosine triphosphate (ATP), adenosine diphosphate (ADP) and its nucleoside adenosine that, in turn, are able to tune the excessive platelet aggregation.[11]

Curcumin (Cur; 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione or diferuloylmethane), a dietary spice from turmeric, is a polyphenol with many biological properties described so far, such as anti-inflammatory, antioxidant, chemopreventive and neuroprotective.[12–16] In addition to these properties and in particular interest for this

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research, some studies have demonstrated the anti-aggregant activity of Cur. This compound inhibits aggregation induced by arachidonic acid (AA), adrenaline, collagen and platelet-activating factor; alters eicosanoid metabolism down-regulating the release of AA (deacylation of phospholipids) with a concomitantly reduced formation of thromboxane B_2 (TXB_2), 12-hydroxyheptadecatrienoic acid (12-HHT) and 12-hydroeicosatetraenoic acid (12-HETE); and further, inhibited the A-23187-induced mobilization of intracellular Ca^{2+} from the platelets.[17,18]

Considering these facts, the purpose of the present study was to investigate the activity of the enzymes NTPDase, 5'-nucleotidase and ADA in platelets from cigarette smoke-exposed rats treated with Cur, a major component of turmeric, which has been shown to inhibit platelet aggregation.

MATERIALS AND METHODS

Reagents

Nucleotides, Trizma Base, Percoll, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-hydroxyethil)piperazine-N'-(2-ethanesulfonic acid) (HEPES) buffer, adenosine and Coomassie brilliant blue G, Cur (Cur $\geq$ 80%; curcuminoid content $\geq$ 94%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The brand of cigarette used in the experiment was manufactured by Souza Cruz S.A. (Brazil). The cigarette contains 10 mg of tar, 0·9 mg of nicotine and 10 mg of carbon monoxide. All other reagents used in the experiments were of analytical grade and of highest purity.

Animals

Male Wistar rats (90–110 days) from the Central Animal House of the Federal University of Santa Maria (UFSM) were used in this experiment. They were housed five to a cage on a natural day/night cycle at a temperature of 21 °C with free access to water and standard chow *ad libitum*. All animal procedures were approved by the animal ethics committee from the UFSM (protocol under number: 23081.004963/2009-71).

Cigarette smoke exposure and treatment with curcumin

The experimental procedure was divided into two sets of experiments. In the first set, the animals were randomly divided into four groups (ten rats in each group): vehicle (corn oil), Cur 12·5 mg·kg^{-1} body weight, Cur 25 mg·kg^{-1} body weight and Cur 50 mg·kg^{-1} body weight. In the second experimental set, the animals were divided into five groups (ten rats in each group): vehicle (corn oil), smoke exposed, smoke and Cur 12·5 mg·kg^{-1} body weight, smoke and Cur 25 mg·kg^{-1} body weight and smoke and Cur 50 mg·kg^{-1} body weight. Cur was diluted with corn oil, administered by gavage, not exceeding 0·1 ml·kg^{-1} body weight. The treatment with Cur and cigarette smoke was carried out once a day, 5 days each week, during 30 days. First, the Cur or corn oil was administered, and approximately 10 min later, the smoking groups were exposed to the aged and diluted sidestream smoke of commercial cigarettes inside a whole-body smoke exposure. Control animals were placed in an equal chamber for the same amount of time but without exposure to smoke. When the smoke exposure was carried out, the control group was always outside, without any contact with smoke.[19]

Smoke generation

After placing the rats inside the exposure chamber (size 564 × 385 × 371 mm; volume 53 100 ml/1795 oz; plastic material), four cigarettes were lit simultaneously, 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 min. 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 as experienced by non-smokers.[19]

Platelet separation

Platelet-rich plasma (PRP) was prepared as previously described,[20] with the following minor modifications. Total blood was collected by cardiac puncture with 0·120 mol·l^{-1} of sodium citrate as anticoagulant. The total blood-citrate system was centrifuged at 160 *g* during 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 mmol·l^{-1} of HEPES isosmolar buffer. The washed platelets were resuspended in HEPES isosmolar buffer and adjusted to 0·4–0·6 mg of protein per milliliter.

Ectonucleoside triphosphate diphosphohydrolase and 5'-nucleotidase assays

The NTPDase enzymatic assay of the platelets was carried out in a reaction medium containing 5 mmol·l^{-1} of $CaCl_2$, 100 mmol·l^{-1} of NaCl, 4 mmol·l^{-1} of KCl, 5 mmol·l^{-1} of glucose and 50 mmol·l^{-1} of Tris-HCl buffer, pH 7·4, at a final volume of 200 µl as previously described.[20] For adenosine monophosphate (AMP) hydrolysis, the chemical reagents used were the same described for NTPDase activity, except that 5 mmol·l^{-1} of $CaCl_2$ was replaced by 10 mmol·l^{-1} of $MgCl_2$. First, 20 µl of enzyme preparation (8–12 µg of protein) were added to the reaction mixture and pre-incubated at 37 °C for 10 min. The reaction was initiated by the addition of ATP or ADP to obtain a final concentration of 1·0 mmol·l^{-1}, and incubation proceeded for 60 min. For AMP hydrolysis, 5'-nucleotidase activity was carried out as previously described, and the final concentration of the nucleotide AMP added was 2 mmol·l^{-1}. In all cases, reactions were stopped by the addition of 200 µl of 10% trichloroacetic acid (TCA) to provide a final concentration of 5%. Released inorganic phosphate (Pi) was assayed using malachite green as the colorimetric reagent and KH_2PO_4 as standard.[21] Controls were carried out to correct for non-enzymatic hydrolyses

of nucleotides by adding platelets after TCA addition. All samples were run in triplicate. Enzyme specific activities are reported as nanomole Pi released per minute per milligram of protein.

Adenosine deaminase activity determination

Adenosine deaminase was determined as previously described.[22] Briefly, 50 µl of platelets reacted with 21 mmol·l^{-1} of adenosine, pH 6·5, and was incubated at 37 °C for 60 min. This method is based on the direct production of ammonia when ADA acts in excess of adenosine. The protein content used for the platelet experiment was adjusted to 0·7–0·9 mg·ml^{-1}. Results were expressed in units per milligram of protein (U·mg^{-1} of protein). One unit (1 U) of ADA is defined as the amount of enzyme required to release 1 mmol·l^{-1} of ammonia per minute from adenosine at standard assay conditions.

Protein determination

Protein was measured by the Coomassie blue method[23] using serum albumin as standard.

Statistical analysis

The statistical analysis was performed using one-way ANOVA, followed by Newman-Keuls multiple comparison test. $p < 0{\cdot}05$ was considered to represent a significant difference among the analyses used. All data were expressed as mean ± SEM.

RESULTS

Ectonucleoside triphosphate diphosphohydrolase and 5'-nucleotidase activity

Ectonucleoside triphosphate diphosphohydrolase activity from platelets was not modified by Cur *per se* treatment using both ATP [$F(3,36) = 1{\cdot}24$, $p > 0{\cdot}05$, $n = 10$] (Figure 1A) and ADP [$F(3,36) = 0{\cdot}057$, $p > 0{\cdot}05$, $n = 10$] (Figure 1C) as the substrates as well as the 5'-nucleotidase activity [$F(3,36) = 0{\cdot}13$, $p > 0{\cdot}05$, $n = 10$] (Figure 2A). In the hydrolysis of ATP in platelets, the smoke group presented an increase of 121 194 164 and 135% [$F(4,45) = 3{\cdot}20$, $p < 0{\cdot}05$, $n = 10$] (Figure 1B) when compared with the control, Cur 12·5, Cur 25 and Cur 50 mg·kg^{-1}, respectively. Statistical analysis showed a significant control or smoke versus Cur (12·5, 25 or 50 mg·kg^{-1}) interaction [$F(5,72) = 5{\cdot}712$, $p < 0{\cdot}01$, $n = 10$]. In the hydrolysis of ADP in the platelets, the smoke group presented a decrease of 48, 54, 53 and 56% [$F(4,45) = 3{\cdot}79$, $p < 0{\cdot}05$, $n = 10$] (Figure 1D) when compared with the control, Cur 12·5, Cur 25 and Cur 50 mg·kg^{-1}, respectively. Statistical analysis demonstrated a significant control or smoke versus Cur (12·5, 25 or 50 mg·kg^{-1}) interaction [$F(5,72) = 2{\cdot}923$, $p < 0{\cdot}05$, $n = 10$]. In the hydrolysis of AMP in the platelets, the smoke group presented an increase of 159, 151, 170 and 143%

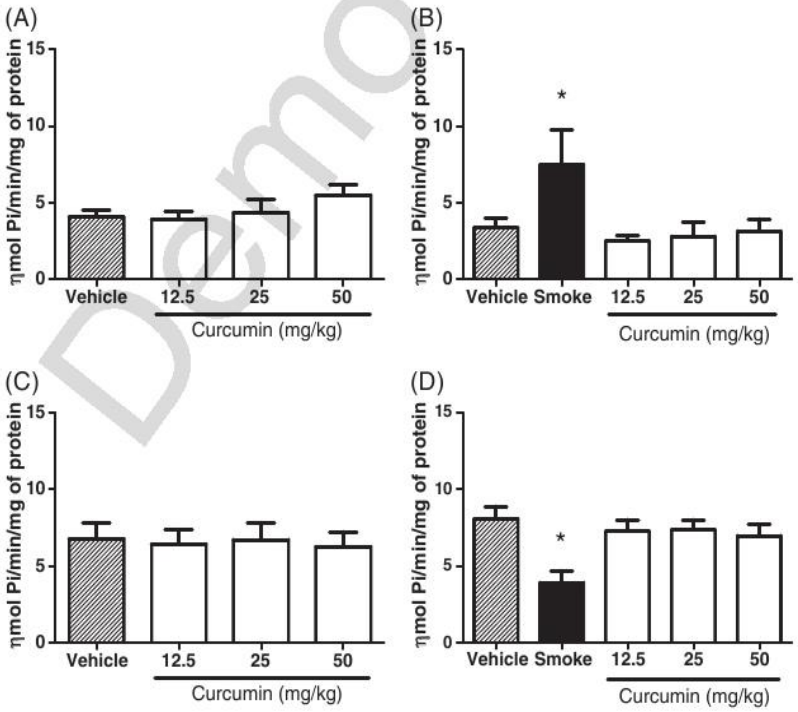

Figure 1. Ectonucleoside triphosphate diphosphohydrolase activity in the platelets from cigarette smoke-exposed rats treated with curcumin (Cur) using adenosine triphosphate (A, B) and adenosine diphosphate (C, D) as substrate [ηanomole inorganic phosphate per minute per milligram of protein (ηmol Pi/min/mg of protein)]. Bars represent means ± SEM ($p < 0{\cdot}05$; $n = 10$). ANOVA-Newman-Keuls multiple comparison test (B, D). *Compared with vehicle and Cur 12·5 and 25 mg·kg^{-1}

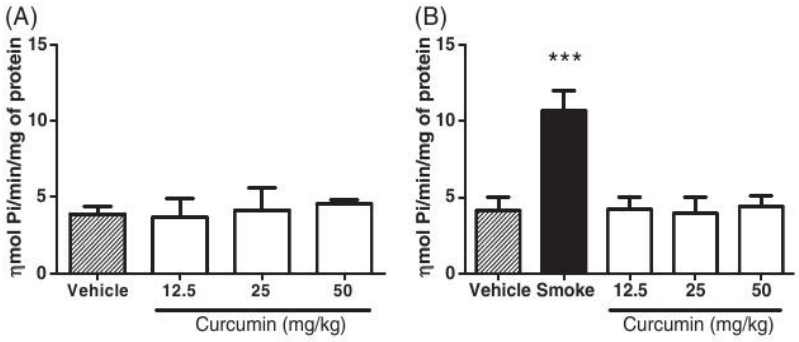

Figure 2. 5'-Nucleotidase activity in platelets from cigarette smoke-exposed rats treated with Cur using adenosine monophosphate as substrate (ηmol Pi/min/mg of protein). Bars represent means ± SEM ($p < 0{\cdot}05$; $n = 10$). ANOVA-Newman-Keuls multiple comparison test (B). *Different from all groups

[$F(4,45) = 7{\cdot}913$, $p < 0{\cdot}001$, $n = 10$] (Figure 2B) when compared with the control, Cur 12·5, Cur 25 and Cur 50 mg·kg^{-1}, respectively. Statistical analysis showed a significant control or smoke versus Cur (12·5, 25 or 50 mg·kg^{-1}) interaction [$F(5,72) = 6{\cdot}603$, $p < 0{\cdot}01$, $n = 10$].

Adenosine deaminase activity

Adenosine deaminase activity from the platelets was not modified by Cur *per se* treatment [$F(3,36) = 0{\cdot}11$, $p > 0{\cdot}05$, $n = 10$] (Figure 3A). In the second set of experiments, we also did not find alterations in the ADA activity among the groups [$F(4,45) = 0{\cdot}21$, $p > 0{\cdot}05$, $n = 10$] (Figure 3B). Statistical analysis did not demonstrate any significant control or smoke versus Cur (12·5, 25 or 50 mg·kg^{-1}) interaction [$F(5,72) = 0{\cdot}056$, $p > 0.05$, $n = 10$].

DISCUSSION

The investigation of NTPDase, 5'-nucleotidase and ADA activity in the platelets from cigarette smoke-exposed rats revealed an increased activity in the ATP and AMP hydrolysis and a decreased ADP hydrolysis, which would lead to an elevated ADP nucleotide concentration in the bloodstream. It is well known that the enhanced level of ADP is related to an increased platelet aggregation, shape change, increased cytosolic calcium and inhibition of stimulated adenylate cyclase.[24] Therefore, the results presented by this manuscript, which remit to high ADP level in the bloodstream and, consequently, an increased platelet aggregation are consistent with the findings of Inoue,[3] which showed that cigarette smokers have more spontaneous platelet aggregation than non-smokers. A previous study performed by our study group demonstrated that cigarette smoke-exposed rats had an increased platelet aggregation,[19] This fact was proposed to be associated with the alterations observed in the ectonucleotidases activities in which ATP hydrolysis was increased, and there was a tendency of ADP hydrolysis decrease, similar to the results presented by this manuscript.

Regarding the increased AMP hydrolysis observed in the cigarette smoke-exposed rats, it remits to an increased extracellular adenosine concentration.[25,26] This molecule has anti-aggregant effects mediated via G-coupled adenosine receptors, specifically the A_{2A} and A_{2B} receptor subtypes[27,28], which upregulate the production of cyclic AMP (cAMP), an inhibitor of platelet activation.[29,30] The increased cAMP activates the protein kinase A, which reduces the release of intracellular Ca^{2+} store,[31] stabilize the cytoskeleton[28] and thus prevents thrombus formation. Because adenosine is generally related to anti-inflammatory autocrine signalling through P1 receptors,[32] we speculate that a possible increase in the bloodstream concentration of adenosine could represent a dynamic physiological mechanism to regulate the vascular response to endothelial damage

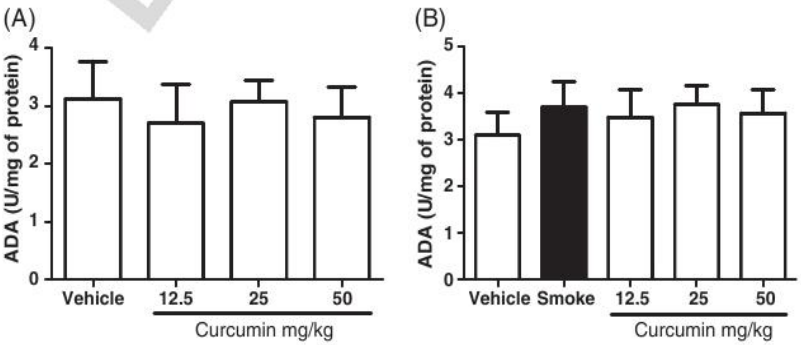

Figure 3. Adenosine deaminase activity in the platelets from cigarette smoke-exposed rats treated with Cur (U·mg^{-1} of protein). Bars represent means ± SEM ($n = 10$). ANOVA-Newman-Keuls multiple comparison test

and to avoid those effects of cigarette-smoke exposure such as platelet aggregation and atherosclerosis development.

Our findings also demonstrated that Cur was effective in preventing alterations in the activities of the enzymes NTPDase and 5'-nucleotidase in platelets from cigarette smoke-exposed rats. According to a previous study,[17] Cur was able to reduce the release of intracellular Ca^{2+} from platelets and to inhibit both phospholipase A_2/C and cyclooxygenase activities, leading to a decreased release of AA with concomitantly reduced formation of TXB_2. Furthermore, these Cur-treated platelets showed a simultaneous increase of lypoxygenase-derived products such as 12-hydroperoxyeicosatetraenoic acid (12-HPETE) (precursor of 12-HETE), which inhibits platelet aggregation, and at higher concentrations, it inhibits thromboxane formation as well.[33] Because the elevated concentration of ADP is related to an increased platelet aggregation, these effects showed by Cur over the enzymes of cigarette smoke-exposed rats could be proposed as one extra anti-platelet effect exerted by this polyphenol.

It is known that the generation of reactive oxygen and nitrogen species also plays a role in the platelet aggregation. The cigarette smoke is composed of two distinct phases: the gas phase and the particulate matter (or tar). The radicals in cigarette tar (10^{17} spin/gram) arise from semiquinone radicals,[34] whereas a single puff contains more than 10^{15} organic radicals in the gas phase.[35] Some of these radicals are the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), nitrogen dioxide (NO_2), NO and peroxynitrite ($ONOO^-$).[36] These oxidants are able to react and damage tissues and biomolecules such as the endothelium and the cellular membranes and organelles, respectively. This injury provided by the subproducts of cigarette smoke might release some signalling molecules and trigger the platelet aggregation via many different pathways.

As discussed above, the generation of reactive species may be involved in the increased platelet aggregation in smokers. Furthermore, some authors reviewed that antioxidants provide some protection against diseases associated with smoking such as cardiovascular diseases. Following this line of thought, we believe that the plenty antioxidant properties already described of Cur[12–14,37] may be involved in the effects observed in this study because it would protect the biomolecule integrity from the attack of oxidants present in the cigarette smoke.

In conclusion, the results of the present study indicate that oral Cur administration at the doses tested is effective in preventing changes in NTPDase and 5'-nucleotidase activities in the platelets from cigarette smoke-exposed rats and that Cur alone did not affect these enzyme activities. The effects provided by Cur probably are a result of its ability to regulate the thrombus formation because the supposed high levels of ADP in the smoke-exposed group are related to an excessive platelet aggregation.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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ARTIGO 2 – THE EFFECT OF CURCUMIN IN THE ECTONUCLEOTIDASES AND ACETYLCHOLINESTERASE ACTIVITIES IN SYNAPTOSOMES FROM THE CEREBRAL CORTEX OF CIGARETTE SMOKE-EXPOSED RATS

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RAPID COMMUNICATION

The effect of curcumin in the ectonucleotidases and acetylcholinesterase activities in synaptosomes from the cerebral cortex of cigarette smoke-exposed rats

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With the evidence that curcumin may be a potent neuroprotective agent and that cigarette smoke is associated with a decline in the cognitive performance as our bases, we investigated the activities of Ecto-Nucleoside Triphosphate Diphosphohydrolase (NTPDase), 5'-nucleotidase and acetylcholinesterase (AChE) in cerebral cortex synaptosomes from cigarette smoke-exposed rats treated with curcumin (Cur). The experimental procedures entailed two sets of experiments. In the first set, the groups were vehicle, Cur 12·5, 25 and 50 $mg \cdot kg^{-1}$; those in the second set were vehicle, smoke, smoke and Cur 12·5, 25 and 50 $mg \cdot kg^{-1}$. Curcumin prevented the increased NTPDase, 5'-nucleotidase and AChE activities caused by smoke exposure. We suggest that treatment with Cur was protective because the decrease of ATP and acetylcholine (ACh) concentrations is responsible for cognitive impairment, and both ATP and ACh have key roles in neurotransmission. Copyright © 2011 John Wiley & Sons, Ltd.

KEY WORDS—curcumin; cigarette smoke; NTPDase; 5'-nucleotidase; acetylcholinesterase

INTRODUCTION

Cigarette smoke is currently associated with the development of many diseases, such as respiratory, cardiovascular and cerebrovascular diseases and tumorigenesis. Some studies have investigated the effects of smoking on the cognitive functions and found a decline in cognitive performance attributed to the effects of cigarette smoke exposure.[1,2]

Acetylcholine (ACh) and adenosine 5'-triphosphate (ATP) are important neurotransmitters, synaptic modulators and paracrine or autocrine signaling substances.[3] The homeostatic control of these molecules in the central nervous system (CNS) is carried out by different enzymes: first, the acetylcholinesterase (AChE, EC 3.1.1.7), which is responsible for the degradation of ACh into inactive metabolites, coline and acetate[4]; second, a group of enzymes called ecto-nucleotidases, a family of enzymes, which catalyse the sequential dephosphorylation of nucleoside triphosphates to nucleoside monophosphates (ATP → adenosine 5'-diphosphate (ADP) → adenosine 5'-monophosphate (AMP)) by NTPDase (CD39, EC 3.6.1.5),[5] and the ecto-5'-nucleotidase (CD73, EC 3.1.3.5), which catalyses the phosphohydrolysis of nucleoside monophosphates (5'-monophosphate → adenosine).[6]

Curcumin (diferuloylmethane), a polyphenol, is an active principle of the perennial herb *Curcuma longa* L. (commonly known as turmeric) and has been proposed to have several biological activities, such as antioxidant,[7,8] anti-inflammatory,[9] anticarcinogenic and antimicrobial,[10,11] thrombosuppressive,[12] cardiovascular (i.e. as protection against myocardial infarction),[13] hypoglycemic[14] and antiarthritic (i.e. as protection against rheumatoid arthritis).[15] Besides these well-documented properties, many studies have demonstrated

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the neuroprotective potential of curcumin in the prevention of cognitive dysfunction,[16–18] neurotoxicity,[19] neuroinflammation and Alzheimer's and Parkinson's diseases,[20] as well as the promotion of neuroplasticity and neurogenesis.[20,21]

With the evidence that cigarette smoke is associated with the decline in cognitive performance and that the enzymes NTPDase, 5'-nucleotidase and AChE activities, which regulate the levels of important neurotransmitters, possibly can be altered as our bases, we aimed to investigate whether curcumin is able to attenuate the effects of cigarette smoke because its neuroprotective effects are widely discussed.

MATERIALS AND METHODS

Reagents

Nucleotides, Trizma Base, Percoll, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES) buffer, Coomassie brilliant blue G and curcumin (curcumin ≥ 80%; curcuminoid content ≥ 94%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The brand of cigarette used in the experiment was manufactured by Souza Cruz S.A., Brazil. The cigarette contained 10 mg of tar, 0.9 mg of nicotine and 10 mg of carbon monoxide. All other reagents used in the experiments were of analytical grade and of highest purity.

Animals

Male Wistar rats (90–110 days) from the Central Animal House of the Federal University of Santa Maria (UFSM) were used in this experiment. They were housed five to a cage on a natural day/night cycle at a temperature of 21 °C with free access to water and standard chow *ad libitum*. All animal procedures were approved by the Animal Ethics Committee from the UFSM (protocol under number: 23081.004963/2009-71).

Cigarette smoke exposure and treatment with curcumin

The experimental procedure entailed two sets of experiments. In the first set, the animals were randomly divided into four groups (four rats in each group): vehicle (corn oil) and Cur 12·5, 25 and Cur 50 $mg \cdot kg^{-1}$ body weight. In the second experimental set, the animals were divided into five groups (10 rats in each group): vehicle (corn oil); smoke exposed; smoke and Cur 12.5 $mg \cdot kg^{-1}$ body weight; smoke and Cur 25 $mg \cdot kg^{-1}$ body weight; and smoke and Cur 50 $mg \cdot kg^{-1}$ body weight. Curcumin was diluted with corn oil, administered by oral gavage, not exceeding 0·1 $ml \cdot kg^{-1}$ body weight. The treatment with curcumin and cigarette smoke was carried out once a day, 5 days each week, during 30 days. First, the curcumin or corn oil was administered, and approximately 10 min later, the smoking groups were exposed to the aged and diluted sidestream smoke of commercial cigarettes inside a whole-body smoke exposure chamber. Control animals were placed in an identical chamber for the same amount of time but without exposure to smoke. While the smoke exposure was carried out, the control group was outside, without any contact with smoke.[22]

Smoke generation

After placing the rats inside the exposure chamber (size 564 × 385 × 371 mm; volume 53 100 ml/1795 oz; plastic material), four cigarettes were lit, 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 min. 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, thus preventing 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 as experienced by non-smokers.[22]

Synaptosome preparation

The cerebral cortex was homogenized in 10 volumes of an ice-cold medium (Medium I), consisting of 320 $mmol \cdot l^{-1}$ of sucrose, 0·1 $mmoll^{-1}$ of ethylenediaminetetraacetic acid (EDTA) and 5 $mmol \cdot l^{-1}$ of HEPES, with a pH of 7·5, in a motor-driven Teflon-glass homogenizer. The synaptosomes were isolated as described by Nagy and Delgado-Escueta[23] using a discontinuous Percoll gradient. The pellet was suspended in an isosmotic solution, and the final protein concentration was adjusted to 0·4–0·6 $mg \cdot ml^{-1}$. Synaptosomes were prepared fresh daily, maintained at 0–4 °C throughout the procedure and used for enzymatic assays.

NTPDase and 5'-nucleotidase assays

The NTPDase enzymatic assay of the synaptosomes was carried out in a reaction medium containing 5 $mmol \cdot l^{-1}$ of KCl, 1·5 $mmol \cdot l^{-1}$ of $CaCl_2$, 0·1 $mmol \cdot l^{-1}$ of EDTA, 10 $mmol \cdot l^{-1}$ of glucose, 225 $mmol \cdot l^{-1}$ of sucrose and 45 $mmol \cdot l^{-1}$ of Tris-HCl buffer, pH 8·0, in a final volume of 200 µl as described in a previous work from our laboratory.[24] Twenty microliters of enzyme preparation (8–12 µg of protein) were added to the reaction medium and pre-incubated at 37 °C for 10 min. The reaction was initiated by the addition of ATP or ADP to obtain a final concentration of 1·0 $mmol \cdot l^{-1}$, and incubation proceeded for 20 min in either case. The 5'-nucleotidase activity was determined essentially by the method of Heymann *et al.*[25] in a reaction medium containing 10 $mmol \cdot l^{-1}$ of $MgSO_4$ and 100 $mmol \cdot l^{-1}$ of Tris-HCl buffer, pH 7·5, in a final volume of 200 µl. Twenty microliters of enzyme preparation (8–12 µg of protein) were added to the reaction medium and pre-incubated at 37 °C for 10 min. The reaction was initiated by the addition of AMP to a final concentration of 2·0 $mmol \cdot l^{-1}$ and proceeded for 20 min. In all cases, the reaction was stopped by the addition of 200 µl of 10% trichloroacetic acid (TCA) to obtain a final concentration of 5%. Then, the tubes were chilled on ice for 10 min, and the released inorganic phosphate (Pi) was

assayed using the method of Chan *et al.*[26] using malachite green as a colorimetric reagent and KH_2PO_4 as standard. Controls were carried out by adding the synaptosomal fraction after TCA addition to correct for non-enzymatic nucleotide hydrolysis. All samples were run in triplicate. Enzyme activities are reported as nanomole Pi released per minute per milligram of protein.

AChE activity

The AChE enzymatic assay was determined using a modification of the spectrophotometric method of Ellman *et al.*[27] as previously described by Rocha *et al.*[28] The reaction medium (2 ml final volume) contained 100 mmol·l^{-1} of K^+-phosphate buffer, pH 7·5, and 1 mmol·l^{-1} of 5,5'-dithiobisnitrobenzoicacid. The method is based on the formation of the yellow anion, 5,5'dithio-bis-acid-nitrobenzoic, measured by absorbance at 412 ηm during 2-min incubation at 25 °C. The enzyme (40–50 µg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0·8 mmol·l^{-1} of acetylthiocholine iodide. All samples were run in duplicate or triplicate, and enzyme activity was expressed in micromole AcSCh per hour per milligram of protein.

Protein determination

Protein was measured by the Coomassie blue method according to Bradford[29] using serum albumin as standard.

Statistical analysis

Data were analysed using a one-way or two-way ANOVA. *Post hoc* analyses were carried out by the Student–Newman–Keuls test, when appropriate. $p < 0{\cdot}05$ was considered to represent a significant difference among the analyses. All data were expressed as mean ± SEM.

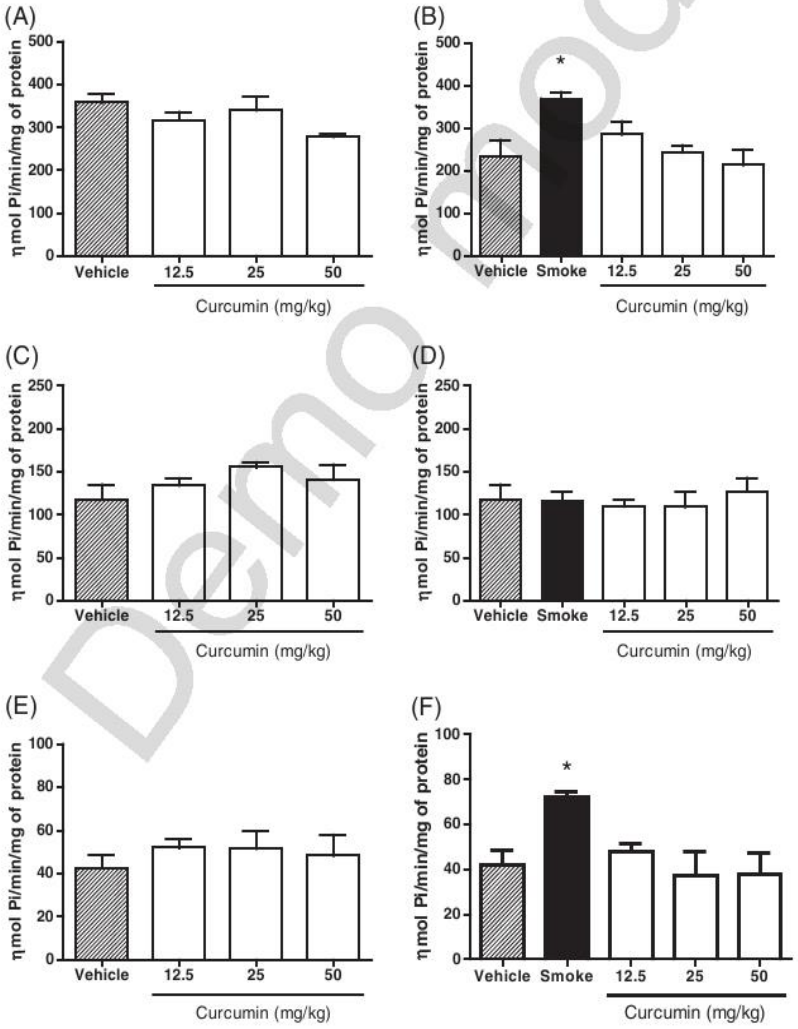

Figure 1. NTPDase activity in cerebral cortex synaptosomes from cigarette smoke-exposed rats treated with curcumin using ATP (A, B) and ADP (C, D) as substrate [nanomole of inorganic phosphate per minute per milligram (nmol Pi/min/mg) of protein]. 5'-Nucleotidase activity in cerebral cortex synaptosomes from cigarette smoke-exposed rats treated with curcumin using AMP (E, F) as substrate (nmol Pi/min/mg of protein). Bars represent means ± SEM. ($n = 4$–8). ANOVA–Newman–Keuls multiple comparison test. *Statistical difference from the other groups ($p < 0{\cdot}05$)

RESULTS

NTPDase and 5'-nucleotidase activity

NTPDase and 5'-nucleotidase activities from synaptosomes were not modified by curcumin *per se* treatment (Figure 1A, 1C and 1E). In the hydrolysis of ATP in synaptosomes of the cerebral cortex, the smoke group showed an increase compared with the control (59%), Cur 12·5 (29%), Cur 25 (51%) and Cur 50 mg·kg^{-1} (72%) groups ($p < 0{\cdot}05$) (Figure 1B). ADP hydrolysis in synaptosomes of the cerebral cortex did not present any difference among the groups (Figure 1D). 5'-nucleotidase activity in synaptosomes of the cerebral cortex of the smoke group was increased compared with the control (70%), Cur 12·5 (50%), Cur 25 (93%) and Cur 50 mg·kg^{-1} (90%) ($p < 0{\cdot}05$) groups (Figure 1F). In relation to AMP hydrolysis, statistical analysis showed a significant control or smoke versus curcumin (12·5, 25 and 50 mg·kg^{-1}) interaction ($p < 0{\cdot}05$).

AChE activity

The results obtained for AChE activity in cerebral cortex synaptosomes are presented in Figure 2. As can be observed, AChE activity was not modified by curcumin *per se* treatment (Figure 2A), and AChE activity was significantly increased in the smoke group ($p < 0{\cdot}05$) compared with the control (35%), Cur 12·5 (28%), Cur 25 (18%) and Cur 50 mg·kg^{-1} (15%) groups (Figure 2B). The statistical analysis showed a significant control or smoke versus curcumin (12·5, 25 and 50 mg·kg^{-1}) interaction ($p < 0{\cdot}05$).

DISCUSSION

The purpose of this study was to investigate the effects of curcumin, a strong candidate for the prevention and treatment of neurodegenerative diseases,[30] on NTPDase, 5'-nucleotidase and AChE activities in cerebral cortex synaptosomes in a model of passive cigarette smoke exposure. Some studies have demonstrated that, among its noxious properties, chronic tobacco smoking has a negative effect on cognitive processes including memory functions[1,2] and also induces subtle anatomical and chemical brain changes in normal adults.[31]

The investigation of AChE activity in synaptosomes from cerebral cortex of cigarette smoke-exposed rats demonstrated an increase in the enzyme activity, which possibly means that the neurotransmitter ACh acts very briefly in the receptors located on the postsynaptic membrane. The central cholinergic system plays an undisputed key role in the regulation of learning and memory, which are the constituents of cognitive behavior.[32] Thus, alterations in AChE activity, as well as in the ACh neurotransmitter levels, are neurochemically associated with cognitive deficits.[33,34]

Furthermore, regarding ectonucleotidase activity, an increase was observed in NTPDase (with ATP as substrate) and 5'-nucleotidase in synaptosomes from cerebral cortex of cigarette smoke-exposed rats. ATP is a potent signaling molecule exerting its short-term physiological actions through the interaction with the ionotropic P2X receptors, abundantly expressed in the CNS, which elicit a direct influx of extracellular Ca^{2+} leading to a secondary activation of voltage-gated Ca^{2+} channels. These receptors have an established role in neurotransmission, co-transmission, neuromodulation, glial communication and trophic actions,[35] making clear the role of purines in neurological homeostasis, especially the ATP molecule.

Our findings demonstrated that curcumin was effective in preventing the enhancement of AChE activity induced by cigarette smoke exposure in cerebral cortex synaptosomes from rats treated with curcumin. Some recent studies have reported that curcumin possesses AChE inhibitory activity in the CNS. Kuhad and Chopra[34] demonstrated that orally administered curcumin (60 mg·kg^{-1} body weight) was effective to attenuate cholinergic dysfunction and increased AChE activity in diabetic rats. Agrawal *et al.*[36] achieved similar results with oral doses of curcumin (200 mg·kg^{-1} body weight) against enhanced AChE activity in streptozotocin-induced dementia in rats. AChE activity in cerebral cortex synaptosomes was not influenced by curcumin *per se* at the doses studied.

The treatment with curcumin also was effective in preventing alterations in ectonucleotidase activities in cerebral cortex synaptosomes from cigarette smoke-exposed rats. The

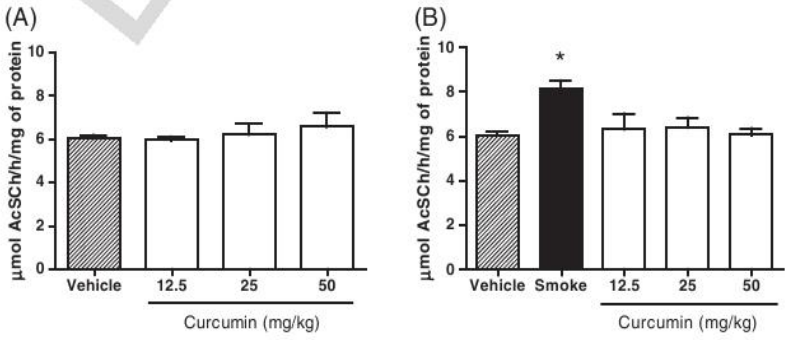

Figure 2. Acetylcholinesterase activity in cerebral cortex synaptosomes from cigarette smoke-exposed rats treated with curcumin (micromole AcSCh per hour per milligram of protein). Bars represent means ± SEM. ($n = 4$–5). ANOVA–Newman–Keuls multiple comparison test. (B) *Statistical difference from the other groups ($p < 0{\cdot}05$)

observed activation of NTPDase (hydrolysis of ATP) activity leads to a decrease in the synaptic ATP levels, which could lead to a disturbance in neurological homeostasis. The ectonucleotidase activity in cerebral cortex synaptosomes also was not influenced by curcumin alone at the doses studied.

Results of the present study indicate that curcumin has the ability to prevent the alterations of the cholinergic system in the cerebral cortex synapses of cigarette smoke-exposed rats. Additionally, the treatment with curcumin also prevents the alterations of the ectonucleotidases activities, successfully preventing alterations in the nucleotide concentrations in the CNS. These findings suggest the therapeutic potential of curcumin in the treatment of neurological disorders promoted by cigarette smoke exposure.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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ARTIGO 3 – CURCUMIN PROTECTS AGAINST CIGARETTE SMOKE-INDUCED COGNITIVE IMPAIRMENT AND INCREASED ACETYLCHOLINESTERASE ACTIVITY IN RATS

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Curcumin protects against cigarette smoke-induced cognitive impairment and increased acetylcholinesterase activity in rats

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ABSTRACT

Cigarette smoke, a widely spread habit, is associated with a decline in cognitive function and studies have demonstrated that curcumin (Cur), an Indian spice, possesses a strong neuroprotective potential. Considering the relevance of investigating dietary compounds this study aimed to investigate the effect of Cur on memory and acetylcholinesterase (AChE) activity in brain structures and blood of cigarette smoke-exposed rats. Male Wistar rats were treated with curcumin and cigarette smoke, once a day, 5 days each week, for 30 days. The experimental procedures were divided in two sets of experiments. In the first, the animals were divided into 4 groups: Vehicle (corn oil), Cur 12.5 mg/kg, Cur 25 mg/kg and Cur 50 mg/kg. In the second, the animals were divided into 5 groups: Vehicle (corn oil), Smoke, Smoke plus Cur 12.5 mg/kg, Smoke plus Cur 25 mg/kg and Smoke plus Cur 50 mg/kg. Treatment with Cur significantly prevented the decreased latency and cholinergic alterations in cigarette smoke-exposed rats. These AChE alterations could suggest a role in the memory impairment promoted by cigarette smoke-exposure and point toward the potential of Cur to modulate cholinergic neurotransmission and, consequently, improve cognition deficits induced by smoke. This study suggests that the dietary compound Cur may be involved in cholinergic system modulation and as a consequence exert an effect on learning and memory.

1. Introduction

Several studies have investigated the effects of smoking on cognitive function. In the majority of recent studies, a decline in cognitive function is attributed to the effects of cigarette smoke exposure [1,2], and a dose–response relationship with the amount smoked has been observed [3]. Many authors have studied inhibitors of acetylcholinesterase (AChE), a key enzyme involved in cognitive function, and verified an improvement in global cognitive functioning [4] by increasing the neurotransmitter acetylcholine concentration at cholinergic synapses located throughout the brain [5].

Curcumin (diferuloylmethane) is the major constituent in the most important fraction of the coloring agents found in the rhizomes of turmeric (*Curcuma longa* L.). Turmeric is a perennial herb that belongs to the Zingiberaceae family and is distributed throughout the tropical and subtropical regions of the world; it has been widely cultivated in Asiatic countries, mainly India and China [6]. Curcumin is a polyphenol employed in old Hindu medicine [7], and in traditional Indian and Chinese medicine, it is used for the treatment of many disorders [4,8].

In addition to the well-documented anti-inflammatory, antioxidant and chemopreventive (i.e., growth-inhibitory effects on cancer cells) properties [9–12], many studies have demonstrated the neuroprotective potential of curcumin in the prevention of cognitive dysfunction [13–15], neurotoxicity [16], neuroinflammation and Alzheimer's and Parkinson's disease [17], as well as in the promotion of neuroplasticity and neurogenesis [17,18]. The neuroprotective effects promoted by curcumin are

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thought to be partially related to the regulation of important enzymes and molecules involved in inflammation, such as cyclooxygenase-2 (COX-2), lipoxygenase, nuclear factor-kappa B (NF-κB) and cytokines [19]. Furthermore, some studies have demonstrated that curcumin is able to modulate intracellular signaling pathways through the activity of the Ca^{2+}-dependent protein kinase enzymes A, B, and C (PKA, PKB and PKC), as well as the inositol 1,4,5-triphosphate receptor, both of which are important to the neurotransmission [19]. A previous investigation [20] demonstrated that curcumin prevented the induced rise in brain AChE activity.

In the present study, we investigated the effects of curcumin on AChE activity in different cerebral regions and evaluated the memory parameters of rats exposed to cigarette smoke.

2. Material and methods

2.1. Reagents

Acetylthiocholine iodide, 5,5-dithiobis-2-nitrobenzoic acid (DTNB), tris (hydroxymethyl)-aminomethane GR, Coomassie brilliant blue G, and curcumin (curcumin ≥ 80%; curcuminoid content ≥ 94%) were obtained from Sigma Chemical Co (St. Louis, MO, USA). The brand of cigarette used in the experiment was manufactured by Souza Cruz S.A., Brazil. The cigarette contained 10 mg tar, 0.9 mg nicotine, and 10 mg carbon monoxide. All other reagents used in the experiments were of analytical grade and of highest purity.

2.2. Animals

Male Wistar rats (90–110 days) from the Central Animal House of the Federal University of Santa Maria (UFSM) were used in this experiment. They were housed five to a cage (49 × 34 × 16 cm) on a natural day/night cycle (lights on at 19:00 and off at 7:00) at a constant temperature of 21 °C with free access to water and standard chow *ad libitum*. All animal procedures were approved by the animal Ethics Committee from the UFSM (protocol under number: 23081.004963/2009-71).

2.3. Cigarette smoke exposure and treatment with curcumin

Experimental procedures were divided in two sets of experiments. In the first set, animals were randomly divided into four groups (10 rats in each group): Vehicle (corn oil); Cur 12.5 mg/kg body weight; Cur 25 mg/kg body weight; or Cur 50 mg/kg body weight. In the second experimental set, animals were divided into 5 groups (10 rats in each group): Vehicle (corn oil); smoke exposed; smoke and Cur 12.5 mg/kg body weight; smoke and Cur 25 mg/kg body weight; or smoke and Cur 50 mg/kg body weight. Curcumin was diluted with corn oil, administered by oral gavage, and did not exceed 1.0 ml/kg body weight. The treatment with curcumin and cigarette smoke was carried out once a day, 5 days each week, for 30 days (six weeks). We chose these doses of curcumin and time of treatment based on previous studies of our research group in which we observed that cigarette smoke have effects on the immune and central nervous system and curcumin showed a protective effect [21–23]. First, curcumin or corn oil was administered, and approximately 10 minutes later, the smoking groups were exposed to the aged and diluted sidestream smoke of commercial cigarettes inside a whole-body smoke exposure chamber for 15 minutes. Control animals were placed in an equal chamber for the same amount of time, but without exposure to smoke. While the smoke exposure procedure was performed, the control group was always outside, without any contact with the smoke [24].

2.4. Smoke generation

After placing the rats inside the exposure chamber (size 56.4 × 38.5 × 37.1 cm; plastic material), 4 cigarettes were lit, and a stopwatch was turned on. The cigarettes were fixed in a metal holder, allowing them to be fully burned down within a period of 15 min. After lighting the cigarettes, the chamber was immediately closed, with a small opening (371 × 40 mm) in both extremities for ventilation. The smoke generated inside the chamber was suctioned by a noiseless extractor fan to keep an air flow inside the chamber. A metal grille was placed on top of the cigarette holder to avoid direct contact with the cigarettes and, thus, to prevent the rats from injuring themselves. The inhalation exposure of our study was to aged and diluted sidestream smoke, used as a simulation of environmental tobacco smoke (ETS) as experienced by non-smokers [24].

2.5. Behavioral procedure — inhibitory avoidance

Thirty days after the treatment with smoke and curcumin or vehicle, animals were subjected to training and tested in a step-down inhibitory avoidance apparatus [25]. Briefly, the rats were subjected to a single training session in a step-down inhibitory avoidance apparatus, returned to their home cage and tested for retention 24 h later. The apparatus consisted of a 25 × 25 × 35 cm box with a grid floor, and the left portion was covered by a 7 × 25 cm platform 2.5 cm high. The rat was placed gently on the platform facing the rear left corner, and when the rat stepped down with all four paws on the grid, a 3 s 0.4 mA shock was applied to the grid. Test step-down latency was taken as a measure of retention, and a cut-off time of 600 s was established.

2.6. Behavioral procedure - open field

Immediately after the inhibitory avoidance test session, animals were transferred to an open-field measuring 56 × 40 × 30 cm, with the floor divided into 12 squares measuring 12 × 12 cm each. The open field session lasted 5 min, and during this time, the number of crossing and rearing responses was recorded. This test was carried out to identify motor disabilities, which might influence performance during the inhibitory avoidance test.

2.7. Brain tissue preparation

After the behavioral tests, animals were anesthetized and euthanized. Brain structures were quickly removed from skull, rinsed in ice-cold Tris–HCl buffer (10 mM, pH 7.4), placed on filter paper moistened with the same buffer on top of a Petri dish filled with ice and the following brain regions were dissected: hypothalamus, cerebellum, cerebral cortex, hippocampus and striatum, using consistent anatomical landmarks as criteria for dissection. Placing the brain ventral side up hypothalamus was dissected with the help of a scalpel. Cerebellum was dissected by cutting the cerebellar peduncles at the surface of the brainstem. Cerebral cortex comprised all regions dorsolateral to the olfactory tract, excluding the hippocampus, and was dissected from each hemisphere by peeling it away from the striatum. The brain structures were homogenized in a glass potter in a Tris–HCl solution. Aliquots of resulting brain structure homogenates were stored at −20 °C until utilization. Protein was determined previously in a range that varied for each structure: cerebral cortex (0.7 mg/ml), striatum (0.4 mg/ml), hippocampus (0.8 mg/ml), hypothalamus (0.6 mg/ml) and cerebellum (0.6 mg/ml) as determined by the Coomassie blue method [26], using bovine serum albumin as standard solution.

2.8. Cerebral AChE enzymatic assay

AChE enzymatic assay was determined by a modification of a spectrophotometric method [27], as previously described [28]. The reaction mixture (2 ml final volume) contained 100 mM K^+-phosphate buffer at pH 7.5 and 1 mM DTNB. The method is based on the formation of the yellow anion, 5,5′-dithio-bis-acid-nitrobenzoic, measured by absorbance at 412 nm during a 2-min incubation at 25 °C. The enzyme (40–50 μg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh). All samples were run in duplicate or triplicate, and the enzyme activity is expressed in μmol AcSCh/h/mg of protein.

2.9. Blood sample collection

Blood was collected in vaccutainer tubes using EDTA as an anticoagulant. The samples were hemolysed with phosphate buffer, pH 7.4 containing Triton X 100 (0.03%) and stored at −20 °C for one week.

2.10. Determination of AChE activity in whole blood

AChE enzymatic assay was determined by a modification of a spectrophotometric method [27] as previously described [29]. The specific activity of whole blood AChE was calculated as the AChE activity per mol of hemoglobin, and the results were expressed as mU/μmol of hemoglobin.

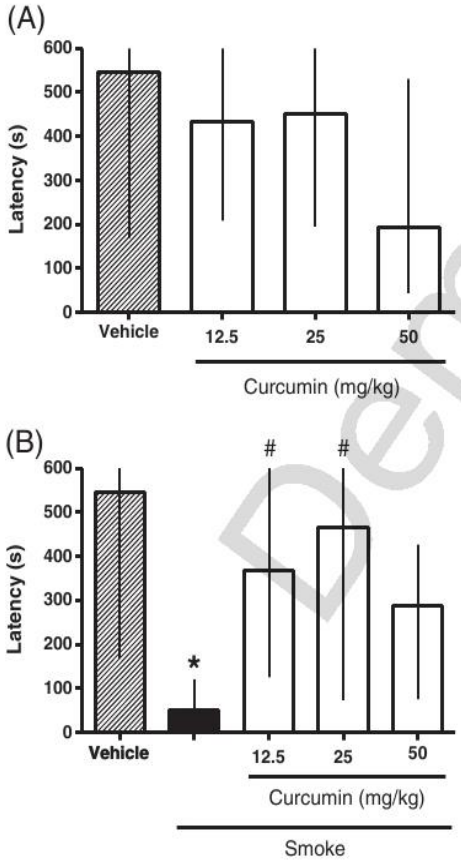

Fig. 1. Effect of oral administration of vehicle (corn oil) or curcumin (Cur, 12.5, 25 or 50 mg/kg body weight) (A) and effect of oral administration of vehicle (corn oil) or curcumin (12.5, 25 or 50 mg/kg body weight) in cigarette smoke-exposed rats (B) on the inhibitory avoidance task performance of adult rats measured as the test step-down latencies. Data are the median ± interquartile range for 10 animals in each group. *P<0.05 compared with the vehicle group and #P<0.05 compared with the Smoke group by Dunn's Multiple Comparison Test.

Table 1
Effect of oral administration of vehicle (corn oil) or curcumin (Cur, 12.5, 25 or 50 mg/kg) 5 days each week, during 30 days on the latency to training on the inhibitory avoidance apparatus and on the behavior of rats (number of crossing and rearing responses) in the open-field immediately after the inhibitory avoidance testing session.

Group	Lat. training (s)	Crossing	Rearing	N
Vehicle	8.50 ± 3.09	12.20 ± 2.26	7.60 ± 1.60	10
Cur 12.5	6.22 ± 2.00	13.56 ± 2.56	7.33 ± 1.54	10
Cur 25	9.57 ± 2.56	16.71 ± .25	8.86 ± 2.46	10
Cur 50	10.40 ± 2.05	9.80 ± 1.80	5.40 ± 0.90	10

Data are means ± SEM. N, number of animals in each group.

2.11. Statistical analysis

Statistical analysis of test step-down latencies was carried out by the Kruskal–Wallis test followed by a Dunn's multiple comparison test. The relationships between AChE activity, crossing, rearing and latency to training responses were analyzed by one-way ANOVA, followed by Newman–Keuls Multiple Comparison Test. $P<0.05$ was considered to represent a significant difference in all experiments.

3. Results

3.1. Behavioral tests

Fig. 1 shows the effect of administration of curcumin alone (12.5, 25 or 50 mg/kg body weight) and its administration in cigarette smoke-exposed rats on step-down latencies. Post hoc analysis of testing showed that curcumin alone did not modify step-down latencies ($P>0.05$) (Fig. 1A). However, smoke exposure decreased step-down latencies, and curcumin (12.5 and 25 mg/kg) prevented this effect ($P<0.05$) (Fig. 1B). Statistical analysis of the training session showed no difference between groups (Tables 1 and 2).

Because motivational disparities in the training session may account for differences in inhibitory avoidance at testing, experiments were performed to assess whether smoking or curcumin affected locomotor ability of the animals. Post hoc analysis of open-field data revealed that pharmacological treatment did not alter the number of crossing or rearing responses in a subsequent open-field test session (Tables 1 and 2).

3.2. Activity of AChE in brain

Table 3 shows the effect of administration of curcumin alone (12.5, 25 or 50 mg/kg body weight) on AChE activity in the cerebellum, hippocampus, striatum, hypothalamus and cerebral cortex. Post hoc analysis showed that AChE activity was significantly increased by Cur (50 mg/kg) in the cerebellum, hippocampus and striatum (Table 3) ($P<0.05$) when compared to the other groups. However, AChE activity was significantly decreased by Cur (50 mg/kg) in the hypothalamus (Table 3) ($P<0.05$) when compared to the other

Table 2
Effect of oral administration of vehicle (corn oil), smoke, and smoke plus curcumin (12.5, 25 or 50 mg/kg) 5 days each week, during 30 days on the latency to training on the inhibitory avoidance apparatus and on the behavior of rats (number of crossing and rearing responses) in the open-field immediately after the inhibitory avoidance testing session.

Group	Lat. training (s)	Crossing	Rearing	N
Vehicle	14.60 ± 3.72	12.30 ± 2.76	6.00 ± 1.43	10
Smoke	14.20 ± 3.08	8.70 ± 1.92	4.90 ± 1.36	10
Smoke-Cur12.5	11.90 ± 3.43	16.70 ± 4.38	9.10 ± 2.32	10
Smoke-Cur 25	13.89 ± 3.34	7.80 ± 2.09	4.20 ± 1.64	10
Smoke-Cur 50	9.20 ± 2.66	16.00 ± 2.48	8.60 ± 1.58	10

Data are means ± SEM. N, number of animals in each group.

Table 3
Effect of oral administration of vehicle (corn oil) and curcumin (12.5, 25 or 50 mg/kg) 5 days each week, during 30 days on AChE activity (mean ± S.E.M.) in the five brain structures and whole blood of rats (n = 10).

Group	CE	CO	HP	HY	ST	WB
Vehicle	3.19 ± 0.17	8.05 ± 0.67	7.99 ± 0.31	5.44 ± 0.43	15.09 ± 0.92	64.62 ± 3.39
Cur 12.5	3.15 ± 0.17	7.52 ± 0.89	8.52 ± 0.33	5.79 ± 0.13	15.34 ± 1.28	63.93 ± 4.09
Cur 25	3.32 ± 0.12	8.81 ± 1.56	8.84 ± 0.61	5.05 ± 0.30	13.63 ± 1.50	68.20 ± 5.11
Cur 50	3.91 ± 0.19*	8.40 ± 0.89	11.44 ± 0.11*	3.90 ± 0.15**	20.60 ± 1.67*	69.75 ± 2.58

Cur: curcumin; CE: cerebellum; CO: cerebral cortex; HP: hippocampus; HY: hypothalamus; ST; striatum; WB: whole blood.
* P<0.05 and **P<0.01 compared with all groups.

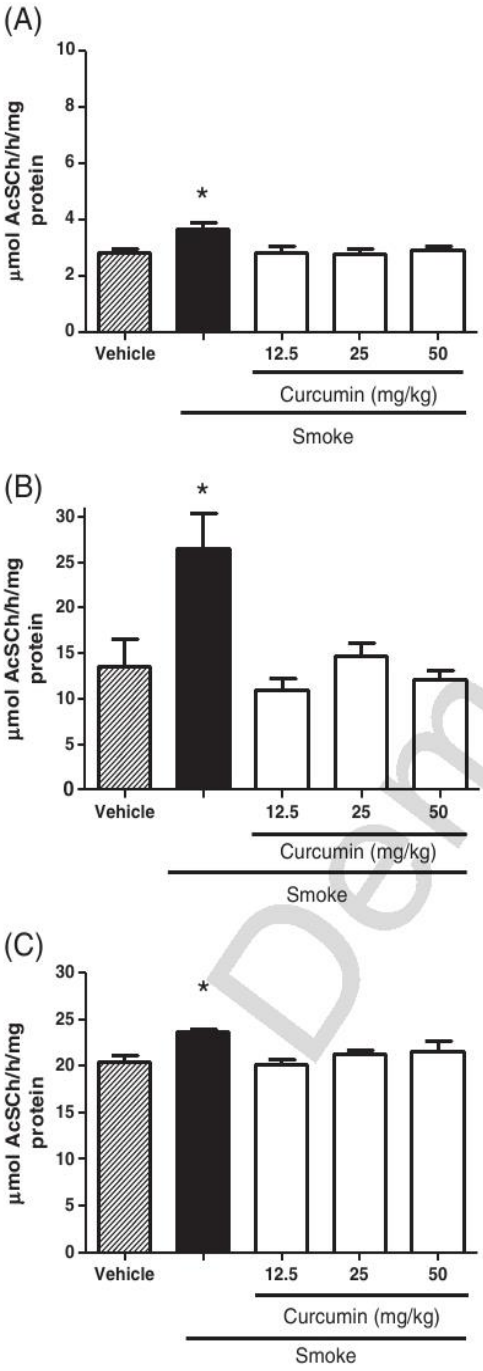

Fig. 2. AChE activity in the cerebellum (A), cerebral cortex (B) and hippocampus (C) of cigarette smoke exposed rats and those treated with curcumin (12.5, 25 or 50 mg/kg) plus smoke. Data are means ± Standard Error of the Mean (SEM) for 10 animals in each group. *P<0.05 compared to all groups.

groups. No significant effect of Cur was found on AChE activity in cerebral cortex (Table 3).

Figs. 2–4 show the effect of the administration of curcumin (12.5, 25 and 50 mg/kg body weight) and smoke on AChE activity in the cerebellum, cerebral cortex, hippocampus, striatum and hypothalamus. AChE activity in these cerebral regions was significantly increased in the smoke-exposed group (P<0.05), compared to the control group (Figs. 2–4). However, treatment with curcumin (12.5, 25 or 50 mg/kg) prevented this increase in the cerebellum, cerebral cortex and hippocampus (Figs. 2A–C), although treatment with Cur (50 mg/kg) did not prevent the increase of AChE activity induced by smoke in the striatum (Fig. 3) and hypothalamus (P<0.05) (Fig. 4).

3.3. Activity of AChE in whole blood

Table 3 shows the effect of administration of curcumin alone (12.5, 25 and 50 mg/kg body weight) on AChE activity in whole blood. Post hoc analysis showed that curcumin alone did not affect AChE activity (P>0.05). However, when curcumin (12.5, 25 or 50 mg/kg) was given to the smoke-exposed animals, it was effective in preventing the increase in AChE activity induced by smoke (Fig. 5) (P<0.05).

4. Discussion

Cigarette smoke exposure is associated with cognitive impairment [30,31], which could be a consequence of neurochemical alterations in the Central Nervous System (CNS). An important agent with a fundamental role in learning and memory processes is the AChE enzyme [32,33]. Studies have demonstrated that some compounds present in cigarette smoke, such as nitric oxide and cyanide, are related to an enhanced AChE activity and memory impairment [34,35]. Therefore, we suggest that the up-regulation of this enzyme could be one of the

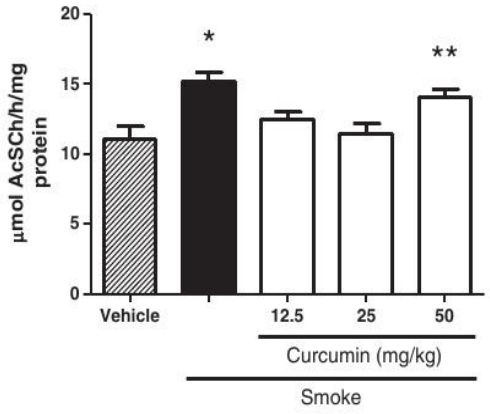

Fig. 3. AChE activity in striatum of cigarette smoke-exposed rats and those treated with curcumin (12.5, 25 and 50 mg/kg) plus smoke. Data are means ± SEM for 10 animals in each group. *P<0.05 compared with vehicle and curcumin 12.5 and 25 mg/kg. ** P<0.05 compared with vehicle.

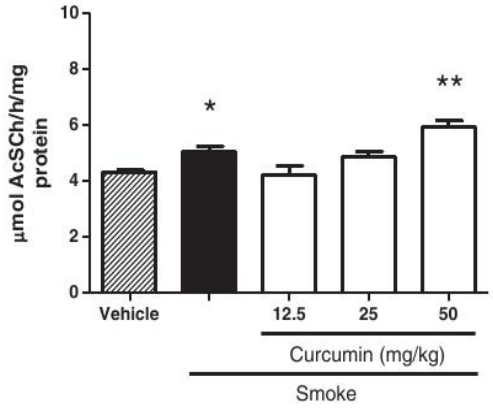

Fig. 4. AChE activity in the hypothalamus of cigarette smoke-exposed rats and those treated with curcumin (12.5, 25 and 50 mg/kg) plus smoke. Data are means ± SEM for 10 animals in each group. *P<0.05 compared with all groups, except curcumin 25 mg/kg. ** P<0.05 compared with all groups.

pathological bases of the cognitive impairment promoted by cigarette smoke exposure.

The inhibitory avoidance test is a classic behavioral test with a strong aversive component and is used to evaluate learning and memory in rats and mice [36]. In our study, we found a significant decrease in step-down latency in cigarette smoke-exposed rats in the inhibitory avoidance test, suggesting learning and memory impairment in these animals. These results are in agreement with other studies that have also verified cognitive impairment in cigarette smoke-exposed rats [30,31]. However, when smoke-exposed rats were orally treated with Cur (12.5 or 25 mg/kg) 5 days a week for 30 days, the step-down latency in the inhibitory avoidance test was similar to that found for rats from the control group. These findings indicate that treatment with curcumin was able to prevent the learning and memory impairment induced by cigarette smoke exposure.

Our results are in agreement with the literature; a number of studies have demonstrated that different doses of curcumin, both orally and as an intragastric infusion, ranging from 3 to 300 mg/kg, for different periods (2–6 weeks), have the potential to ameliorate the cognitive impairment caused by different types of agents, such phenytoin [14], scopolamine [37], $AlCl_3$ associated with D-galactose [13] and human immunodeficiency virus type 1 glycoprotein 120 V3 loop peptide [15]. It is important to mention that curcumin alone did not affect the step-down latency.

A major concern in shock-motivated learning tests, particularly in those that investigate the effect of drugs given before the test is given, is whether the pharmacological treatment affects locomotor activity. To rule out this possibility, we assessed locomotor behavior immediately after the inhibitory avoidance test session to identify any motor disability that might have influenced the inhibitory avoidance performance. Our results demonstrated that in the control, smoke-exposed and smoke-exposed groups treated with curcumin, there was no difference in locomotor activity, and thus, the number of crossing or rearing responses in the open-field session was not affected by this parameter. These data exclude the possibility that locomotor activity may have contributed to the alteration in step-down latencies in the inhibitory avoidance test in smoke-exposed rats.

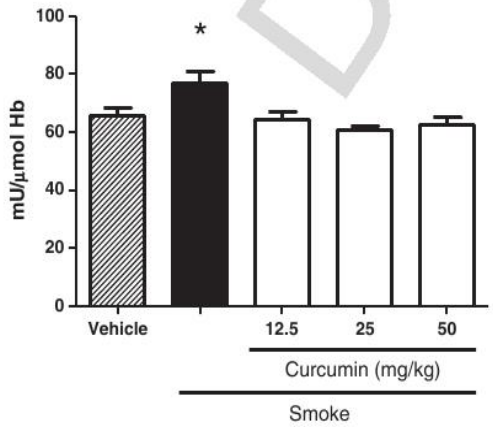

Fig. 5. AChE activity in whole blood of cigarette smoke-exposed rats and those treated with curcumin (12.5, 25 and 50 mg/kg) plus smoke. Data are means ± SEM for 10 animals in each group. *P<0.05 compared with all groups.

The importance of the cholinergic system in learning and memory processes is undeniable, and thus, alteration to AChE activity, as well as in the acetylcholine neurotransmitter levels, is neurochemically associated with cognitive deficits [38,39]. In this study, we found increased AChE activity in the blood and in all cerebral structures of the smoke-exposed groups. According to the study of Anbarasi et al. [40], chronic exposure to cigarette smoke significantly decreases membrane-bound ATPases activity, which alters ion homeostasis and leads to an increase in Ca^{2+} and Na^{+} levels within the cell. The concurrent augment of Ca^{2+} and Na^{+} concentrations causes a hyperpolarization of neuronal cell membrane and consequently the release of more neurotransmitters such as ACh. We believe that the increased AChE activity might be a compensatory response to these biochemical events.

Our findings demonstrate that curcumin was effective in preventing the enhancement of AChE activity induced by smoke in the blood and all the cerebral structures studied. This preventive effect of curcumin produced an amelioration of the smoke-induced cognitive impairment. A number of papers have provided results that corroborate our data, reporting that curcumin possess an AChE inhibitory activity [41,42], which leads to a larger window of time in which acetylcholine can stimulate post-synaptic muscarinic receptors, consequently acting as a memory enhancer.

The central cholinergic system, particularly in the hippocampus, plays an undisputed key role in the regulation of learning and memory, which are the primary constituents of cognitive behavior [43]. In line with this role, the normal AChE activity in the smoke-exposed groups treated with 12.5, 25 or 50 mg/kg of curcumin, especially in the hippocampus, may be the major factor responsible for maintaining cognitive function by enhancing cholinergic activity.

AChE activity in the cerebral structures was not influenced by curcumin alone at the doses of 12.5 or 25 mg/kg; however, most of the groups showed statistically significant alterations in enzyme activity at the dose of 50 mg/kg. Corroborating our findings, a previous study [20] also found that curcumin did not show any effect *per se* on brain AChE activity at the dose of 20 mg/Kg. We speculate that curcumin may prevent the excitotoxicity promoted by cigarette smoke-exposure, which might explain the finding that curcumin is able to reduce AChE activity when it is stimulated, while it does not modify basal AChE activity. Although the majority of the curcumin doses were effective in preventing the increase in AChE activity in the cerebral structures promoted by the cigarette smoke exposure, the dose of 50 mg/kg was not effective in reversing this increase in the striatum and hypothalamus.

It was [42] demonstrated that curcumin, orally administered at a dose of 60 mg/kg, significantly attenuated cholinergic dysfunction and was effective in preventing the increased AChE activity observed in diabetic rats. A previous study [41] demonstrated that curcumin, orally administered at a dose of 200 mg/kg, was also effective against enhanced AChE activity in streptozotocin-induced dementia in rats.

The authors [37] who evaluated AChE activity in frontal cortex and hippocampus of rats treated with curcuminoids at doses of 3 or 10 mg/kg found reduced AChE activity. Nevertheless, attempts to obtain similar results with curcumin were unsuccessful. These data, unlike the results shown in the present paper, may be due to the lower

doses administered (3 and 10 mg/kg), but further investigations regarding the effects of curcumin on the Central CNS, and especially on AChE activity, should be encouraged.

The effects produced by AChE activity in whole blood in the different groups was similar to the effects obtained in the cerebral regions, in both the first and second set of experiments. This pattern of AChE activity was found previously by our research group [44]; It has been proposed previously [45] that whole blood could have similar functional properties as synaptic AChE, and therefore, the whole blood AChE activity could reflect the status at the synaptic site. If this hypothesis is true, as we believe it to be, then we can assume that by assessing peripheral blood AChE activity we can extrapolate data for the CNS.

5. Conclusions

In summary, the present study demonstrates that curcumin was effective in preventing cigarette smoke-induced cognitive impairment and increased AChE activity in rats. This study suggests the potential of adjuvant curcumin therapy in ameliorating cognitive impairment caused by cigarette smoke exposure.

Conflicts of interest statement

There are no actual or potential conflicts of interest.

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ARTIGO 4 - FREE AND NANOENCAPSULATED CURCUMIN PREVENTS CIGARETTE SMOKE-INDUCED COGNITIVE IMPAIRMENT AND REDOX IMBALANCE

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Free and nanoencapsulated curcumin prevents cigarette smoke-induced cognitive impairment and redox imbalance

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ABSTRACT

Cigarette smoke-exposure promotes neurobiological changes associated with neurocognitive abnormalities. Curcumin, a natural polyphenol, have shown to be able to prevent cigarette smoke-induced cognitive impairment. Here, we investigated possible mechanisms involved in curcumin protection against cigarette smoke-induced cognitive impairment and, due to its poor bioavailability, we investigated the potential of using curcumin-loaded lipid-core nanocapsules (C-LNC) suspension. Rats were treated with curcumin and cigarette smoke, once a day, 5 days each week, for 30 days. Animals were divided into ten groups: I, control (vehicle/corn oil); II, curcumin 12.5 mg/kg; III, curcumin 25 mg/kg; IV, curcumin 50 mg/kg; V, C-LNC 4 mg/kg; VI, tobacco exposed; VII, curcumin 12.5 mg/kg along with tobacco exposure; VIII, curcumin 25 mg/kg along with tobacco exposure; IX, curcumin 50 mg/kg along with tobacco exposure; X, C-LNC 4 mg/kg along with tobacco exposure. Cigarette smoke-exposure impaired object recognition memory ($P < 0.001$), indicated by the low recognition index, increased biomarkers of oxidative/nitrosative stress such as TBARS ($P < 0.05$) and NOx ($P < 0.01$), decreased antioxidant defenses such as NPSH content ($P < 0.01$) and SOD activity ($P < 0.01$) and inhibited the activities of enzymes involved in ion homeostasis such as Na^+,K^+-ATPase and Ca^{2+}-ATPase. Both curcumin formulations (free and nanoencapsulated) prevented the memory impairment, the redox imbalance and the alterations observed in the ATPases activities. Maintenance of ion homeostasis and redox balance is involved in the protective mechanism of curcumin against tobacco-induced cognitive impairment. Our results suggest that curcumin is a potential therapeutic agent for neurocognition and that C-LNC may be an alternative to its poor bioavailability.

1. Introduction

Epidemiological data indicates that nearly 20% of worldwide population, estimated in 1.4 billion of people, uses products derived from tobacco and, as a consequence, around 6 million of people died only in 2011 (Eriksen, Mackay, & Ross, 2012). Cigarette smoke contains over 4000 different chemicals including many carcinogenic compounds (Genbacev-Krtolica, 2005). Besides these compounds, cigarette is also a source of reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\cdot}$), hydroxyl radical ($HO^{\cdot}$) and hydrogen peroxide (H_2O_2); and reactive nitrogen species (RNS) such as nitric oxide ($NO^{\cdot}$), peroxynitrite ($ONOO^-$) and peroxynitrate (O_2NOO^-) (Pryor & Stone, 1993).

Studies demonstrate that the reactive species generated by the exposure to these compounds and by combustion of cigarettes

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cause oxidative damage in biological macromolecules (Moriarty et al., 2003) and trigger an inflammatory cascade which can lead to the development and/or facilitate the development of many diseases that involve the central nervous system (CNS) (Almeida et al., 2008; McQuaid, Cunnea, McMahon, & Fitzgerald, 2009). Furthermore, chronic exposure to cigarette smoke alters ion homeostasis (Anbarasi, Vani, Balakrishna, & Devi, 2005), which also can contribute to neurological diseases and memory impairment since it is associated with neuronal injury or death (Xiao, Wei, Xia, Rothman, & Yu, 2002).

Curcumin (diferuloylmethane), a polyphenol, is an active principle of the perennial herb *Curcuma longa* L. (commonly known as turmeric) (Goel, Kunnumakkara, & Aggarwal, 2008). Studies demonstrate its cytoprotective potential against the oxidative damage in neuronal cells (Scapagnini et al., 2006) and neuroprotective in the prevention of cognitive dysfunction (Jaques et al., 2012; Pan, Qiu, Lu, & Dong, 2008; Reeta, Mehla, & Gupta, 2009; Tang et al., 2009), neurotoxicity (Sethi, Jyoti, Hussain, & Sharma, 2009), as well as in the promotion of neuroplasticity and neurogenesis (Begum et al., 2008; Kim et al., 2008).

Biological activity of curcumin, however, is severely limited due to its poor bioavailability (Kelloff et al., 1996). Facing this problem, many study groups have employed nanotechnology to improve oral bioavailability of curcumin and the effects of nanoparticles formulations of curcumin have been promising (Ray, Bisht, Maitra, Maitra, & Lahiri, 2011; Shaikh, Ankola, Beniwal, Singh, & Kumar, 2009; Thangapazham, Puri, Tele, Blumenthal, & Maheshwari, 2008).

In the present study, we investigated the effects of curcumin and C-LNC on ionic and oxidative stress parameters in cerebral cortex of rats exposed to cigarette smoke and also investigated their memory performance.

2. Materials and methods

2.1. Reagents

Ouabain octahydrate (⩾95%, HPLC, Sigma O3125), adenosine 5′-triphosphate disodium salt hydrate (⩾99%, Sigma A2383), 5-5′-dithiobis(2-nitrobenzoic acid) (⩾98%, TLC, Sigma D8130), (−)-epinephrine(+)bitartrate salt (Sigma E4375), malonaldehyde bis-dimethyl acetal (MDA, 99%, Aldrich 108383), 2-thiobarbituric acid (sodium derivative, Aldrich S564508) and (E,E)-1,7-bis(4-Hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (diferuloylmethane; curcumin; Sigma C1386) were obtained from Sigma–Aldrich Chemical Co (St. Louis, MO, USA). The brand of cigarette used in the experiment was manufactured by Souza Cruz S.A., Brazil. The cigarette contained 10 mg tar, 0.9 mg nicotine, and 10 mg carbon monoxide. All other reagents used in the experiments were of analytical grade and of highest purity.

2.2. Animals

Male Wistar rats (90–110 days) from the Central Animal House of the Federal University of Santa Maria (UFSM) were used in this experiment. They were housed five to a cage (49 × 34 × 16 cm) on a natural day/night cycle (lights on at 19:00 and off at 7:00) at a constant temperature of 21 °C with free access to water and standard chow *ad libitum*. All animal procedures were approved by the Animal Ethics Committee from the UFSM (protocol under number: 23081.004963/2009-71).

2.3. Cigarette smoke exposure and treatment with curcumin

Animals were randomly divided into ten groups (5 rats in each group): I, control (vehicle/corn oil); II, curcumin 12.5 mg/kg; III, curcumin 25 mg/kg; IV, curcumin 50 mg/kg; V, curcumin-loaded lipid-core nanocapsules (C-LNC) suspension 4 mg/kg; VI, tobacco exposed; VII, curcumin 12.5 mg/kg along with tobacco exposure; VIII, curcumin 25 mg/kg along with tobacco exposure; IX, curcumin 50 mg/kg along with tobacco exposure; X, C-LNC 4 mg/kg along with tobacco exposure. In their home cages, rats were housed in same-treatment groups. Free curcumin was diluted with corn oil, administered by oral gavage, and did not exceed 1.0 mL/animal. C-LNC was also administered by oral gavage, and did not exceed 2.5 mL/animal. The treatment with curcumin was carried out once a day, 5 days each week, for 30 days (6 weeks). We chose these doses of curcumin and time of treatment based on previous studies of our research group in which we observed that cigarette smoke have effects on the immune and central nervous system and curcumin showed a protective effect (Jaques, Rezer, Goncalves, et al., 2011; Jaques, Rezer, Ruchel, et al., 2011; Jaques, Ruchel, et al., 2011; Jaques et al., 2012). Furthermore, this protocol was an attempt to mimic the occupational exposure of people which work 5 days a week. First curcumin or corn oil was administered, and approximately 10 min later, the smoking groups were exposed to the aged and diluted sidestream smoke of commercial cigarettes inside a whole-body smoke exposure chamber for 15 min. They were exposed to smoke in groups of 5 rats, the whole group. Control animals were placed in an equal chamber for the same amount of time, but without exposure to smoke. While the smoke exposure procedure was performed, the control group was always outside, without any contact with smoke (Thome et al., 2009).

2.4. Smoke generation

After placing the rats inside the exposure chamber (size 56.4 × 38.5 × 37.1 cm; plastic material), 4 cigarettes were lit, and a stopwatch was turned on. The cigarettes were fixed in a metal holder, allowing them to be fully burned down within a period of 15 min. After lighting the cigarettes, the chamber was immediately closed, with a small opening (371 × 40 mm) in both extremities for ventilation. The smoke generated inside the chamber was suctioned by a noiseless extractor fan to keep an air flow inside the chamber. A metal grille was placed on top of the cigarette holder to avoid direct contact with the cigarettes and, thus, to prevent the rats from injuring themselves. The inhalation exposure of our study was to aged and diluted sidestream smoke, used as a simulation of environmental tobacco smoke as experienced by non-smokers (Thome et al., 2009).

2.5. Preparation of C-LNC

C-LNC were obtained by the interfacial deposition of polymer method (Jager et al., 2009). The organic phase contained poly(ε-caprolactone) as a biodegradable polymer (1.0 g), sorbitan monoestearate (0.383 g), curcumin (0.05 g) and grape seed oil as lipid-core (1.65 mL). These hydrophobic constituents were dissolved in 267 mL of acetone, a water miscible organic solvent, and injected into the 534 mL of aqueous phase containing polysorbate 80 (0.766 g). Then, acetone was eliminated and the aqueous phase concentrated by evaporation under reduced pressure to obtain 100 mL. The formulations were prepared and kept protected from light.

2.6. Characterization of lipid-core nanocapsules

2.6.1. Drug content, encapsulation efficiency and pH

Curcumin was assayed by validated liquid chromatography (LC) method. The mobile phase was composed by acetonitrile: 0.1% trifluoracetic acid (50/50 v/v), (adjusted with pH 3.0 with triethylamine) eluted at the flow rate of 0.6 mL min^{-1}. The column used

was a RP-18 Sigma–Aldrich (150 mm × 4.6 mm × 5 μm particle size, 110 Å pore diameter) and curcumin was detected at 427 nm. Drug content (mg mL^{-1}) was determined after dissolution of nanoparticles in acetonitrile followed by centrifugation at 4120*g* during 10 min. An aliquot of supernatant was withdrawn, diluted in mobile phase, filtrated in 0.45 μm membrane (Millex GV, Millipore, Ireland) and 20 μL was injected in LC. Encapsulation efficiency was determined after separation of free curcumin from the particles by ultrafiltration-centrifugation technique (Ultrafree Microcon 10,000 MW, Millipore, Ireland) at 4120*g* during 10 min. The ultrafiltrate was diluted with acetonitrile (1:1 v/v) and the free curcumin was quantified by LC. Encapsulation efficiency (%) was calculated by the difference between the total and free drug concentration. The pH measurements were determined directly in the suspensions using a calibrated potentiometer (VB-10, Denver Instrument, USA).

2.6.2. Particle size distribution, polydispersity index and zeta potential

Particle size distribution was analyzed by photon correlation spectroscopy (Zetasizer Nano ZS®, Malvern Instruments, UK). The samples were previously diluted (500×) with ultrapure water and analyzed at 25 °C. The polydispersity indices (PDI) were determined by the same technique. The zeta potential was estimated after dilution of the samples in 10 mM NaCl aqueous solution (500×) by electrophoretic mobility (Zetasizer Nano ZS®, Malvern Instruments, UK). Measurements were taken at 25 °C. The samples were analyzed in triplicate batches ($n = 3$).

2.7. Behavioral study

2.7.1. Object recognition memory task

Thirty days after the treatment with smoke and curcumin or vehicle, animals were subjected to the object recognition memory task to evaluate the long-term memory (LTM), performed in three consecutive days as previously described (Balderas et al., 2008) with some modifications briefly outlined. On the first day animals were positioned into the open field arena without any objects for 5 min. On the second day the training trial was conducted (sample phase), in which rats were placed in the arena facing the wall opposite the objects for 10 min and were allowed to freely explore two identical objects (A_1 and A_2). Memory was tested 24 h later (LTM). On memory test, rats were allowed to explore freely one copy of the previously presented object (familiar, A_3) together with a new one (B_1) for 5 min. The object recognition index was calculated as follows: time of exploration of novel object/(time of exploration of familiar object + time of exploration of novel object) (Ennaceur & Delacour, 1988). A recognition index equal to 0.5 reflects no preference for any of the objects. An index higher than 0.5 shows preferences for novel objects.

2.7.2. Open field test

Immediately after the object recognition test session, animals were transferred to an open-field arena measuring 56 × 40 × 30 cm, with the floor divided into 12 squares measuring 12 × 12 cm each. The open field session lasted 5 min, and during this time, the number of crossing and rearing responses was recorded. This test was carried out to identify motor disabilities, which might influence performance during the object recognition test.

2.8. Biochemical studies

2.8.1. Preparation of tissue homogenate

After the behavioral tests, animals were anesthetized and euthanized. Brain was quickly removed from skull, rinsed in ice-cold Tris-HCl buffer (10 mM, pH 7.4), placed on filter paper moistened with the same buffer on top of a Petri dish filled with ice and cerebral cortex was dissected using consistent anatomical landmarks as criteria for dissection. Cerebral cortex comprised all regions dorsolateral to the olfactory tract, excluding the hippocampus, and was dissected from each hemisphere by peeling it away from the striatum. After dissection, cerebral cortex was homogenized (1:10, weight/volume; w/v) in a glass potter in a Tris-HCl buffer (10 mM, pH 7.4) and centrifuged at 2000*g* for 10 min. Aliquots of the supernatants were stored at −20 °C until utilization.

2.8.2. Na^+,K^+-ATPase activity

Na^+,K^+-ATPase activity was measured as previously described (Wyse et al., 2000) with minor modifications (Carvalho et al., 2012). Briefly, the assay medium consisted of 30 mM Tris–HCl buffer (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$ and 100 μg of protein in the presence or absence of 1 mM ouabain, in a final volume of 350 μL. The reaction was started by the addition of adenosine triphosphate (ATP) to a final concentration of 3 mM. After 30 min at 37 °C, the reaction was stopped by the addition of 70 μL of 50% (w/v) trichloroacetic acid (TCA). Saturating substrate concentrations were used, and reaction was linear with protein and time. Appropriate controls were included in the assays for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate (Pi) released was quantified colorimetrically, as previously described (Fiske & Subbarow, 1925), using NaH_2PO_4 as reference standard. Specific Na^+,K^+-ATPase activity was calculated by subtracting the ouabain-insensitive activity from the overall activity (in the absence of ouabain) and expressed in nmol of Pi/min/mg of protein.

2.8.3. Ca^+,K^+-ATPase activity

Ca^{2+}-ATPase activity was measured as previously described (Rohn, Hinds, & Vincenzi, 1993) with minor modifications (Trevisan et al., 2009). Briefly, the assay medium consisted of 30 mM Tris–HCl buffer (pH 7.4), 0.1 mM EGTA, 6 mM $MgCl_2$ and 200 μg of protein in the presence or absence of 1 mM $CaCl_2$, in a final volume of 200 μL. The reaction was started by the addition of ATP to a final concentration of 3 mM. After 60 min at 37 °C, the reaction was stopped by the addition of 70 μL of 50% (w/v) TCA. Saturating substrate concentrations were used, and reaction was linear with protein and time. Appropriate controls were included in the assays for non-enzymatic hydrolysis of ATP. The amount of Pi released was quantified colorimetrically, as previously described (Fiske & Subbarow, 1925), using NaH_2PO_4 as reference standard. Specific Ca^{2+}-ATPase activity was calculated by subtracting the difference between the activity in the presence and absence of calcium in the assay medium and expressed in nmol of Pi/min/mg of protein.

2.8.4. Thiobarbituric acid reactive substances

As an index of lipid peroxidation, we used TBARS formation during an acid-heating reaction as previously described (Ohkawa, Ohishi, & Yagi, 1979) with some modifications. Briefly, 200 μL of homogenized tissue supernatant (1:10 w/v) samples were mixed with 500 μL of 2.5 M acetic acid pH 3.4, 500 μL of 0.8% thiobarituric acid, 200 μL of 8.1% sodium dodecyl sulfate (SDS) and 100 μL of distilled water. This mixture was then heated in a boiling water bath for 120 min. A malondialdehyde (MDA) solution was used as reference standard. TBARS were determined by the absorbance at 532 nm and were expressed as malondialdehyde equivalents (nmol MDA/mg of protein).

2.8.5. Nitrites and nitrates (NOx)

In consequence of its very short half-life, determination of $NO^{\cdot}$ itself is very difficult. So, to estimate the level of $NO^{\cdot}$, its end products nitrite/nitrate (NOx) are often measured. For NOx determina-

tion, an aliquot (200 uL) was homogenized (1:1) in 200 mM Zn_2SO_4 and acetonitrile. After, the homogenate was centrifuged at 16,000g for 30 min at 4 °C and supernatant was separated for analysis of NOx content as previously described (Miranda, Espey, & Wink, 2001). A nitrite solution was used as reference standard. NOx were determined by the absorbance at 570 nm and were expressed as μmol/g of tissue.

2.8.6. Non-protein thiols (NPSH)

Tissue non-protein thiols were determined as previously described (Ellman, 1959). Briefly, an aliquot of the homogenized tissue supernatant (1:10 w/v) was diluted (1:1) with 10% TCA, vortexed, and centrifuged at 2000g for 10 min. Subsequently, the supernatant was reacted with 250 μM DTNB in a final volume of 2 mL and the absorbance was read at 412 nm. A cysteine solution was used as reference standard. NPSH were expressed as μmol SH/g of tissue.

2.8.7. Superoxide dismutase (SOD) activity

This method is based on reaction autoxidation adrenaline to adenochrome. The intermediate in this reaction is superoxide, which is scavenged by SOD. Results were expressed as U SOD/mg of protein. One SOD unit was defined as the enzyme amount to cause 50% inhibition of adrenaline autoxidation (Misra & Fridovich, 1972).

2.9. Quantification of proteins

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

2.10. Statistical analysis

Data were analyzed by a one- or two-way ANOVA. *Post hoc* analyses were carried out by the Dunnett's Multiple Comparison Test. TBARS and NPSH levels were log-transformed to achieve normal distribution of data. A probability of $P < 0.05$ was considered significant. All data are expressed as mean ± Standard Error of the Mean (SEM).

3. Results

3.1. Characterization of C-LNC

C-LNC were successfully obtained by interfacial deposition of polymer method. The physicochemical characteristics of the nanocapsule suspensions are shown in Table 1. Formulation presented drug content close to its theorical value (0.49 mg mL^{-1}) and slight acid pH. As expected for lipophilic substances, the encapsulation efficiency was high, close to 100%. Analysis by PCS showed that the nanocapsule suspension have nanometric mean size (202 nm) and a controlled size distribution (PDI value of 0.11). The zeta potential values were negative and close to neutrality (−7.34). These values are frequent to lipid-core nanocapsules coated with polysorbate 80 and sufficient to guarantee the stability of the formulation (da Silva, Contri, Jornada, Pohlmann, & Guterres, 2012; Ourique et al., 2010).

Table 1
Physicochemical characteristics of C-LNC.

Drug content (mg mL^{-1})	EE (%)	pH	Particle size (nm)	PDI	Zeta potential (mV)
0.49 ± 0.02	100 ± 0.0	5.81 ± 0.03	202 ± 5.51	0.11 ± 0.01	−7.34 ± 0.14

Data are means ± Standard Error of the Mean (SEM). $n = 3$. EE, Encapsulation efficiency, PDI, Polydispersity index.

3.2. Behavioral study

3.2.1. Object recognition memory task

One-way ANOVA showed that during the training trial the groups exhibited similar time exploring each of the two identical objects, represented by the recognition index (Table 2, see *F* and *P* values within the table). The different doses of curcumin *per se* (12.5, 25, 50 mg/kg free and 4 mg/kg nanoencapsulated) exhibited similar profile (Table 3, see *F* and *P* values within the table). Two-way ANOVA did not demonstrate any significant control or smoke versus curcumin interaction on the training trials [$F(7,40) = 0.31$, $P > 0.05$].

Fig. 1 shows the effect of curcumin free (12.5, 25 and 50 mg/kg) and nanoencapsulated (4 mg/kg) along with smoke on the test recognition index. Statistical analysis (one-way ANOVA) revealed significant difference among the groups in the test recognition index [Fig. 1, $F(5,24) = 8.68$, $P < 0.001$]. *Post hoc* analysis showed that cigarette smoke-exposure decreases the recognition index ($P < 0.001$). The recognition index decreased from 0.85 ± 0.04 in the control group to 0.35 ± 0.04 in the cigarette smoke-exposed group. Non-encapsulated curcumin at the dose of 25 mg/kg partially prevented (0.55 ± 0.04) the decreased recognition index induced by the cigarette smoke-exposure ($P < 0.05$ compared with control group). However, non-encapsulated curcumin (50 mg/kg) and C-LNC (4 mg/kg) prevented the decrease in the recognition index induced by cigarette smoke-exposure, the values being 0.72 ± 0.05 and 0.74 ± 0.04, respectively (Fig. 1). Curcumin alone (12.5, 25, 50 mg/kg free and 4 mg/kg nanoencapsulated) did not show a significant effect compared with control (Table 3, see *F* and *P* values within the table). Two-way ANOVA showed a significant control or smoke versus curcumin 12.5, 25, 50 mg/kg free and 4 mg/kg nanoencapsulated) interaction [$F(7,40) = 3.93$, $P < 0.01$].

3.2.2. Open field test

Because locomotor and exploratory disparities in the testing session may account for differences in recognition index, experiments were performed to assess whether smoking or curcumin affected locomotor and/or exploratory abilities of the animals. Statistical analysis (one-way ANOVA) showed that neither cigarette smoke-exposure nor pharmacological treatment altered the number of crossing or rearing responses in a subsequent open-field test session (Table 2, see *F* and *P* values within the table). The different doses of curcumin *per se* (12.5, 25, 50 mg/kg free and 4 mg/kg nanoencapsulated) exhibited the same profile on crossing and rearing responses (Table 3, see *F* and *P* values within the table).

Table 2
Effect of oral administration of curcumin free (12.5, 25 and 50 mg/kg) and nanoencapsulated (4 mg/kg) along with smoke on the training trial (sample phase) recognition index and on behavior of rats (number of crossing and rearing responses) in the open-field immediately after the object recognition testing session.

Group	Recognition index (training trial)	Crossing	Rearing
Control	0.57 ± 0.06	38.00 ± 4.30	28.40 ± 2.29
Smoke	0.52 ± 0.14	24.80 ± 8.88	17.00 ± 6.77
Smoke-Cur 12.5	0.52 ± 0.05	33.00 ± 5.07	21.40 ± 4.11
Smoke-Cur 25	0.51 ± 0.08	44.00 ± 5.44	30.40 ± 3.67
Smoke-Cur 50	0.50 ± 0.04	42.00 ± 3.51	24.60 ± 4.19
Smoke-C-LNC	0.53 ± 0.06	39.80 ± 4.26	27.40 ± 2.77
Statistical analysis	$F(5,24) = 0.08$ $P > 0.05$	$F(5,24) = 1.62$ $P > 0.05$	$F(5,24) = 1.38$ $P > 0.05$

One-way ANOVA – Dunnett's Multiple Comparison Test. Data are means ± Standard Error of the Mean (SEM) for 5 animals in each group.

Table 3
Effect of oral administration of curcumin free (12.5, 25 and 50 mg/kg) and nanoencapsulated (4 mg/kg) *per* se on the training and test recognition indexes, on crossing and rearing responses in the open field immediately after the object recognition test session, on the activities of Na^+,K^+-ATPase and Ca^{2+}-ATPase, and on parameters of oxidative stress (TBARS, NOx, NPSH and SOD).

Group	Training	Test	Crossing	Rearing	Na^+,K^+-ATPase
Control	0.57 ± 0.06	0.85 ± 0.04	38.00 ± 4.30	1.45 ± 0.04	140.44 ± 16.11
Cur 12.5	0.41 ± 0.09	0.56 ± 0.09	40.00 ± 9.41	1.45 ± 0.16	127.56 ± 12.81
Cur 25	0.50 ± 0.07	0.62 ± 0.09	35.40 ± 2.91	1.42 ± 0.05	130.20 ± 11.85
Cur 50	0.51 ± 0.06	0.73 ± 0.08	37.80 ± 4.99	1.36 ± 0.03	125.89 ± 21.47
C-LNC	0.56 ± 0.04	0.79 ± 0.04	39.60 ± 4.37	1.31 ± 0.10	113.80 ± 11.56
Statistical analysis	$F(4,20) = 2.07$	$F(4,20) = 2.78$	$F(4,20) = 0.10$	$F(4,20) = 0.44$	$F(4,20) = 0.39$
	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$
	Ca^{2+}-ATPase	TBARS	NOx	NPSH	SOD
Control	40.03 ± 3.08	0.85 ± 0.02	64.98 ± 8.97	1.13 ± 0.04	20.75 ± 0.51
Cur 12.5	35.03 ± 3.48	0.87 ± 0.05	75.74 ± 14.6	1.08 ± 0.02	18.08 ± 1.06
Cur 25	30.79 ± 2.35	0.85 ± 0.02	61.68 ± 6.87	1.09 ± 0.04	16.64 ± 1.59
Cur 50	31.98 ± 3.04	0.77 ± 0.01	66.94 ± 11.8	1.12 ± 0.06	20.09 ± 1.35
C-LNC	36.20 ± 1.58	0.77 ± 0.06	58.12 ± 5.76	1.05 ± 0.01	20.44 ± 0.92
Statistical analysis	$F(4,20) = 0.55$	$F(4,20) = 0.11$	$F(4,20) = 0.78$	$F(4,20) = 0.61$	$F(4,20) = 0.08$
	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$

One-way ANOVA – Dunnett's Multiple Comparison Test. Data are means ± Standard Error of the Mean (SEM) for 5 animals in each group.

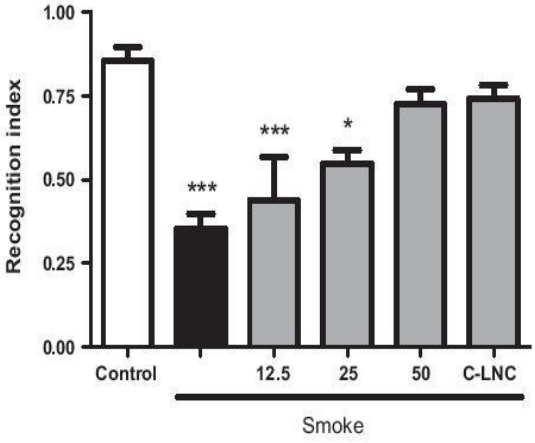

Fig. 1. Recognition index on test trial (memory test) in object recognition memory task of cigarette smoke-exposed rats and those treated with curcumin free (12.5, 25 and 50 mg/kg) and nanoencapsulated (4 mg/kg) along with smoke. One-way ANOVA – Dunnett's Multiple Comparison Test. Data are means ± Standard Error of the Mean (SEM) for 5 animals in each group. $^{*}(P < 0.05)$ and $^{***}(P < 0.001)$ compared with control.

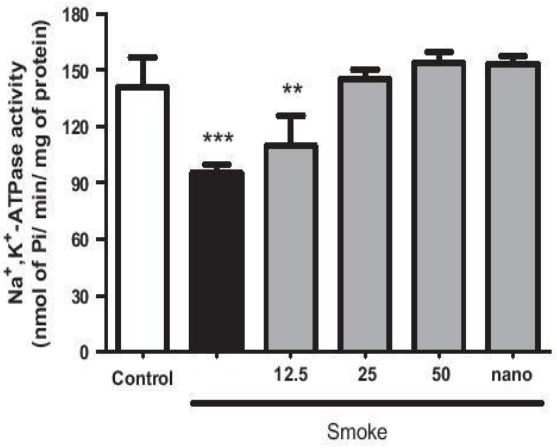

Fig. 2. Na^+,K^+-ATPase activity in cerebral cortex homogenates of cigarette smoke-exposed rats and those treated with curcumin free (12.5, 25 and 50 mg/kg) and nanoencapsulated (4 mg/kg) along with smoke. One-way ANOVA - Dunnett's Multiple Comparison Test. Data are means ± Standard Error of the Mean (SEM) for 5 animals in each group. $^{**}(P < 0.01)$ and $^{***}(P < 0.001)$ compared with control.

Two-way ANOVA did not demonstrate any significant control or smoke versus curcumin interaction on crossing [$F(7,40) = 1.17$, $P > 0.05$] or rearing [$F(7,40) = 1.68$, $P > 0.05$] responses.

3.3. Na^+,K^+-ATPase activity

Fig. 2 shows the effect of curcumin free (12.5, 25 and 50 mg/kg) and nanoencapsulated (4 mg/kg) along with smoke on Na^+,K^+-ATPase activity of cerebral cortex. Statistical analysis (one-way ANOVA) indicated significant difference among the groups [$F(5,24) = 10.04$, $P < 0.001$]. *Post hoc* analysis showed that cigarette smoke-exposure caused a significant decrease compared to the control group ($P < 0.001$). The cerebral cortex Na^+,K^+-ATPase activity decreased from 140.44 ± 16.11 nmol of Pi/min/mg of protein in the control group to 94.95 ± 4.65 nmol of Pi/min/mg of protein in the cigarette smoke-exposed group. Non-encapsulated curcumin administered along with cigarette smoke at the dose of 12.5 mg/kg partially prevented (109.26 ± 16.12 nmol of Pi/min/mg of protein) the decreased cerebral cortex Na^+,K^+-ATPase activity observed in the cigarette smoke-exposed group ($P < 0.01$ compared with control group). Non-encapsulated curcumin at the doses of 25 and 50 mg/kg and C-LNC at the dose of 4 mg/kg administered along with cigarette smoke-exposure prevented the decrease in cerebral cortex Na^+,K^+-ATPase activity, the values being 144 ± 4.47, 153.62 ± 5.74 and 153 ± 3.71 nmol of Pi/min/mg of protein, respectively (Fig. 2). The different doses of curcumin *per se* (12.5, 25, 50 mg/kg free and 4 mg/kg nanoencapsulated) did not show a significant effect compared with control (Table 3, see *F* and *P* values within the table). Two-way ANOVA indicated a significant control or smoke versus curcumin (12.5, 25, 50 mg/kg free and 4 mg/kg nanoencapsulated) interaction [$F(7,40) = 3.74$, $P < 0.05$].

3.4. Ca^+-ATPase activity

Fig. 3 shows the effect of curcumin free (12.5, 25 and 50 mg/kg) and nanoencapsulated (4 mg/kg) along with smoke on Ca^{2+}-ATPase activity of cerebral cortex. There was significant difference in the cerebral cortex Ca^{2+}-ATPase activity [$F(5,24) = 5.61$, $P < 0.01$]. *Post hoc* analysis revealed that cigarette smoke-exposure caused a significant decrease compared to the control group ($P < 0.01$). The cerebral cortex Ca^{2+}-ATPase activity decreased from

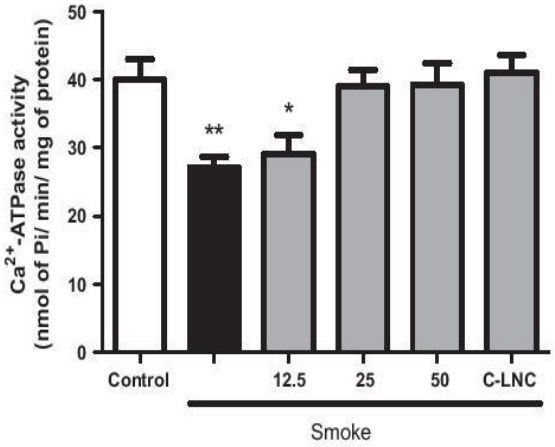

Fig. 3. Ca^{+}-ATPase activity in cebebral cortex homogenates of cigarette smoke-exposed rats and those treated with curcumin free (12.5, 25 and 50 mg/kg) and nanoencapsulated (4 mg/kg) along with smoke. One-way ANOVA – Dunnett's Multiple Comparison Test. Data are means ± Standard Error of the Mean (SEM) for 5 animals in each group. $^{*}(P < 0.05)$ and $^{**}(P < 0.01)$ compared with control.

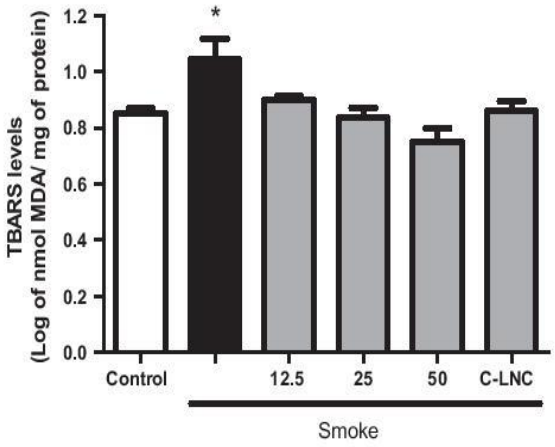

Fig. 4. Thiobarbituric acid reactive substances (TBARS) levels in cerebral cortex homogenates of cigarette smoke-exposed rats and those treated with curcumin free (12.5, 25 and 50 mg/kg) and nanoencapsulated (4 mg/kg) along with smoke. One-way ANOVA – Dunnett's Multiple Comparison Test. Data are means ± Standard Error of the Mean (SEM) for 5 animals in each group. $^{*}(P < 0.05)$ compared with control.

40.03 ± 3.08 nmol of Pi/min/mg of protein in the control group to 27.10 ± 1.51 nmol of Pi/min/mg of protein in the cigarette smoke-exposed group. Non-encapsulated curcumin administered along with cigarette smoke at the dose of 12.5 mg/kg partially prevented (29.16 ± 2.79 nmol of Pi/min/mg of protein) the decreased cerebral cortex Ca^{2+}-ATPase activity observed in the cigarette smoke-exposed group ($P < 0.05$ compared with control group). Non-encapsulated curcumin at the doses of 25 and 50 mg/kg and C-LNC at the dose of 4 mg/kg administered along with cigarette smoke-exposure prevented the decrease in cerebral cortex Ca^{2+}-ATPase activity, the values being 39.12 ± 2.10, 39.33 ± 3.02 and 41.10 ± 2.16 nmol of Pi/min/mg of protein, respectively (Fig. 3). The different doses of curcumin *per se* (12.5, 25, 50 mg/kg free and 4 mg/kg nanoencapsulated) did not show a significant effect compared with control (Table 3, see *F* and *P* values within the table). Two-way ANOVA indicated a significant control or smoke versus curcumin (12.5, 25, 50 mg/kg free and 4 mg/kg nanoencapsulated) interaction [$F(7,40) = 4.27$, $P < 0.01$].

3.5. TBARS

TBARS content was measured as an index of lipid peroxidation in the cerebral cortex (Fig. 4). One way-ANOVA showed a statistically significant difference among the groups [$F(5,24) = 5.56$, $P < 0.01$]. Subsequently *post hoc* analysis showed that cigarette smoke-exposure caused a significant increase of lipid peroxidation as indicated by the rise in TBARS levels as compared to the control group ($P < 0.05$). The cerebral cortex TBARS levels increased from 0.85 ± 0.02 log of nmol MDA/mg of protein in the control animals to 1.05 ± 0.07 log of nmol MDA/mg of protein in the cigarette smoke-exposed animals. When curcumin was administered along with cigarette smoke, curcumin produced a significant reduction of cerebral cortex TBARS as compared to cigarette smoke-exposed group ($P < 0.05$), the values being 0.89 ± 0.01, 0.83 ± 0.03, 0.74 ± 0.04 and 0.86 ± 0.03 log of nmol MDA/mg of protein, respectively, in the curcumin free 12.5, 25, 50 mg/kg and C-LNC 4 mg/kg co-administered animals. The different doses of curcumin *per se* (12.5, 25, 50 mg/kg free and 4 mg/kg nanoencapsulated) did not show a significant effect compared with control (Table 3, see *F* and *P* values within the table). Two-way ANOVA did not show any significant control or smoke versus curcumin (12.5, 25, 50 mg/kg free and 4 mg/kg nanoencapsulated) interaction [$F(7,40) = 2.50$, $P > 0.05$].

3.6. NOx

NOx was measured as an estimation of $NO^{\cdot}$ content (Fig. 5). There was difference in the cerebral cortex NOx content [$F(5,24) = 9.73$, $P < 0.001$]. *Post hoc* analysis revealed that cigarette smoke-exposure caused a significant increase compared to the control group ($P < 0.01$). The cerebral cortex NOx content increased from 64.98 ± 8.97 μmol/g of tissue in the control group to 128.88 ± 21.29 μmol/g of tissue in the cigarette smoke-exposed group. When curcumin was administered along with cigarette smoke, curcumin produced a significant reduction of cerebral cortex NOx as compared to cigarette smoke-exposed group, the values being 60.22 ± 6.19, 50.36 ± 8.39, 33.78 ± 6.16 and 30.88 ± 8.04 μmol/g of tissue, respectively, in the curcumin free 12.5, 25 and 50 mg/kg and C-LNC 4 mg/kg co-administered animals (Fig. 5). The different doses of curcumin *per se* (12.5, 25 and 50 mg/kg free and 4 mg/kg nanoencapsulated) did not show a significant effect compared with control (Table 3, see *F* and *P* values within the table). The two-way ANOVA indicated a significant control or smoke versus curcumin (12.5, 25 and 50 mg/kg free and 4 mg/kg nanoencapsulated) interaction [$F(7,40) = 6.34$, $P < 0.001$].

3.7. NPSH

Fig. 6 shows the effect of curcumin free (12.5, 25 and 50 mg/kg) and nanoencapsulated (4 mg/kg) along with smoke on the cerebral cortex NPSH level. One-way ANOVA showed a statistically significant difference among the groups [$F(5,24) = 4.32$, $P < 0.01$]. Subsequently *post hoc* analysis showed that cigarette smoke-exposure caused a significant decrease of NPSH compared to the control group ($P < 0.01$). The cerebral cortex NPSH level decreased from 1.13 ± 0.04 log of μmol SH/g of tissue in the control animals to 0.88 ± 0.07 log of μmol SH/g of tissue in the cigarette smoke-exposed animals. When curcumin was administered along with cigarette smoke, curcumin prevented the reduction of cerebral cortex NPSH as compared to cigarette smoke-exposed group, the values being 1.11 ± 0.03, 1.10 ± 0.04, 1.08 ± 0.02 and 1.05 ± 0.04 log of μmol SH/g of tissue, respectively, in the curcumin free 12.5, 25 and 50 mg/kg and C-LNC 4 mg/kg co-administered animals (Fig. 6). The different doses of curcumin *per se* (12.5, 25 and 50 mg/kg free and 4 mg/kg nanoencapsulated) did not show a significant effect compared with control (Table 3, see *F* and *P* values within the Table). Two-way ANOVA showed a significant control

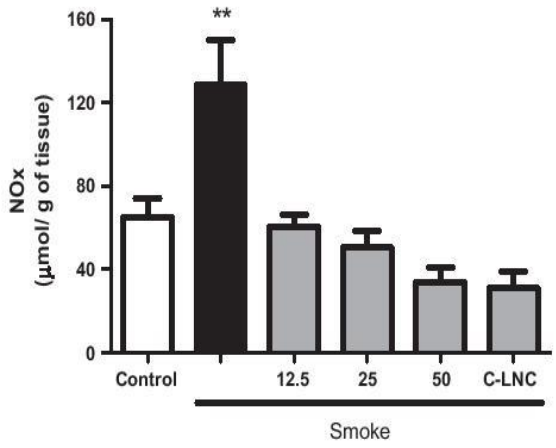

Fig. 5. Nitrites and nitrates (NOx) levels in cerebral cortex homogenates of cigarette smoke-exposed rats and those treated with curcumin free (12.5, 25 and 50 mg/kg) and nanoencapsulated (4 mg/kg) along with smoke. One-way ANOVA – Dunnett's Multiple Comparison Test. Data are means ± Standard Error of the Mean (SEM) for 5 animals in each group. **($P < 0.01$) compared with control.

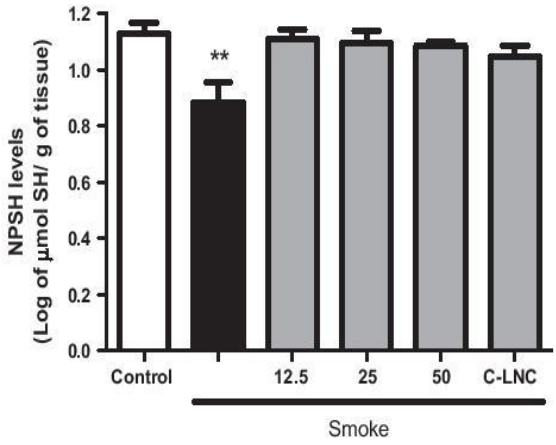

Fig. 6. Non-protein sulfhydryl groups (NPSH) levels in cerebral cortex homogenates of cigarette smoke-exposed rats and those treated with curcumin free (12.5, 25 and 50 mg/kg) and nanoencapsulated (4 mg/kg) along with smoke. One-way ANOVA – Dunnett's Multiple Comparison Test. Data are means ± Standard Error of the Mean (SEM) for 5 animals in each group. **($P < 0.01$) compared with control.

or smoke versus curcumin (12.5, 25 and 50 mg/kg free and 4 mg/kg nanoencapsulated) interaction [$F(7,40) = 3.60$, $P < 0.05$].

3.8. SOD activity

Fig. 7 shows the effect of curcumin free (12.5, 25 and 50 mg/kg) and nanoencapsulated (4 mg/kg) along with smoke in the cerebral cortex SOD activity. One-way ANOVA indicated significant difference among the groups in the cerebral cortex SOD activity [$F(5,24) = 3.70$, $P < 0.05$]. *Post hoc* analysis showed that cigarette smoke-exposure caused a significant decrease compared to the control group ($P < 0.01$). The cerebral cortex SOD activity decreased from 20.75 ± 0.51 U SOD/mg of protein in the control group to 14.53 ± 2.02 U SOD/mg of protein in the cigarette smoke-exposed group. When curcumin was administered along with cigarette smoke, curcumin prevented SOD decrease as compared to cigarette smoke-exposed group, the values being 20.28 ± 1.24, 19.50 ± 1.05, 18.32 ± 0.77, 17.75 ± 0.86 U SOD/mg of protein, respectively, in the curcumin free 12.5, 25, 50 mg/kg and C-LNC 4 mg/kg co-administered animals. The different doses of curcumin *per se* (12.5, 25, 50 mg/kg free and 4 mg/kg nanoencapsulated) did not

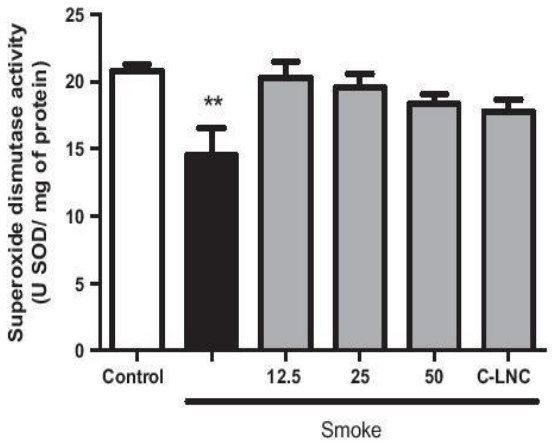

Fig. 7. Superoxide dismutase (SOD) activity in cerebral cortex homogenates of cigarette smoke-exposed rats and those treated with curcumin free (12.5, 25 and 50 mg/kg) and nanoencapsulated (4 mg/kg) along with smoke. One-way ANOVA – Dunnett's Multiple Comparison Test. Data are means ± Standard Error of the Mean (SEM) for 5 animals in each group. **($P < 0.01$) compared with control.

show a significant effect compared with control (Table 3, see *F* and *P* values within the table). Two-way ANOVA revealed a significant control or smoke versus curcumin (12.5, 25 and 50 mg/kg free and 4 mg/kg nanoencapsulated) interaction [$F(7,40) = 4.77$, $P < 0.01$].

4. Discussion

In this study we proposed to measure the activities of Na^+,K^+-ATPase and Ca^{2+}-ATPase and to investigate oxidative stress parameters in cerebral cortex of rats passively exposed to cigarette smoke in an attempt to understand the possible mechanisms involved in curcumin protection against cigarette smoke-induced cognitive impairment. As an index of cigarette smoke exposure, the serum level of cotinine, a metabolite of nicotine, was quantified in the control and the cigarette smoke-exposed group. Average serum cotinine level at 1 h after smoke exposure was around 280 ng/mL (data not showed) which is comparable to average cotinine level reported for smokers (around 300 ng/mL) (Ypsilantis, Politou, Anagnostopoulos, Kortsaris, & Simopoulos, 2012). Furthermore, in spite of the poor bioavailability of curcumin, we investigated the potential of C-LNC in this prevention.

Many research groups have been investigating the neurocognitive consequences of cigarette smoke-exposure and have verified diverse effects such as deficits in accuracy of working memory (Jacobsen et al., 2005), poorer performance for audio-verbal learning and/or memory (Fried, Watkinson, & Gray, 2006), information processing speed (Spilich, June, & Renner, 1992), general intelligence (Weiser, Zarka, Werbeloff, Kravitz, & Lubin, 2010), cognitive flexibility (Kalmijn, van Boxtel, Verschuren, Jolles, & Launer, 2002), and general intellectual abilities (Deary et al., 2003). Moreover, neurobiological effects of cigarette smoking such as abnormal increase of global brain atrophy (Kubota et al., 1987) characterized by increased ventricular and sulcal volumes (Longstreth et al., 2000), smaller volume in left dorsal cingulated cortex and lower tissue density in the cerebellum (Brody et al., 2004) have also been presented. However, the mechanisms involved in the promotion of these neurocognitive and neurobiological abnormalities associated with cigarette smoke are still unclear (Durazzo, Meyerhoff, & Nixon, 2010). In this study, the evaluation of LTM in rats passively exposed to cigarette smoke by the object recognition memory task revealed impaired memory, as indicated by the low recognition index (below 0.5) corroborating with the aforementioned literature and supporting our recent findings (Jaques et al., 2012).

As previously demonstrated in a recent study conducted by our group, curcumin free had shown to be effective against cigarette smoke-induced cognitive impairment evaluated by the step down latencies in the inhibitory avoidance task (Jaques et al., 2012). In addition to the referred study, some other groups have shown the potential of curcumin free administered orally in a wide range of doses (3–300 mg/kg) and periods (2–6 weeks) to prevent the cognitive impairment caused by different types of agents such as phenytoin (Reeta et al., 2009), scopolamine (Ahmed & Gilani, 2009), $AlCl_3$ associated with D-galactose (Pan et al., 2008), and human immunodeficiency virus type 1 glycoprotein 120 V3 loop peptide (Tang et al., 2009). In this study, curcumin free prevented cigarette smoke-induced cognitive impairment, although the lowest dose was not effective as the higher doses (25 and 50 mg/kg). This study was the first designed to investigate the performance of a formulation containing nanoencapsulated curcumin on the effects of cigarette smoke exposure on memory and parameters involved in the homeostasis of CNS. Interestingly, C-LNC in a dose 4-fold lower than the effectless one (12.5 mg/kg) presented similar results compared to the highest dose (50 mg/kg) of non-encapsulated curcumin. This dose was more than 12-fold higher than the dose of nanoencapsulated curcumin, strongly suggesting that this formulation has the potential of increase the oral bioavailability of curcumin.

Several studies have been employing nanotechnology based carriers to increase the bioavailability of curcumin and found promising results in the research of human cancer therapy (Bisht et al., 2007; Nair, Thulasidasan, Deepa, Anto, & Kumar, 2012; Yallapu et al., 2010) and pathologies involving the CNS (Mathew et al., 2012; Ray et al., 2011). A recent study employing the same lipid-core nanocapsules used in this research showed a higher drug concentration in brain, liver and kidney tissues after daily intraperitoneal (i.p.) injection or oral gavage administration, than that observed for the free drug (Frozza et al., 2010). Furthermore, it was demonstrated that nanoencapsulation of curcumin improves its oral bioavailability by at least 9-fold compared to curcumin administered as a suspension with piperine, an absorption enhancer (Shaikh et al., 2009). In addition, a recently published study (Tsai, Chien, Lin, & Tsai, 2011) demonstrated that after intravenous (i.v.) administration of curcumin-loaded PLGA nanoparticles (C-NPs), the half-life ($t_{1/2}$) and the mean residence time (MRT) of curcumin in the brain tissue were significantly increased over conventional curcumin. Pharmacokinetics analysis in the different brain regions revealed that the $t_{1/2}$ and the MRT were increased in the hippocampus and cerebral cortex (Tsai et al., 2011), both regions of vital importance in the cognition and memory processes (Izquierdo & Medina, 1995).

After the evaluation of memory parameters, we aimed to elucidate some mechanisms involved in the memory impairment caused by the cigarette smoke-exposure and by what routes curcumin exerts its protective effect. With that purpose we assessed the activity of the membrane-bound ATPases Na^+,K^+-ATPase and Ca^{2+}-ATPase, and some parameters of oxidative stress.

The measurement of Na^+,K^+-ATPase and Ca^{2+}-ATPase activities in cerebral cortex of rats exposed to cigarette smoke revealed decreased activities for both enzymes which leads to increased Na^+ and Ca^{2+} and depletion of K^+ in the citosol (Anbarasi et al., 2005). This disturbance in the electrolyte balance alters the membrane potential and impulse propagation causing an excessive release and stimulation of receptors by excitatory neurotransmitters such as glutamate, acetylcholine (ACh), and adenosine triphosphate (ATP) and, as a consequence, there could be cellular damage and death (Xiao et al., 2002). The inhibition of Na^+,K^+-ATPase has been associated with neuronal death related to disrupted ion homeostasis involving both apoptotic and necrotic components (Xiao et al., 2002). Furthermore, a prior study (Yang & Liu, 2003) also demonstrated that cigarette smoke extract (CSE) inhibited mouse brain mitochondrial ATPase and increased Ca^{2+} in mitochondria, which was also associated with tissue injury and death.

Previous studies indicate that ROS and free radicals are involved in the inhibition of Ca^{2+}-ATPase (Rohn, Hinds, & Vincenzi, 1996) and Na^+,K^+-ATPase (Franzon et al., 2003; Rohn et al., 1996). Corroborating with these data, in this study, the group of animals exposed to cigarette smoke presented an increase in biomarkers of oxidative and nitrosative stress such as TBARS and NOx, which probably was involved in the inhibition of the membrane-bound enzymes and, consequently, would have contributed to the breakdown of ion homeostasis. Furthermore, the group of animals exposed to cigarette smoke had a depletion of non-enzymatic and enzymatic antioxidant defenses represented by NPSH content and SOD activity, respectively, which rendered brain tissue more susceptible to oxidative damage by radical species generated endogenously, by cellular metabolism, or exogenously, by cigarette smoke exposure. A decrease in enzyme-based free radical scavengers (e.g., SOD, catalase, glutathione reductase, glutathione peroxidase) and non-enzyme based radical scavengers (e.g., reduced glutathione, low molecular weight thiols, and vitamins A, C and E) in brain was already associated with cigarette smoke exposure (Anbarasi, Vani, Balakrishna, & Devi, 2006; Mendez-Alvarez, Soto-Otero, Sanchez-Sellero, & Lopez-Rivadulla Lamas, 1998).

Our findings demonstrate that administration of both curcumin free and nanoencapsulated was effective in preventing the decrease of Na^+,K^+-ATPase and Ca^{2+}-ATPase induced by cigarette smoke exposure, although the dose of 12.5 mg/kg had shown a partial prevention. Remarkably, in spite of its low dose (4 mg/kg), the C-LNC presented results similar to the higher doses, which we believe to be associated with an increased bioavailability. In regard the oxidative stress parameters evaluated, all the treatment doses of curcumin (both free and nanoencapsulated) were effective in preventing the cigarette smoke-induced changes in the antioxidant defense systems, thus contributing to prevent the increase of markers of cellular damage mediated by ROS and RNS. All these effects provided by the treatment with curcumin may be attributed to its well known systemic antioxidant properties, especially in the brain tissue (Lim et al., 2001; Scapagnini et al., 2006), and its ability to improve membrane homeostasis (Sharma, Ying, & Gomez-Pinilla, 2010). We suggest that curcumin protection of membranes from ROS and RNS-mediated damage was of potential usefulness in the prevention of ion homeostasis breakdown and probably in the prevention and treatment of certain disease process involving the CNS.

5. Conclusions

In summary, the present study shows that cigarette smoke-exposure breaks the ion homeostasis which is related to excitotoxicity and, consequently, oxidative/nitrosative stress and neurocognitive impairment. In addition, the effects promoted by curcumin revealed that the maintenance of ion homeostasis and redox balance is part of its protective mechanism against smoke-induced cognitive impairment. Furthermore, these results strongly suggest that the use of lipid-core nanocapsules may be an alternative to improve this protection.

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DISCUSSÃO

A exposição à fumaça do cigarro é um fator de risco para o desenvolvimento de diversas doenças como a doença pulmonar obstrutiva crônica (DPOC), câncer de pulmão (SOPORI, 2002), déficits encefálicos (GALLINAT et al., 2006). De interesse específico para este estudo, a exposição à fumaça do cigarro é um dos fatores fortemente associados com doenças cardiovasculares como a doença arterial coronariana e a aterosclerose (CASTELLI et al., 1981; GLANTZ; PARMLEY, 1991; GUARINO et al., 2011; STEENLAND et al., 1996). O dano inferido ao tecido endotelial, em virtude da exposição ao cigarro, também está associado à ativação dos trombócitos ou plaquetas em consequência da ligação ao colágeno, à outras proteínas expostas da matriz extracelular e da subsequente liberação de mediadores solúveis armazenados em vesículas (SEMPLE et al., 2011). Diversos estudos indicam que a agregabilidade plaquetária é mais acentuada em individuos fumantes e que estes apresentam níveis elevados de diversas moléculas envolvidas no processo de agregação como, por exemplo, o ADP e o FAP, de especial interesse para o contexto da discussão deste estudo (DOTEVALL et al., 1987; FITZGERALD et al., 1983; FUSEGAWA; HANDA, 2000; IMAIZUMI, 1991; MIYAURA et al., 1992; PERNERSTORFER et al., 1998; THOME et al., 2009).

A partir da quantificação da atividade das enzimas E-NTPDase, E-5'-NT e E-ADA em plaquetas de ratos expostos de forma passiva à fumaça do cigarro observou-se um aumento na hidrólise dos nucleotídeos ATP e AMP, e uma redução na hidrólise do nucleotídeo ADP, o que permite a suposição de que há aumento do nível circulante de ADP na corrente sanguínea. Algumas das consequências bem conhecidas do aumento do nível de ADP no plasma sanguíneo são: aumento da agregação plaquetária, alterações conformacionais, aumento do conteúdo de Ca^{2+} citosólico e inibição da enzima adenilato ciclase (PARK; HOURANI, 1999). Desta forma, os resultados apresentados neste estudo, os quais remetem a um aumento nos níveis circulantes de ADP na corrente sanguínea e, consequentemente, um aumento da agregação plaquetária são consistentes com resultados previamente descritos na literatura (INOUE, 2004), os quais mostram que indivíduos fumantes possuem uma maior agregação espontânea do que indivíduos não fumantes. Um estudo desenvolvido pelo nosso grupo de pesquisa demonstra que ratos expostos de forma passiva à fumaça do cigarro possuem acentuada agregabilidade

plaquetária (THOME et al., 2009). Nesta pesquisa mencionada, estes achados foram associados às alterações observadas na atividade das ectonucleotidades, onde houve um aumento na hidrólise do nucleotídeo ATP e uma tendência à redução na hidrólise do nucleotídeo ADP, padrão semelhante ao observado neste estudo.

O aumento na hidrólise do nucleotídeo AMP, observado no grupo de ratos expostos de forma passiva à fumaça do cigarro, é associado a uma elevação nos níveis circulantes de adenosina (BECKER et al., 2010; SAUCEDO et al., 2010). Esta molécula possui efeitos anti-agregantes mediados por receptores metabotrópicos de adenosina, especificamente os receptores A2A e A2B (JOHNSTON-COX et al., 2011; YANG, D. et al., 2010), os quais regulam positivamente a produção de cAMP, um inibidor da agregação plaquetária (LINDEN et al., 2008; PAUL et al., 1990). O aumento nos níveis intracelulares de cAMP ativa a proteína kinase A (PKA), a qual reduz a liberação intracelular das reservas de Ca^{2+} (SIM et al., 2004), estabiliza a estrutura do citoesqueleto (YANG, D. et al., 2010) e, assim, previne a formação do trombo. Uma vez que o nucleosídeo adenosina é associado com a sinalização autócrina antiinflamatória via receptores do tipo P1 (JUNGER, 2011), se especula que um possível aumento no nível circulante desta molécula possa representar um mecanismo fisiológico dinâmico para regular a resposta vascular ao dano endotelial e evitar os efeitos exacerbados da exposição à fumaça do cigarro como, por exemplo, a agregação plaquetária e o desenvolvimento da aterosclerose.

Atualmente, além das funções clássicas atribuídas às plaquetas como a hemostase e a trombogênese, existe uma gradual compreensão de que elas desempenham uma importante função na modulação da resposta imune inata e adaptativa (ELZEY et al., 2003; HENN et al., 1998; KISSEL et al., 2006; SEMPLE et al., 2011; SPRAGUE et al., 2008; VON HUNDELSHAUSEN; WEBER, 2007). Além disso, estudos demonstram que plaquetas ativadas estão envolvidas na etiologia de diversas doenças que acometem o SNC (CIABATTONI et al., 2007; HORSTMAN et al., 2010; PUTNAM, 1935; SHEREMATA et al., 2008). Dentre as possibilidades que justificam esta relação pode-se citar a sua capacidade, juntamente com os linfócitos, de aumentar a permeabilidade da BHE (HORSTMAN et al., 2010). Especificamente, o FAP, um dos principais agonistas liberados pelas plaquetas e um potente mediador pró-inflamatório, seria secretado pela cooperação entre plaquetas e leucócitos e facilitaria o rompimento da BHE, uma vez que uma das funções conhecidas e mais proeminentes do FAP é a ruptura das junções endoteliais

(ADAMSON et al., 2008; BRKOVIC; SIROIS, 2007; JIANG et al., 2008; KNEZEVIC et al., 2009). Uma vez que quando há liberação de ADP a partir dos grânulos plaquetários, também há liberação do FAP, supõe-se que concomitante ao aumento do nível circulante de ADP no grupo de animais expostos de forma passiva à fumaça do cigarro também ocorra um aumento do nível circulante do FAP. As observações de Imaizumi (1991) corroboram com esta suposição, uma vez que demonstra, pela primeira vez, que a exposição à fumaça do cigarro está associada com um aumento na liberação do FAP (IMAIZUMI, 1991). Em conformidade a esta presunção, investigamos o envolvimento dos efeitos oriundos da exposição passiva à fumaça do cigarro com alterações neuroquímicas e neurocognitivas.

Muitos estudos demonstram que a exposição crônica à fumaça do cigarro está envolvida com o declínio na precisão da memória de trabalho (JACOBSEN et al., 2005), na memória áudio-verbal (FRIED et al., 2006), na velocidade de processamento de informações (SPILICH et al., 1992), na inteligência geral (WEISER et al., 2010), na velocidade psicomotora e na flexibilidade cognitiva (KALMIJN et al., 2002) e em habilidades intelectuais gerais (DEARY et al., 2003). Além disso, têm sido reportados efeitos neurobiológicos como a atrofia encefálica (KUBOTA et al., 1987) caracterizada por aumento no volume dos sulcos e ventrículos (LONGSTRETH et al., 2000), redução no volume do córtex cingulado dorsal esquerdo e menor densidade tecidual no cerebelo (BRODY et al., 2004). Contudo, os mecanismos envolvidos na promoção destes efeitos neurocognitivos e neurobiológicos associados com a exposição à fumaça do cigarro ainda são pouco claros (DURAZZO et al., 2010). Os efeitos neurocognitivos são sugestivos de alterações no córtex cerebral (DE GROOT et al., 2000; YLIKOSKI et al., 1993), que pode ser resultante de lesões em pequenos vasos, uma vez que já foram descritas alterações vasculares e no fluxo sanguíneo cerebral de fumantes crônicos (ROGERS et al., 1984) e estas alterações estão diretamente relacionadas com o desempenho cognitivo (MEYER et al., 1988).

Neste estudo foi avaliada a memória de longa duração (LTM, *long term memory*) através de dois testes comportamentais. Primeiramente, foi realizado o teste da esquiva inibitória, um modelo comportamental clássico com um forte componente aversivo utilizado para a avaliação do aprendizado e memória em ratos e camundongos (CAHILL et al., 1986). Neste estudo, observou-se uma redução na latência de descida da plataforma no grupo de ratos expostos de forma passiva à

fumaça do cigarro no teste da esquiva inibitória, o que sugere um comprometimento nos processos de aprendizado e memória nestes animais. Estes resultados estão em concordância com outros estudos que também descrevem comprometimento cognitivo em ratos expostos à fumaça do cigarro (CZUBAK et al., 2008; NOWAKOWSKA et al., 2006). De forma complementar, foi realizado o teste do reconhecimento de objetos, um teste que mensura a preferência dos animais por um objeto desconhecido (BALDERAS et al., 2008). A avaliação da memória através do teste do reconhecimento de objetos revelou uma redução na preferência por objetos desconhecidos no grupo de ratos expostos de forma passiva à fumaça do cigarro, indicada pelo declínio no índice de reconhecimento. Este resultado sugere um comprometimento nos processos de aprendizado e memória, o que corrobora com os resultados obtidos no princípio deste estudo.

Uma das principais preocupações em testes cognitivos que envolvem fortes componentes aversivos como o choque, e particularmente estudos que investigam o efeito de drogas administradas antes da realização do teste, é se o tratamento farmacológico afeta a atividade locomotora e exploratória. Com o intuito de excluir esta possibilidade, investigou-se através do teste do campo aberto as atividades locomotora e exploratória imediatamente após a seção de teste da esquiva inibitória e do reconhecimento de objetos para identificar se a presença de alguma inabilidade motora poderia ter influenciado a performace dos animais nos testes realizados. Pode-se perceber que nenhum dos grupos apresentou diferença no número de cruzamentos (atividade locomotora) e respostas de levantar (atividade exploratória) em relação ao grupo controle. Desta forma, estes resultados permitem a exclusão da possibilidade de que a atividade locomotora possa ter exercido algum tipo de influência nos resultados obtidos.

Após a avaliação dos parâmetros cognitivos foram investigados alguns dos possíveis mecanismos envolvidos no comprometimento observado nos testes de aprendizado e memória causado pela exposição passiva à fumaça do cigarro. Com este propósito foi avaliado: a atividade de enzimas envolvidas no mantenimento de gradientes iônicos transmembrana; o balanço redox através da avaliação de marcadores de dano oxidativo e nitrosativo, e avaliação das defesas antioxidantes; e outras enzimas que desempenham funções vitais para o SNC.

Os impulsos nervosos que percorrem os neurônios e estão envolvidos nos processos cognitivos possuem natureza eletroquímica, pois requerem a formação de

um gradiente iônico através da membrana plasmática neuronal, ou seja, a capacidade de manter um desequilíbrio nas concentrações intra e extracelulares de íons como Na^+, K^+ e Ca^{2+} (ERECINSKA; SILVER, 1994). A medida das atividades das enzimas Na^+,K^+-ATPase e Ca^{2+}-ATPase em córtex cerebral de ratos expostos de forma passiva à fumaça do cigarro revelou uma redução na atividade de ambas enzimas, o que possivelmente leva ao aumento nas concentrações intracelulares dos íons Na^+ e Ca^{2+} e a depleção de K^+. O desequilíbrio no balanço eletrolítico altera o potencial transmembrana e a propagação dos impulsos nervosos causando um estímulo excessivo de receptores neuronais em virtude da liberação acentuada de neurotransmissores excitatórios como o glutamato, a ACh e o ATP. Em consequência destes eventos, pode haver dano e morte celular (XIAO et al., 2002). A inibição da atividade da Na^+,K^+-ATPase está associada com morte neuronal devido à quebra da homeostase iônica envolvendo componentes apoptóticos e necróticos (XIAO et al., 2002). Além disso, já foi demonstrado (YANG, Y. M.; LIU, 2003) que o extrato da fumaça do cigarro inibe a ATPase mitocondrial encefálica de camundongos e aumenta a concentração de Ca^{2+} nesta organela, o que também associa-se com dano tecidual e morte celular.

Estudos indicam que as EROs e radicais livres estão envolvidos na inibição das enzimas Ca^{2+}-ATPase (ROHN et al., 1996) e Na^+,K^+-ATPase (FRANZON et al., 2003; ROHN et al., 1996). Em conformidade com estes achados, neste estudo o grupo de animais expostos de forma passiva à fumaça do cigarro apresentou um aumento em biomarcadores de estresse oxidativo e nitrosativo como o TBARS e NOx, respectivamente, o que provavelmente estaria associado à inibição observada nas atividades das ATPases e, consequentemente teria contribuído com o desarranjo da homeostase iônica. Além disso, o grupo de animais expostos de forma passiva à fumaça do cigarro apresentaram uma depleção nas defesas antioxidantes não enzimáticas e enzimáticas representadas pelo conteúdo de NPSH e pela atividade da enzima SOD, respectivamente. O efeito observado nas defesas antioxidantes tornam o tecido encefálico mais vulnerável ao dano oxidativo causado por espécies reativas geradas de forma endógena pelo metabolismo celular, ou exógenamente, pela exposição à fumaça do cigarro. Estes resultados corroboram com os achados de autores que descrevem que há uma associação entre a exposição à fumaça do cigarro e o declínio nas defesas antioxidantes enzimáticas (e.g., SOD, catalase, glutationa redutase, glutationa peroxidase) e sequestradores

não enzimáticos (e.g., redução nos níveis de glutationa, tióis de baixa massa molecular, e vitaminas A, C e E) no encéfalo (ANBARASI et al., 2006; MENDEZ-ALVAREZ et al., 1998).

Acredita-se que a ativação das plaquetas em indivíduos expostos à fumaça do cigarro também contribua com estes efeitos, pois em consequência da ativação plaquetária há liberação dos diversos fatores trombogênicos e pró-inflamatórios e a consequente redução na seletividade da BHE, como discutido previamente, o que acarreta em um aumento na migração de células inflamatórias para o tecido neural, uma das principais características de diversas desordens neuroimunes (MAZZONE et al., 2010). Neste contexto, concomitante com o processo neuroinflamatório, ocorre um aumento na produção de EROs e ERNs o que, por sua vez, contribui para a exacerbação dos danos teciduais e celulares.

Para uma melhor compreensão acerca dos mecanismos envolvidos nos efeitos cognitivos causados pela exposição passiva à fumaça do cigarro investigou-se a atividade da enzima AChE, a qual desempenha um papel fundamental em várias funções vitais para o SNC, especialmente o aprendizado e a memória (DAS et al., 2002; MESULAM et al., 2002; SATO et al., 2004; WINKLER et al., 1995). Estudos demonstram que alguns compostos presentes na fumaça do cigarro, como o $NO^{\bullet}$ e o cianeto, estão relacionados com um aumento na atividade da enzima AChE e prejuízos cognitivos (JEYARASASINGAM et al., 2000; OWASOYO; IRAMAIN, 1980). Desta forma, sugere-se que o aumento na atividade desta enzima possa ser uma das bases patológicas do comprometimento cognitivo causado pela exposição à fumaça do cigarro.

De fato, neste estudo, observamos um aumento na atividade da enzima AChE em cerebelo, córtex cerebral, hipocampo, estriado, hipotálamo e sangue total de ratos expostos de forma passiva à fumaça do cigarro. Os efeitos observados na atividade da AChE em sangue total nos diferentes grupos foi similar àqueles obtidos nas regiões encefálicas. É interessante mencionar que já foi descrito anteriormente que a AChE eritrocítica (predominante no sangue) tem demonstrado similares propriedades funcionais e estruturais àquelas da AChE encefálica e, desta forma, a atividade enzimática observada em sangue total poderia refletir o status do sítio sináptico (THIERMANN et al., 2005). Além da facilidade para a obtenção de amostra para a quantificação da atividade da enzima AChE eritrocítica, uma justificativa para esta alternativa é que foi proposto que ela pode apresentar níveis de atividade

alterados mesmo antes das colinesterases encefálicas (MATTSSON et al., 2001). Este padrão na atividade da enzima AChE já foi observado anteriormente em nosso grupo de pesquisa (SCHMATZ et al., 2009).

Assim como o sistema colinérgico, o sistema purinérgico tem uma participação chave em processos que envolvem o SNC como a neurotransmissão (BURNSTOCK, 1976; BURNSTOCK, 2007), a neuromodulação (BURNSTOCK, 2007) e a plasticidade sináptica (WIERASZKO; EHRLICH, 1994), além dos processos de tromborregulação, hemostase e função plaquetária (ZIMMERMANN, 1999), discutidos anteriormente. Desta forma, investigou-se a atividade de enzimas purinérgicas envolvidas na regulação dos níveis extracelulares dos nucleotídeos e do nucleosídeo de adenina que, por sua vez, são moléculas sinalizadoras. Observou-se um aumento na hidrólise de ATP pela enzima E-NTPDase e na hidrólise de AMP pela enzima E-5'-NT em sinaptossomas de córtex cerebral de ratos expostos de forma passiva à fumaça do cigarro. O ATP é uma potente molécula sinalizadora, a qual exerce seus efeitos fisiológicos de curta duração através da ligação com receptores ionotrópicos do tipo P2X, abundantemente expressos no SNC, o que desencadeia o influxo de íons Ca^{2+} levanto a ativação secundária de canais de cálcio dependentes de voltagem (VGCC, *voltage-gated calcium channels*). Estes receptores possuem uma função bem estabelecida na neurotransmissão, co-transmissão, neuromodulação, comunicação glial e efeitos tróficos (APOLLONI et al., 2009), o que demonstra claramente a importância das purinas no mantenimento da homeostase neural. O aumento observado na atividade da enzima E-5'-NT no grupo de ratos expostos de forma passiva à fumaça do cigarro pode representar uma elevação nos níveis pericelulares de adenosina. Sabe-se que a molécula de adenosina pode modular a liberação de uma ampla variedade de neurotransmissores, incluindo o glutamato, a ACh, a noradrenalina, e a dopamina (DI IORIO et al., 1998), assim como o ATP, uma vez que ele é co-liberado com os demais neurotransmissores (ZIMMERMANN, 2008). Um importante evento adaptativo de neuroplasticidade da via das ectonucleotidases é a redução dos níveis de ATP, um neurotransmissor excitatório, e o aumento dos níveis de adenosina, um composto neuroprotetor.

Corroborando com os resultados deste trabalho, um artigo publicado recentemente indica que a exposição crônica à fumaça do cigarro reduz significativamente a atividade de ATPases ancoradas à membrana, o que leva à

quebra da homeostase iônica e ao aumento nos níveis dos íons Ca^{2+} e Na^{+} intracelulares (ANBARASI et al., 2006). O aumento simultâneo na concentração citosólica destes íons é capaz de gerar uma hiperpolarização da membrana celular neuronal e, consequentemente, acentuar a liberação de neurotransmissores como a ACh e o ATP. Se a molécula de ATP é liberada em grandes quantidades e durante um período extenso, ela pode promover um aumento dramático nos níveis de Ca^{2+} intracelulares mediados por receptores do tipo P2X, o que pode representar danos celulares e teciduais semelhantes àqueles causados pelo excesso de glutamato (EDWARDS et al., 1992). Por outro lado, o mantenimento de níveis elevados do neurotransmissor ACh está associado com a dessensibilização e a internalização de seus receptores, em especial o mAChR que está intimamente associado com processos cognitivos (INTROINI-COLLISON et al., 1996). Neste sentido, acredita-se que o aumento observado na atividade das enzimas AChE e E-NTPDase (ATP como substrato) no grupo de animais expostos de forma passiva à fumaça do cigarro seja um mecanismo de resposta aos eventos bioquímicos que levam ao aumento de seus respectivos substratos. Além disso, associa-se o aumento da cinética enzimática da E-NTPDase e E-5'-NT com o aumento dos níveis sinápticos de adenosina, o que pode representar uma resposta à quebra da homeostase e aos efeitos causados pelo aumento dos níveis de ATP.

Uma vez que o córtex cerebral possui um papel crucial na modulação do aprendizado e da memória, tanto as alterações hemostáticas quanto as alterações observadas nesta estrutura como o desequilíbrio redox, a alteração na atividade de enzimas envolvidas no mantenimento do gradiente iônico transmembrana e as alterações nos sistemas colinérgico e purinérgico de ratos expostos de forma passiva à fumaça do cigarro possivelmente estão associadas com os efeitos neurocognitivos descritos neste estudo.

Como citado anteriormente, dentre as alternativas para o tratamento de diversas doenças destaca-se o emprego de compostos naturais extraídos das plantas, denominados fitoterápicos. Levando em consideração que a exposição à fumaça do cigarro altera parâmetros tromborregulatórios e causa alterações neurobiológicas, neuroquímicas e neurocognitivas optou-se por empregar um composto com propriedades anti-agregantes e neuroprotetoras como a curcumina no tratamento dos animais expostos de forma passiva à fumaça do cigarro.

Nossos dados demonstram que a curcumina foi eficaz na prevenção das alterações observadas nas atividades das enzimas E-NTPDase e E-5'-NT em plaquetas de ratos expostos de forma passiva à fumaça do cigarro. De acordo com um estudo realizado anteriormente (SRIVASTAVA et al., 1995), a curcumina foi capaz de promover a redução da liberação de Ca^{2+} intracelular das plaquetas e de inibir as atividades das enzimas fosfolipase A_2/C e cicloxigenase, levando à redução da liberação de AA com a concomitante redução da formação de TXB_2. Além disso, as plaquetas tratadas com a curcumina apresentaram a liberação simultânea de produtos derivados da atividade da enzima lipoxigenase como os ácidos monohidroperóxidoeicosateraenoicos (12-HPETE; precursor do 12-HETE), o qual inibe a agregação plaquetária e, em altas concentrações, inibe também a formação de tromboxano (AHARONY et al., 1982). Como apresentado anteriormente nesta discussão, as alterações observadas no grupo de ratos expostos de forma passiva à fumaça do cigarro são indicativas de um aumento nos níveis circulantes de ADP. Desta forma, uma vez que a alta concentração plasmática de ADP é associada com o aumento da agregação plaquetária, estes efeitos exercidos pelo tratamento com a curcumina na atividade das enzimas purinérgicas em plaquetas é proposto como mais uma das suas propriedades antiagregantes.

Sabe-se que além dos produtos da liberação dos grânulos plaquetários outros fatores como a geração de EROs ERNs também estão envolvidos na indução da agregação plaquetária. A fumaça do cigarro é composta de duas diferentes fases: a fase gasosa e a fase particulada. Os radicais presentes na fase particulada (10^{17} spin/grama) são provenientes do $Q^{\bullet-}$ (PRYOR et al., 1983), enquanto uma única tragada contém mais de 10^{15} radicais orgânicos na fase gasosa (PRYOR, 1992). Dentre estas espécies reativas encontram-se o $O_2^{\bullet-}$, o H_2O_2, o $HO^{\bullet}$, o dióxido de nitrogênio (NO_2), o $NO^{\bullet}$, o $ONOO^-$ e o O_2NOO^- (PRYOR; STONE, 1993). Estes oxidantes são capazes de reagir e causar danos aos tecidos e biomoléculas. Por sua vez, os danos causados pelos subprodutos da fumaça do cigarro podem levar à liberação de moléculas sinalizadoras e desencadear a agregação plaquetária através de diversas vias. Acredita-se que as propriedades antioxidantes descritas da curcumina (GOEL et al., 2008; ILBEY et al., 2009; KAMAT et al., 2008; KOWLURU; KANWAR, 2007) possam estar associadas com os efeitos observados neste estudo, uma vez que ela protegeria a integridade das biomoléculas da ação dos oxidantes presentes na fumaça do cigarro.

Em decorrência do efeito protetor da curcumina contra o aumento da liberação de ADP e o consequente estímulo para a agregabilidade plaquetária, também se esperaria que houvesse a redução da liberação do FAP, que por sua vez aumenta a permeabilidade da BHE. Desta forma, em consequência destes eventos, uma das hipóteses seria a atenuação ou prevenção dos efeitos nocivos oriundos da exposição passiva à fumaça do cigarro observados no SNC e também representado pelos efeitos cognitivos.

O tratamento com a curcumina (12.5 ou 25 mg/kg) no grupo de ratos expostos de forma passiva à fumaça do cigarro preveniu a redução observada nas latências de descida da plataforma. Estes resultados indicam que o tratamento com a curcumina foi capaz de prevenir o comprometimento cognitivo e de memória induzido pela exposição passiva à fumaça do cigarro. Posteriormente a obtenção destes resultados investigamos o potencial da formulação de curcumina anteriormente utilizada, doravante denominada curcumina livre, e também uma formulação de curcumina nanoencapsulada, no teste comportamental do reconhecimento de objetos. Percebemos que tanto a curcumina livre nas doses de 25 mg/kg (efeito parcial) e 50 mg/kg quanto a nanoencapsulada na dose de 4 mg/kg preveniram o déficit cognitivo promovido pela exposição passiva à fumaça do cigarro, indicada pelo baixo índice de reconhecimento (abaixo de 0.5). Corroborando com os nossos estudos, outros grupos de pesquisa já descreveram o potencial da curcumina livre administrada por via oral em uma amplo expectro de doses (3-300 mg/kg) e de tempo (2-6 semanas) na prevenção do déficit cognitivo causado por diferentes tipos de agentes como, por exemplo, a fenitoína (REETA et al., 2009), a escopolamina (AHMED; GILANI, 2009), o cloreto de alumínio ($AlCl_3$) associado com D-galactose (PAN et al., 2008), e a glicoproteína 120 do vírus da imunodeficiência humana (HIV) (TANG et al., 2009).

O estudo desenvolvido com a formulação de curcumina nanoencapsulada foi realizado primeiramente para avaliar o desempenho desta nova formulação disponível sobre os efeitos da exposição passiva à fumaça do cigarro sobre a memória e parâmetros envolvidos na homeostase do SNC. Interessantemente, as nanocápsulas carregadas com a curcumina (C-LNC, *curcumin-loaded nanocapsules*) em uma dose cerca de 4 vezes mais baixa do que a dose inefetiva de curcumina livre (12.5 mg/kg) apresentou resultados semelhantes aos da dose mais alta (50 mg/kg) de curcumina livre. Esta dose foi equivalente a cerca de 12 vezes mais

curcumina quando comparada com a dose da curcumina nanoencapsulada, o que é fortemente sugestivo de que esta formulação possui o potencial de aumentar a biodisponibilidade oral da curcumina.

Diversos estudos têm empregado carreadores nanométricos com o objetivo de aumentar a biodisponibilidade da curcumina e resultados promissores têm sido descritos nas áreas de pesquisa da terapia contra o câncer humano (BISHT et al., 2007; NAIR et al., 2012; YALLAPU et al., 2010) e também patologias envolvendo o SNC (MATHEW et al., 2012; RAY et al., 2011). Um estudo recente utilizando as mesmas nanocápsulas de núcleo lipídico empregadas nesta pesquisa mostrou um aumento na concentração do resveratrol nos tecidos encéfalico, hepático e renal após injeções intraperitoneais (i.p.) ou administrações orais diárias quando comparado com a droga administrada em sua forma livre (FROZZA et al., 2010). Além disso, foi demonstrado que a encapsulação da curcumina aumenta a sua biodisponibilidade oral em uma taxa de pelo menos 9 vezes comparada com a curcumina administrada na forma de suspensão associada à piperina, um promotor de absorção (SHAIKH et al., 2009). Adicionalmente, um estudo publicado recentemente (TSAI et al., 2011) demonstra que após a administração intravenosa (i.v.) de nanopartículas de poliéster de ácido láctico-co-glicólico (PLGA) carregadas com curcumina, a meia vida ($t_{1/2}$) e o tempo médio de residência (MRT, *mean residence time*) da curcumina no tecido encefálico aumentou em relação à formulação convencional da droga. Análises farmacocinéticas em diferentes regiões encefálicas revelam que a $t_{1/2}$ e o MRT aumentaram no hipocampo e no córtex cerebral (TSAI et al., 2011), ambas regiões de vital importância nos processos cognitivos e de memória (IZQUIERDO; MEDINA, 1995).

Nossos resultados demonstram que a administração de ambas as formulações de curcumina, livre e nanoencapsulada, foram eficazes na prevenção da redução das atividades das enzimas Na^+,K^+-ATPase e Ca^{2+}-ATPase em córtex cerebral induzida pela exposição passiva à fumaça do cigarro, embora a dose de 12,5 mg/kg tenha apresentado prevenção parcial. Notavelmente, apesar da baixa dose administrada (4 mg/kg), as C-LNC apresentaram resultados similares àqueles encontrados com as doses mais altas de curcumina livre, o que acredita-se estar associado com um aumento na biodisponibilidade da droga. No que diz respeito aos parâmetros de estresse oxidativo avaliados, todas as doses de curcumina (livre e nanoencapsulada) foram eficazes em prevenir as alterações induzidas pela

exposição passiva à fumaça do cigarro nos sistemas de defesa antioxidantes e, desta forma, contribuindo com a prevenção do aumento observado nos marcadores de dano celular mediado por EROs e ERNs em córtex cerebral. Todos estes efeitos promovidos pelo tratamento com a curcumina podem ser atribuídos às suas propriedades antioxidantes sistêmicas, especialmente no tecido encefálico (LIM et al., 2001; SCAPAGNINI et al., 2006), e à sua habilidade de estabelecer a homeostase nas membranas celulares (SHARMA et al., 2010). Sugere-se que à proteção conferida pela curcumina contra o dano causado pelas EROs e ERNs às membranas celulares e subcelulares foi de grande utilidade na prevenção da quebra da homeostase iônica e provavelmente seria útil na prevenção e tratamento de processos patológicos envolvendo o SNC.

Anteriormente ao estudo realizado com a formulação de curcumina nanoencapsulada havíamos investigado o potencial da curcumina livre na prevenção dos efeitos da exposição passiva à fumaça do cigarro sobre os sistemas colinérgico e purinérgico. A curcumina livre demonstrou ser capaz de prevenir o aumento na atividade da enzima AChE induzida pela fumaça do cigarro em sangue total, em sinaptossomas de córtex cerebral e em homogenatos de cerebelo, córtex cerebral, hipocampo, estriado e hipotálamo. No entanto, embora as diferentes doses testadas de curcumina livre tenham sido eficazes na prevenção do aumento promovido pela exposição passiva à fumaça do cigarro na atividade da enzima nas estruturas encefálicas, a dose de 50 mg/kg não foi eficaz em reverter este efeito no estriado e no hipotálamo. Uma vez que o sistema colinérgico está intimamente envolvido na regulação do aprendizado e da memória (IZQUIERDO; MEDINA, 1995), a normalização na atividade da enzima AChE observada no grupo de ratos expostos de forma passiva à fumaça do cigarro e tratados com a curcumina livre nas doses de 12.5, 25 e 50 mg/kg, especialmente no córtex cerebral e hipocampo pode ser um dos importantes fatores envolvidos no mantenimento da função cognitiva.

Recentemente, foi demonstrado que a curcumina livre administrada por via oral na dose de 60 mg/kg atenuou a disfunção colinérgica e foi eficaz na prevenção do aumento na atividade da enzima AChE em ratos diabéticos (KUHAD; CHOPRA, 2007). Além disso, também foi demonstrado que a curcumina livre administrada pela mesma via na dose de 200 mg/kg preveniu o aumento na atividade da enzima AChE em ratos induzidos à demência pela streptozotocina (AGRAWAL et al., 2010).

A atividade da enzima AChE em sangue total e em sinaptossomas de córtex cerebral não apresentou alteração com o uso da curcumina livre *per se* nas mesmas doses empregadas no tratamento dos grupos expostos de forma passiva à fumaça do cigarro. Todavia, embora a atividade desta enzima nos homogenatos de estruturas encefálicas não tenha apresentado alteração com a administração da curcumina livre *per se* nas doses de 12.5 ou 25 mg/kg, foram observadas alterações na dose de 50 mg/kg. Corroborando com os resultados deste trabalho, um estudo recente também demonstrou que a curcumina não apresentou nenhum efeito *per se* na atividade da enzima AChE encefálica na dose de 20 mg/kg (RINWA et al., 2010).

O tratamento com a curcumina livre também foi eficaz em prevenir as alterações observadas nas atividades das enzimas E-NTPDase e E-5'-NT em sinaptossomas de córtex cerebral de ratos expostos de forma passiva à fumaça do cigarro. As atividades destas enzimas em sinaptossomas de córtex cerebral também não foram influenciadas pelo uso da a curcumina livre *per se* nas mesmas doses utilizadas no tratamento dos animais expostos à fumaça do cigarro.

De uma forma geral, o conjunto de dados apresentados neste estudo indica que a exposição passiva à fumaça do cigarro causa um aumento no nível circulante de ADP e, consequentemente, do FAP que por sua vez aumenta a permeabilidade da BHE, tornando o tecido encefálico mais vulnerável ao dano causado pelas espécies reativas, como observado. A redução nas atividades das enzimas Na^{+}-K^{+}-ATPase e Ca^{2+}-ATPse está associada com a despolarização da membrana neuronal e a consequente liberação excessiva de neurotransmissores excitatórios como o glutamato, ACh e o ATP. Acredita-se que o aumento observado nas atividades das enzimas AChE e E-NTPDase em nível de SNC esteja associado com uma resposta ao aumento dos níveis sinápticos de seus substratos (ACh e ATP, respectivamente) e que o aumento na atividade da enzima E-5'-NT seja uma adaptação para a promoção do aumento dos níveis pericelulares de adenosina, uma molécula neuroprotetora.

Por outro lado, especula-se que os efeitos conferidos pelo tratamento com a curcumina na prevenção do aumento nos níveis circulantes de ADP e FAP mantenham a integridade da BHE e impeçam alguns dos efeitos oriundos da exposição passiva à fumaça do cigarro como a excitotoxicidade. Desta forma, não haveria o aumento na liberação de ACh, ATP e nem do conteúdo de adenosina, o que explicaria os níveis basais das atividades das enzimas AChE, E-NTPDase e E-

5’-NT. Além disso, o mantenimento da hemostase e da homeostase em nível de SNC promovido pelo tratamento com a curcumina estaria associado com a prevenção dos efeitos cognitivos causados pela exposição passiva à fumaça do cigarro. Esta hipótese explicaria os nossos achados que indicam que a curcumina previne as alterações observadas nas atividades enzimáticas, embora não modifique a atividade basal destas enzimas quando administrada *per se*.

CONCLUSÃO

Tendo em vista os resultados obtidos no presente estudo, pode-se concluir que:

- O tratamento com a curcumina livre previne o aumento na atividade das enzimas E-NTPDase (ATP como substrato) e E-5'-NT, e a redução na atividade da enzima E-NTPDase (ADP como substrato) em plaquetas de ratos expostos de forma passiva à fumaça do cigarro.

- A administração de curcumina livre previne o aumento nas atividades das enzimas E-NTPDase, E-5'-NT e AChE em sinaptossomas de córtex cerebral de ratos expostos de forma passiva à fumaça do cigarro.

- A curcumina livre previne o aumento na atividade da enzima AChE em cerebelo, córtex cerebral, hipocampo, estriado, hipotálamo e sangue periférico de ratos expostos de forma passiva à fumaça do cigarro. O tratamento também previne o déficit cognitivo observado no grupo de ratos exposto de forma passiva à fumaça do cigarro.

- O uso das formulações de curcumina livre e nanoestruturada previne a redução na atividade das enzimas Na^+,K^+-ATPAse e Ca^{2+}-ATPase e o desequilíbrio redox em córtex cerebral de ratos expostos de forma passiva à fumaça do cigarro. O tratamento com ambas as formulações também previne o déficit cognitivo observado no grupo de ratos exposto de forma passiva à fumaça do cigarro.

- Por fim, estes resultados indicam que a administração da curcumina através de nanocápsulas de núcleo lipídico possa ser uma alternativa para o aumento de sua eficácia, provavelmente pelo aumento da biodisponibilidade da curcumina administrada de forma oral.

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