UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

AVALIAÇÃO DE RESPOSTAS BIOQUÍMICAS, COMPORTAMENTAIS, HEMATOLÓGICAS E DE BIOACUMULAÇÃO EM RATOS EXPOSTOS AO CÁDMIO E TRATADOS COM N-ACETILCISTEÍNA

JAMILE FABBRIN GONÇALVES

Porto Alegre
2012

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JAMILE FABBRIN GONÇALVES

Orientadora: Profa. Dra. Maria Rosa Chitolina Schetinger

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“Algumas pessoas têm amor por você, outras têm raiva. O que sentem nem sempre depende de seu comportamento. As reações delas às vezes são justas, outras vezes são injustas. Dê sem contabilizar. E esteja atento às necessidades delas.

Pouco importa o julgamento dos outros. Os seres são tão contraditórios que é impossível atender às suas demandas, satisfazê-los. Tenha em mente simplesmente ser autêntico e verdadeiro”

Dalai Lama

Dedico esta tese a minha filha que ainda gero no ventre

e que em breve será a razão maior da minha existência.

A mamãe já te ama muito, *Manuela Gonçalves dos Anjos*!!!

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APRESENTAÇÃO

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A **Introdução** apresenta o embasamento teórico que nos levou a formular a proposta de trabalho. Os **Objetivos** – geral e específicos – estão dispostos no corpo da tese e em maiores detalhes inseridos dentro de cada trabalho científico. Os **Capítulos** contêm os artigos publicados e o manuscrito submetido realizados durante o período do doutorado. Para a realização desse trabalho foi necessária colaboração de quatro grupos de pesquisa: Laboratório de Enzimologia Toxicológica, Laboratório de Neurociências e Comportamento, Laboratório de Biotecnologia Vegetal e Laboratório de Análises Químicas Industriais e Ambientais da Universidade Federal de Santa Maria.

O tópico **Discussão** apresenta uma interpretação geral dos resultados obtidos nos diferentes trabalhos. Nas seções **Conclusões** e **Perspectivas** há uma abordagem geral das conclusões da tese e as possibilidades de futuros trabalhos a partir dos resultados obtidos na presente tese.

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ACh: acetilcolina

AChE: acetilcolinesterase

AChRs: receptores específicos de acetilcolina

ADP: adenosina difosfato

AMP: adenosina monofosfato

ATP: adenosina trifosfato

BChE: butirilcolinesterase

ChAT: colina acetiltransferase

EROs: espécies reativas de oxigênio

Hb: hemoglobina

Hct: hematócrito

mAChRs: receptores muscarínicos de acetilcolina

nAChRs: receptores nicotínicos de acetilcolina

NTPDases: nucleosídeo trifosfato difosfoidrolases

SNC: sistema nervoso central

SNP: sistema nervoso periférico

RBC: hemácias

TBARS: substâncias reativas ao ácido tiobarbitúrico

VAChT: transportador vesicular da acetilcolina

WBC: leucócitos

RESUMO

A poluição ambiental por metais pesados tem aumentado muito devido às ações antropogênicas tais como as atividades industriais e o uso de fertilizantes fosfatados na agricultura. Entre os metais tóxicos encontrados no meio ambiente, o cádmio (Cd) é um dos que apresenta maior interesse clínico, uma vez que as intoxicações por esse metal são de difícil tratamento. O Cd pode afetar vários órgãos como o fígado, rins, pulmões, ovários, ossos, testículos e cérebro. A toxicologia do Cd é extremamente complexa e tem sido amplamente estudada, mas ainda não está completamente esclarecida. Sendo assim, o objetivo da presente tese foi investigar os efeitos da intoxicação por Cd sobre parâmetros comportamentais e memória, bioquímicos, hematológicos e a bioacumulação desse metal em diferentes tipos celulares de ratos expostos ao cloreto de Cd ($CdCl_2$) ou ao Cd proveniente de batatas contaminadas, e ainda utilizar o antioxidante N-acetilcisteína (NAC) no tratamento deste tipo de intoxicação buscando-se avaliar o seu possível efeito protetor. Para tal, foram realizados dois experimentos: a) ratos machos Wistar adultos foram expostos oralmente a 2 mg/kg de Cd e/ou 150 mg/kg de NAC, um dia sim e outro não, durante um período experimental de 30 dias; b) ratos machos Wistar recém desmamados ingeriram, durante 5 meses, dieta a base de bolo contendo 1, 5 ou 25 mg/kg de $CdCl_2$ ou Cd presente em tubérculos de batatas crescidas na presença de 10 μM de $CdCl_2$. A partir desses experimentos pode-se concluir que: a) A intoxicação por $CdCl_2$ causa aumento na concentração de Cd nas estruturas cerebrais (cerebelo, estriado, hipotálamo, hipocampo e córtex cerebral), nos níveis de peroxidação lipídica e na atividade da enzima AChE nas diferentes estruturas cerebrais estudadas ocasionando prejuízos à memória dos ratos. Além disso, a NAC é capaz de diminuir os níveis de peroxidação lipídica e subsequentemente restaurar a atividade da AChE modulando assim, a neurotransmissão colinérgica e melhorando os processos cognitivos. Sendo assim, sugere-se que a NAC possa ser um fármaco promissor em terapias alternativas contra a neurotoxicidade induzida pelo Cd; b) A exposição ao $CdCl_2$ promove um aumento na concentração de Cd no plasma, baço e timo, causa danos hematológicos, não altera a atividade da enzima NTPDase em linfócitos, diminui a atividade da AChE em linfócitos e sangue total bem como da BChE em soro de ratos. Além disso, a NAC foi eficaz em diminuir os efeitos danosos do Cd provavelmente por diminuir os níveis de Cd nos órgãos linfóides, reverter ou amenizar os danos hematológicos e relacionados aos leucócitos mesmo sem alterar a atividade das enzimas colinesterases; c) O $CdCl_2$ aumenta a hidrólise de nucleotídeos de adenina em sinaptossomas de córtex cerebral e diminui em plaquetas de ratos. Provavelmente, o aumento na atividade das enzimas NTPDase e 5'-nucleotidase no SNC causado pelo Cd seja uma resposta compensatória do organismo uma vez que a rápida hidrólise de ATP e ADP favorece a produção de adenosina, uma molécula neuroprotetora. Por outro lado, em plaquetas o Cd causou uma diminuição na atividade da NTPDase e, não alterou a atividade da 5'-nucleotidase sugerindo que a possível elevação no nível de ATP e ADP conduza a um estado hipercoagulável nos ratos intoxicados por esse metal. Além disso, a NAC restaura a atividade dessas enzimas no SNC, mas não apresenta interferência sobre elas em plaquetas; d) A dieta prolongada com $CdCl_2$ ou com Cd proveniente de batatas contaminadas ocasiona aumento no comportamento de ansiedade e prejuízo à memória de ratos o que provavelmente, seja resultado de uma concentração aumentada de Cd e de uma atividade aumentada da AChE e diminuída da Na^+,K^+-ATPase em diferentes estruturas cerebrais devido a esse metal. Em linhas gerais, os resultados

obtidos na presente tese demonstram que apesar de todos os mecanismos propostos pelos quais o Cd poderia afetar o comportamento continuarem a gerar controvérsia, é evidente que a exposição ao $CdCl_2$ tanto a curto quanto a longo prazo, bem como ao Cd proveniente de batatas contaminadas interfere nas funções cerebrais aumentando a concentração de Cd, diminuindo ou aumentando a atividade da AChE, aumentando a lipoperoxidação bem como diminuindo a atividade da Na^+,K^+-ATPase em diferentes estruturas cerebrais, consequentemente sendo prejudicial ao comportamento animal, como memória e ansiedade. O Cd afeta sistematicamente o organismo dos animais estando presente tanto no plasma, quanto nos órgãos linfóides e no encéfalo alterando, assim, a atividade das enzimas NTPDase, 5'-nucleotidase, AChE e BChE de diferentes tipos celulares. Além disso, a NAC é capaz de reverter ou amenizar vários efeitos danosos causados pelo Cd sugerindo que este fármaco possa ser considerado após estudos adicionais um importante aliado em terapias contra a intoxicação por esse metal.

Palavra-chave: AChE, batata, BChE, Cd, NAC, NTPDase.

ABSTRACT

The environmental pollution by heavy metals has increased greatly due to anthropogenic activities such as industrial activities and the use of phosphate fertilizers in agriculture. Among the toxic metals found in the environment, cadmium (Cd) is a metal that presents the greatest clinical interest, since poisoning by this metal is difficult to treat. Cd may affect various organs such as liver, kidneys, lungs, ovaries, bones, testes and brain. The toxicology of Cd is extremely complex and has been widely studied, but it is not yet fully elucidated. Therefore, the objective of this thesis was to investigate the effects of Cd intoxication on memory as well as behavioral, biochemical, hematological and bioaccumulation parameters of this metal in different cell types of rats exposed to Cd chloride ($CdCl_2$) or Cd from contaminated potatoes. Also, we investigated the antioxidant N-acetylcysteine (NAC) in the treatment of this type of poisoning seeking to evaluate its possible protective effect. Two experiments were performed: i) adult male Wistar rats were exposed orally to 2 mg/kg Cd and/or 150 mg/kg NAC, every other day for 30 days; ii) weaned male Wistar rats fed for 5 months, cake-based diet containing 1, 5 or 25 mg/kg $CdCl_2$ or Cd present in tubers of potatoes grown in the presence of 10 μM of $CdCl_2$. From these experiments we conclude: Firstly, $CdCl_2$ intoxication caused an increase in the Cd concentration in brain structures (cerebellum, striatum, hypothalamus, hippocampus and cerebral cortex), in the levels of lipid peroxidation, and in the AChE activity of different brain structures studied causing damage to memory of rats. NAC was able to reduce the levels of lipid peroxidation and subsequently restore the AChE activity, modulating thereby the cholinergic transmission and improving the cognitive processes. Thus, NAC may be a promising drug for alternative therapies against neurotoxicity induced by Cd. Secondly, the exposure to $CdCl_2$ increased the Cd concentration in plasma, spleen and thymus, caused damages in the hematological parameters, did not alter the NTPDase activity in lymphocytes as well as decreased the AChE activity in lymphocytes and whole blood and the BChE activity in serum of rats. NAC was effective in reducing the harmful effects of Cd probably by decreasing the levels of Cd in the lymphoid organs, reversing or minimizing the hematological damage and related to leukocytes even without changing the activity of cholinesterases. Thirdly, $CdCl_2$ increases the hydrolysis of adenine nucleotides in synaptosomes of cerebral cortex and decreases in platelets of rats. The increase in the NTPDase and 5'-nucleotidase activities in CNS caused by Cd could be a compensatory response of the organism since the rapid hydrolysis of ATP and ADP favors the production of adenosine, a neuroprotective molecule. On the other hand, Cd decreased the NTPDase activity and did not alter 5'-nucleotidase activity in platelets suggesting that the possible increase in the ATP and ADP levels could lead to a hypercoagulable state in rats intoxicated with this metal. NAC restores the activity of these enzymes in the CNS, but had no interference in platelets. Finally, the prolonged diet with $CdCl_2$ or Cd from contaminated potatoes resulted in an increased anxiety-like behavior and damaged memory of rats which was likely to be a result of the increased concentration of Cd, the increased AChE activity and decreased Na^+,K^+-ATPase activity in the different brain structures studied. In general, the results obtained in this thesis show that despite all the proposed mechanisms by which Cd could affect the behavior still generate controversy, it is clear that the exposure to $CdCl_2$ at both short

and long terms as well as to Cd from contaminated potatoes interferes the brain function by increasing Cd concentration, decreasing or increasing the AChE activity, increasing lipid peroxidation and decreasing Na^+,K^+-ATPase activity in several brain structures, thus being detrimental to the animal behavior influencing memory and anxiety. Cd affected systematically the animal body since it is present in plasma, lymphoid organs and brain, changing the activity of the enzymes NTPDase, 5'-nucleotidase, AChE and BChE of different cell types. In addition, NAC was able to reverse or ameliorate several deleterious effects caused by Cd suggesting that this drug may be, after additional studies, considered an important factor in therapies against intoxication by this metal.

Keywords: AChE, potato, BChE, Cd, NAC, NTPDase.

1. INTRODUÇÃO

1.1. Cádmio

O cádmio (Cd) é um elemento químico pertencente ao grupo 12 da tabela periódica sendo, portanto, classificado como um metal de transição, tendo sido o seu primeiro registro feito em 1817 por Friedrich Strohmeyer. Apresenta cor prata claro, ductilidade e maleabilidade. Possui peso molecular 112,41 e número atômico 48, com ponto de fusão e ebulição iguais a 321ºC e 767,2ºC, respectivamente, e densidade de 8,65 g/cm^3 (BERNARD & LAUWERYS, 1984). É um elemento não essencial encontrado na natureza em baixas concentrações estando associado geralmente ao zinco, chumbo ou cobre (OSPAR Commission, 2002). O Cd ocorre naturalmente no meio ambiente através de processos como o intemperismo das rochas, a erosão do solo, e a partir de eventos singulares como as queimadas florestais e as erupções vulcânicas (RAO et al., 2010) sendo que a atividade vulcânica é considerada a maior fonte natural de emissão de Cd à atmosfera, cerca de 820 toneladas/ano (OSPAR Commission, 2002). As rochas sedimentares possuem maiores concentrações de Cd em relação às rochas ígneas e metamórficas (COOK & MORROW, 1995) e a principal fonte mineral de Cd é a *greenockita*, rica em sulfeto de cádmio (CdS) (CHASIN e CARDOSO, 2003).

Entretanto, a partir do século XX devido ao processo de industrialização, o Cd teve sua concentração muito aumentada no ambiente devido às atividades antropogênicas, tais como a mineração, a aplicação de fertilizantes, a utilização de adubação com lodo de esgoto e de efluentes industriais contaminados, a fabricação de baterias níquel-Cd, os processos de galvanização, as aplicações militares e aeroespaciais e a utilização como estabilizador (OSPAR Commission, 2002; NORDBERG, 2009).

O Cd é encontrado tanto no ar, quanto na água e no solo. As concentrações de Cd no ar a que estão sujeitas as populações são, geralmente, menores que $5x10^{-6}$ mg/m^3, porém concentrações de até $5x10^{-4}$ mg/m^3 foram detectadas no ar de áreas próximas a atividades emissoras (AZEVEDO e CHASIN, 2003). A maior fonte de liberação de Cd à atmosfera é a queima de combustíveis fósseis e a incineração de lixos municipais (OSPAR Commission, 2002). O nível de Cd nas águas apresenta grande variação. Há referências de que as concentrações em mar aberto variam de 0,01 a 0,1 µg/L, e em águas profundas as concentrações são maiores que nas superficiais devido à captação do

metal pelos organismos aquáticos. Existem referências de valores próximos ou maiores que 1 mg/L em águas oriundas de locais próximos a depósitos de minerais (ATSDR, 1997). Entretanto, o nível de Cd na água não contribui significativamente para o total de ingestão de Cd por humanos. Em geral, o limite permitido de concentração de Cd na água potável é em torno de 5 µg/L (JÄRUP et al., 1998). As concentrações do Cd em solos não poluídos são variáveis, dependendo das fontes minerais e do material orgânico. O Cd ocorre em baixas concentrações na crosta terrestre (entre 0,15 e 0,20 mg/kg) podendo alcançar níveis de 1 mg/kg, e em solos de origem vulcânica este valor pode atingir concentrações de 4,5 mg/kg (OSPAR Commission, 2002). Solos altamente poluídos por Cd contendo cerca de 100 mg kg^{-1} deste metal foram encontrados em países como a China e a França (ALLOWAY & STEINNES, 1999; KABATA-PENDIAS & PENDIAS, 2001). A contribuição anual de Cd para o solo é cerca de 2.500 a 15.500 toneladas sendo a deposição atmosférica a principal fonte. Além disto, cerca de 7.500 a 29.500 toneladas de Cd por ano são liberadas em aterros e outros depósitos na forma de produtos descartados que continham esse metal (CADMIUM REVIEW, 2003).

Quando se avalia a exposição humana ao Cd deve-se levar em consideração a rota de exposição ao metal bem como o estilo de vida de cada pessoa uma vez que esses fatores afetam o nível de Cd absorvido. São três as principais formas de exposição ao Cd: cutânea, pulmonar e gastrintestinal. Pouca pesquisa tem sido realizada em relação à absorção dérmica de Cd. Os estudos sobre absorção dérmica sugerem que, embora a absorção por esta via seja lenta, os níveis de Cd podem atingir proporções relevantes nas situações onde soluções concentradas de Cd estejam em contato direto com a pele apresentando uma absorção de 8 a 12% (WESTER et al., 1992; GODT et al., 2006). A absorção pulmonar de Cd é cerca de 40 a 60% do total de Cd inalado. A principal fonte de Cd inalado é através do ato de fumar. Um cigarro contém em torno de 1 a 2 µg de Cd. Uma média de 10% do Cd presente no cigarro é inalada durante a tragada. Considerando-se uma média de 50% de absorção do Cd através da via aérea, pode-se estimar que uma pessoa que fume 20 cigarros diários absorva cerca de 1 µg de Cd por dia (JÄRUP et al., 1998). De fato, em geral, os fumantes possuem níveis sanguíneos de Cd cerca de 4 a 5 vezes maiores em relação aos não fumantes. A absorção gastrintestinal de Cd é apenas 5% do total de Cd ingerido (GODT et al., 2006). Entretanto, a exposição oral assume importantes proporções devido à bioacumulação do

Cd na cadeia alimentar uma vez que a dieta é a principal fonte de Cd para a população em geral (não ocupacional e não fumante).

A contaminação dos alimentos ocorre através da poluição do solo, que, por sua vez, pode ser contaminado através das águas de irrigação, de deposição originária da poluição atmosférica e de adubações através de fertilizantes fosfatados ou fertilizantes de origem de esgotos (GODT et al., 2006). O Cd está presente em praticamente todos os alimentos, mas a sua concentração varia muito em função da origem do alimento, das concentrações de Cd no solo e da disponibilidade do metal no meio em condições de ser incorporado pela planta. Assim, existe uma grande variação na ingestão de Cd pela população devido aos diferentes hábitos alimentares. As pessoas cuja dieta está baseada em vegetais, peixes e frutos do mar, estão mais expostas a esse metal uma vez que estes alimentos acumulam uma maior quantidade de Cd em relação à carne bovina, por exemplo. Além disso, a absorção gastrintestinal de Cd pode ser influenciada por fatores nutricionais uma vez que animais com baixa ingestão de cálcio, ferro, zinco e cobre apresentam maior absorção de Cd (JÄRUP et al., 1998).

De grande importância na história da toxicologia do Cd foi o registro de ocorrência da doença Itai-Itai após a Segunda Guerra Mundial (década de 50) em Fuchu, Japão. A tradução do termo Itai-Itai, em português, significa dói-dói ou também ai-ai e se deve à principal queixa do paciente: dor severa causada por fraturas dos ossos. Essa doença apresentou grande incidência em pessoas que se alimentavam de arroz irrigado pelas águas do Rio Jinzu contaminadas por Cd, uma vez que, a sua nascente era localizada próxima a uma área de mineração. Foi um dos casos mais dramáticos de contaminação alimentar por Cd, sendo caracterizado por osteomalacia, osteoporose e insuficiência renal (JÄRUP et al., 1998; JÄRUP & AKESSON, 2009; NORDBERG, 2009).

Atualmente, muitos estudos tentam estabelecer a média de ingestão diária de Cd proveniente dos alimentos. Em geral, as pessoas que vivem em áreas não contaminadas pelo metal e que não sejam fumantes possuem uma média de ingestão em torno de 10 a 40 µg por dia de Cd a partir da dieta (JÄRUP et al., 1998). Assim, considerando-se uma taxa de absorção gastrintestinal de 5%, acredita-se que cerca de 0,4 a 2,0 µg de Cd seja retido diariamente pelas pessoas a partir da alimentação. A Organização Mundial da Saúde (OMS) tem estabelecido que a ingestão semanal tolerável para Cd é de 7 µg/kg de peso corporal. Esse valor semanal corresponde a uma ingestão tolerável de até 70 µg

de Cd diariamente por um ser humano com 70 kg de peso corporal médio (WHO, 1992).

1.1.1. Efeitos variados do cádmio no organismo

Entre os metais tóxicos encontrados no meio ambiente e utilizados industrialmente, o Cd é um dos que apresenta maior interesse clínico, uma vez que as intoxicações por esse metal são de difícil tratamento (JONES & CHERIAN, 1990). A toxicidade do Cd, tanto em animais experimentais quanto em humanos, é influenciada por um grande número de fatores, tais como a via de administração, a dose, a forma química do metal, a duração da exposição e a idade dos animais (CASALINO et al., 1997). Este elemento é absorvido no organismo em pequenas quantidades, entretanto ele pode se acumular nos tecidos humanos devido a sua longa meia-vida biológica (10-30 anos) (PERRY et al., 1962). Dentro da célula, a maioria do Cd é mantida no citosol (70%), seguido pelo núcleo (15%), sendo que muito pouco é encontrado nas mitocôndrias e no retículo endoplasmático (DIN & FRAZIER, 1985; WAALKES & GOERING, 1990). No citosol o Cd se liga as metalotioneínas (MTs) (VIARENGO, 1985) bem como a grupos sulfidrílicos ou histidínicos (IZATT et al., 1971). Além disso, o Cd interage com todos os componentes dos ácidos nucléicos e com os fosfolipídios de membrana (VALLE & ULMER, 1972).

O Cd pode afetar vários órgãos como o fígado, rins, pulmões, ovários, ossos, testículos e cérebro (SANTOS et al., 2004, 2005a,b, 2006; LUCHESE et al., 2007a, b; BORGES et al., 2008). A intoxicação aguda por Cd produz primariamente injúria hepática e testicular, enquanto a exposição crônica produz dano renal e osteotoxicidade (RIKANS & YAMANO, 2000). Dessa forma, sob condição de exposição mais prolongada ao Cd, este metal se deposita primariamente no fígado, onde ele induz a síntese de MT e se liga a estas moléculas (SHAIKH & LUCIS, 1972), podendo causar efeitos hepatotóxicos. Com o tempo, o complexo Cd-MT hepático é lentamente liberado na circulação (TOHYAMA & SHAIKH, 1981) e posteriormente, após filtração glomerular, este complexo é degradado e os íons Cd liberados se ligam as MTs renais pré-existentes ou àquelas recentemente sintetizadas (CHERIAN et al., 1978). Quando a quantidade de Cd presente no córtex renal excede a capacidade de ligação as MTs, este Cd não ligado à MT é capaz de causar nefrotoxicidade (NOMIYAMA & NOMIYAMA,

1986), provavelmente pela geração de espécies reativas de oxigênio (EROs) (Figura 1) (HASSOUN & STOHS, 1996).

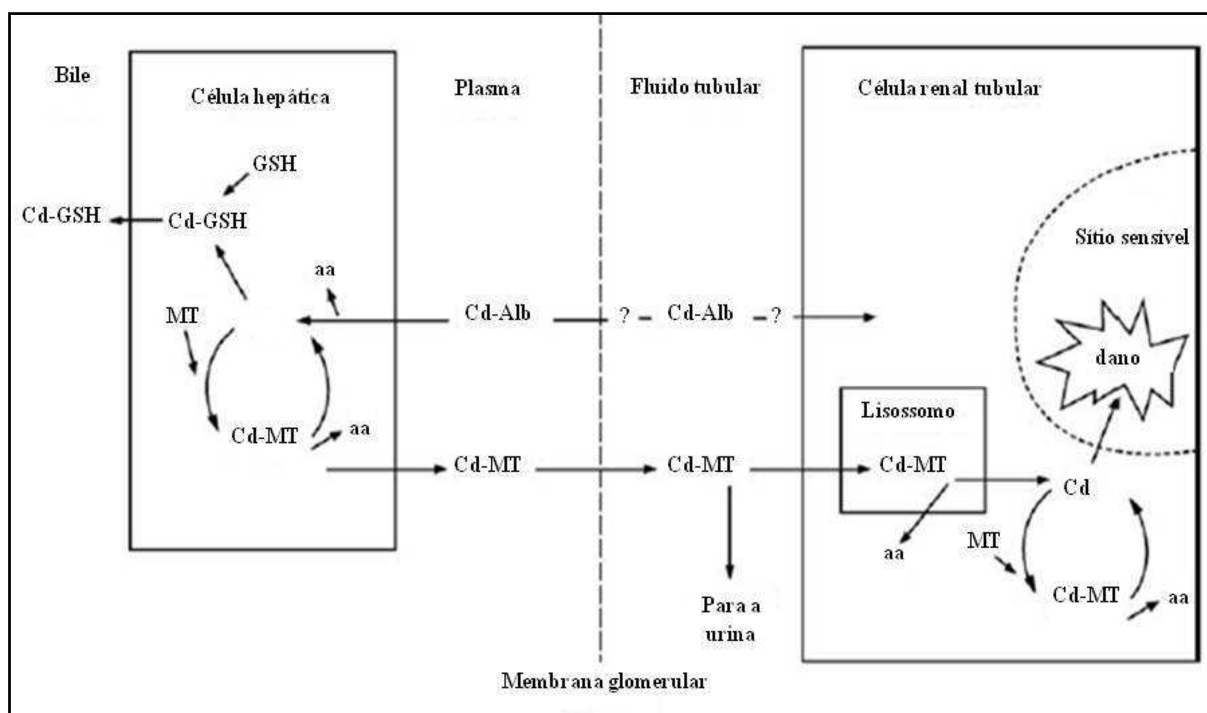


Figura 1. Metabolismo do cádmio (Cd) através do organismo de animais demonstrando a síntese e degradação de metalotioneína no fígado e nos rins. Adaptado de Nordberg (2009). aa = aminoácidos; Alb = albumina; Cd = cádmio; MT = metalotioneínas.

Uma das consequências da exposição ao Cd tanto em plantas quanto em animais é a geração de estresse oxidativo por meio de um desequilíbrio entre o sistema antioxidante e as EROs, as quais podem oxidar proteínas, lipídios, ácidos nucléicos e conduzir a alterações tanto na estrutura quanto nas funções celulares (Figura 2) (HALLIWELL & GUTTERIDGE, 2007; GONÇALVES et al., 2007, 2009). Vários trabalhos relacionados à produção de EROs e à resposta antioxidante de ratos e camundongos expostos ao Cd têm sido realizados (SANTOS et al., 2004, 2005a,b, 2006; LUCHESE et al., 2007a, b; BORGES et al., 2008). Nesses estudos diferentes tecidos (fígado, rins, pulmões, testículos e cérebro) sofreram alterações enzimáticas e aumento da peroxidação lipídica devido à toxicidade do Cd.

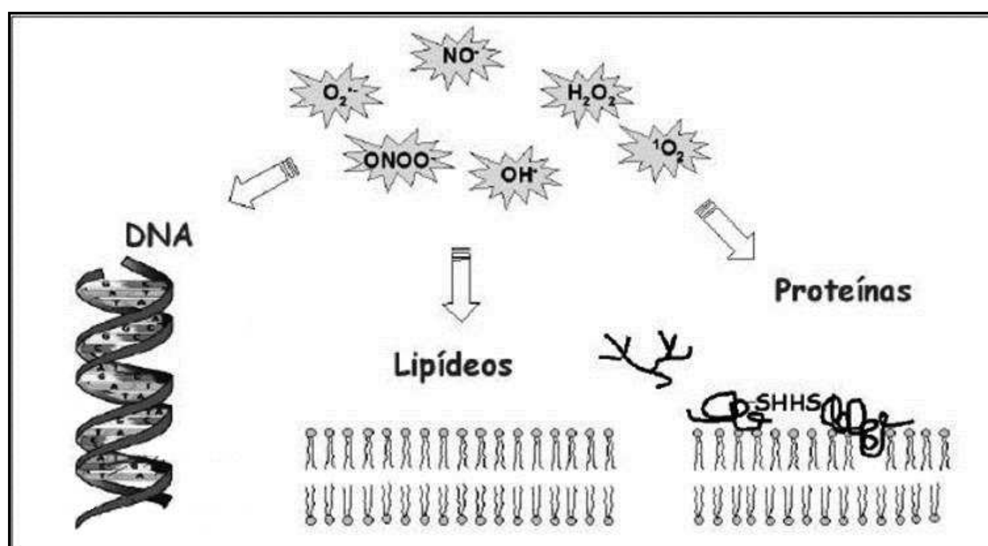


Figura 2. Dano oxidativo em macromoléculas biológicas (TORRES, 2003).

O cérebro, em especial, é susceptível à lipoperoxidação devido a sua alta utilização de oxigênio, abundante suprimento de ácidos graxos poliinsaturados, defesa antioxidante deficiente bem como alto conteúdo de metais de transição como o cobre e o ferro (CALABRESE et al., 2000). Além dos efeitos do Cd estarem associados ao estresse oxidativo, muito tem sido reportado em relação ao Cd como uma neurotoxina. Segundo MINAMI et al. (2001) o Cd ocasionou uma diminuição na liberação de neurotransmissores excitatórios (glutamato e aspartato) no espaço extracelular e um aumento na liberação de neurotransmissores inibitórios (glicina e GABA), sugerindo que esse metal afeta o balanço de excitação-inibição na neurotransmissão sináptica. Alguns estudos têm demonstrado que este metal acumula-se no cérebro alterando a atividade de enzimas colinesterases que são importantes nos processos sinápticos (CARAGEORGIOU et al., 2004, 2005; LUCHESE et al., 2007a, b).

1.1.2. Cádmio e cadeia alimentar

A poluição ambiental por metais pesados tem aumentado muito devido às ações antropogênicas tais como as atividades industriais e o uso de fertilizantes fosfatados na agricultura (BENAVIDES et al., 2005). A poluição do solo é uma ameaça direta ou indireta à saúde pública necessitando de ações remediadoras tal como a fitorremediação. No Brasil, não existem estatísticas da extensão da contaminação do solo, mas sabe-se

que esta ocorre em todo o território nacional, existindo, apenas em São Paulo, em torno de 2.000 locais potencialmente contaminados (ALVES, 1996). O conhecimento sobre as interações entre os metais pesados, as plantas e os animais é muito importante não só à segurança do meio ambiente, mas também para reduzir os riscos associados com a introdução desses elementos na cadeia alimentar (BENAVIDES et al., 2005). Neste sentido, entre os metais pesados, o Cd é um dos mais importantes para se considerar por ser facilmente absorvido pelas raízes e translocado para diferentes partes das plantas (LI et al., 1995). Para o nosso conhecimento, a maioria dos resultados disponíveis em relação ao efeito do Cd sobre o metabolismo tem sido derivada de tratamentos dos animais com sais de Cd, enquanto pouco é conhecido sobre o efeito do Cd orgânico (HAOUEM et al., 2007). Entretanto, visto que os alimentos agrícolas são a principal fonte de Cd aos humanos (SATARUG et al., 2003), experimentos com esse metal naturalmente incorporado nos vegetais são imprescindíveis (HAOUEM et al., 2007). Portanto, ao se considerar essa questão, torna-se muito importante a realização de experimentos nos quais sejam utilizados produtos agrícolas de grande consumo mundial, tais como o trigo, milho, arroz e batata (FAO, 1998).

1.2. Batata (*Solanum tuberosum* L.)

A batata é uma planta dicotiledônea, pertencente à família Solanaceae, gênero *Solanum*, tendo-se registradas mais de 2.000 espécies, das quais pouco mais de 150 produtoras de tubérculos. Entre as cultivadas, a mais importante economicamente produzida no mundo é a espécie *Solanum tuberosum*, que é cultivada em, pelo menos, 140 países (PEREIRA et al., 2003). Esta planta é um vegetal perene, embora habitualmente seja cultivada como bianual na Região Sul do Brasil. O “Plantio da safra” é recomendado em Julho e Agosto, e a “safrinha” é em Fevereiro e Março (BISOGNIN, 1996). Sua parte aérea é herbácea, com altura variável entre 50 e 70 cm, podendo, entretanto, alcançar até 1,5 m na fase adulta. O ciclo vegetativo da cultura pode ser precoce (< 90 dias), médio (90 – 110 dias) ou longo (> 110 dias), dependendo da cultivar (PEREIRA et al., 2003).

A batata é originária da Cordilheira dos Andes e ocupa o quarto lugar mundial em quantidade de produção sendo superada apenas pelo trigo, milho e arroz (FAO, 1998). O cultivo da batata no Brasil foi intensificado na década de 20, sendo hoje considerada a principal hortaliça do país, tanto em área cultivada (cerca de 170.000 ha

ano^{-1}) como em preferência alimentar (LOPES & BUSO, 1997). Essa planta é um dos alimentos mais consumidos no mundo, devido a sua composição, versatilidade gastronômica e tecnológica, assim como pelo baixo preço de comercialização dos tubérculos (COELHO et al., 1999).

A quantidade de produtos industrializados à base de batata tem crescido nos últimos anos em nível mundial, incluindo o Brasil, principalmente para produtos que podem ser consumidos diretamente, como chips e batata palha, ou produtos para serem preparados, como batatas descascadas e cortadas em palitos resfriados ou pré-fritos congelados (ZORZELLA et al., 2003).

No Brasil, a produção de batata concentra-se nos Estados de Minas Gerais, São Paulo, Paraná e Rio Grande do Sul, sendo esses responsáveis por aproximadamente 98% da produção nacional (IBGE, 2004). Segundo estimativas, em 1994, a área plantada no Estado do Rio Grande do Sul ficou distribuída dessa maneira: 80% com a cv. Baronesa, 10% com a cv. Macaca e 10% com outras cultivares. Já em 2000, as estimativas foram de menos de 50% para Baronesa, cerca de 30% para Asterix (introduzida no Rio Grande do Sul na safra 95/96) e 20% para outras cultivares tais como Macaca, Monalisa, Elvira e Achat. Na Região Central do Rio Grande do Sul, praticamente, não se planta mais a cv. Baronesa, devido, principalmente, à dificuldade de comercialização, visto que, entre as batatas de película rosada, a Macaca e a Asterix têm demonstrado melhor aceitação no mercado. A aparência externa da cultivar Asterix é melhor em relação à cor, ao brilho e à uniformidade em comparação à cv. Baronesa. Quanto à Macaca, os principais nichos são as regiões de colonização alemã, onde as suas qualidades culinárias são muito apreciadas (PEREIRA et al., 2003).

De interesse particular para este trabalho é o fato do Cd acumulado nos tubérculos de batata poder representar mais de 50% do total das intoxicações desse metal em humanos (STENHOUSE, 1992). Além disso, os vegetais, em geral, são as maiores fontes de exposição não-ocupacional ao Cd (SATARUG et al., 2003). As plantas de batata são expostas ao Cd principalmente através da deposição atmosférica e da aplicação de fertilizantes fosfatados (Figura 3). Plântulas de batata expostas ao Cd apresentaram redução no crescimento e alterações nas concentrações de nutrientes minerais e nos parâmetros bioquímicos (GONÇALVES et al., 2009 a,b). Em relação às alterações causadas pelo Cd diretamente aos tubérculos de batata, REID et al. (2003) reportaram que as raízes basais foram a principal fonte de Cd para todos os tecidos

dessa hortaliça, e que as raízes contribuíram para aproximadamente 85% do Cd acumulado nos tubérculos. Nesse mesmo trabalho, os autores reportaram que a periderme do tubérculo acumulou altas concentrações de Cd, mas que a absorção deste metal pelo tubérculo foi limitada. Além disso, DUNBAR et al. (2003) confirmaram a importância do floema na canalização dos Cd e dos elementos minerais aos tubérculos de batata.

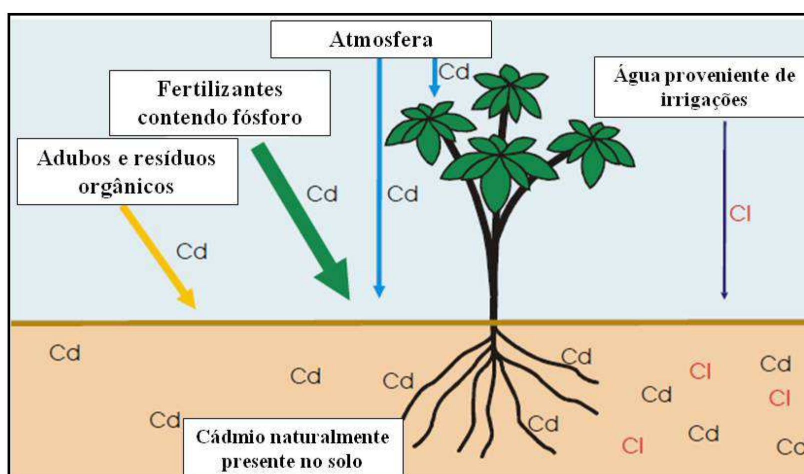


Figura 3. Fontes potenciais de Cd e absorção deste pelos vegetais (http://www.cadmium-management.org.au/documents/Vegenotes.pdf)

1.3. N-acetilcisteína

A N-acetilcisteína (NAC) é um composto tiólico com fórmula química $C_5H_9NO_3S$ e peso molecular 163,2 (Figura 4). É um composto semelhante ao aminoácido cisteína apresentando um grupamento sulfidrílico que é responsável por grande parte da atividade metabólica da NAC, e um substituinte acetil no nitrogênio do grupo amino que garante maior estabilidade da molécula contra oxidação (ATKURI et al., 2007). Além disso, verificou-se que o grupo amida garante maior solubilidade em água e menor toxicidade da NAC se comparada ao aminoácido cisteína, além de permitir a preservação do fármaco no fluido gástrico, pois a maioria dos compostos sulfidrílicos apresenta alta instabilidade nestas condições (BONANOMI & GAZZANIGA, 1980). É uma substância que pode ser administrada por via oral, intravenosa e tópica (ATKURI et al., 2007). Após a ingestão do fármaco, o pico de concentração plasmática pode ser registrado em menos de 1 h tendo uma meia vida

plasmática de aproximadamente 2,5 h e um metabolismo de primeira passagem que torna a sua forma livre totalmente indetectável no plasma após 10-12 h da sua administração (De CARO et al., 1989). Em torno de 16% da NAC é oxidada ao passar pelo trato gastrintestinal, enquanto outros compostos sulfidrílicos apresentam perdas entre 75 e 100% (BONANOMI & GAZZANIGA, 1980). Entretanto, apenas entre 4 e 10% dessa molécula permanece intacta, provavelmente devido a sua ligação dissulfito com as proteínas e desacetilação na mucosa e lúmen gastrintestinal o que contribui para a baixa biodisponibilidade oral da NAC (BORGSTROM et al., 1986; SJODIN et al., 1989).

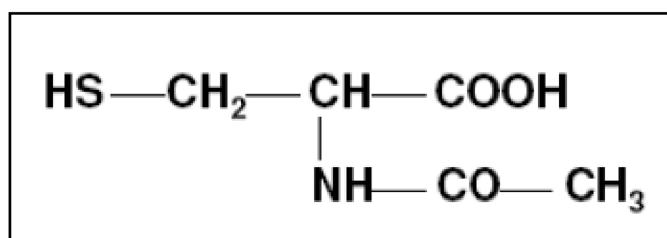


Figura 4. Estrutura química da N-acetilcisteína (http://www.benbest.com/nutrceut/NAC.jpg)

A NAC é considerada uma pró-droga facilmente transportada para o interior das células servindo como fonte para restaurar os níveis de cisteína e glutationa reduzida (GSH) devido a sua habilidade em reduzir a cistina extracelular em cisteína e/ou por ser uma doadora de grupamento sulfidrila. A metabolização desse fármaco libera o aminoácido cisteína que atua como um precursor direto da síntese de GSH (SANTANGELO, 2003). A GSH é um tripeptídeo formado por ácido glutâmico, glicina e cisteína e desempenha uma importante função na manutenção do balanço redox intracelular (HADDAD & HARB, 2005). Além de ser precursora de GSH, a NAC apresenta atividade antioxidante *per se*, por ser capaz de sequestrar radicais hidroxil e superóxido (ARUOMA et al., 1989). Embora já tenha sido verificado que a atividade de enzimas antioxidantes endógenas como a superóxido dismutase e a catalase apresentem uma maior capacidade de varredura de radicais livres se comparada à ação direta da NAC, se sabe que essa molécula pode restaurar o status redox modificado em algumas patologias e intoxicações e, assim, modular a sinalização intracelular (BONANOMI &

GAZZANIGA, 1980; ZAFARULLAH et al., 2003; ATKURI et al., 2007; SADOWSKA et al., 2007).

Em relação ao uso terapêutico, a NAC é utilizada principalmente devido as suas características mucolíticas e destoxificantes na intoxicação com paracetamol (ZAFARULLAH et al., 2003; ATKURI et al., 2007). Seu uso mucolítico é justificado pela capacidade de quebrar as ligações peptídicas das proteínas que constituem o muco, tornando-o menos viscoso e assim mais facilmente eliminado das vias aéreas. Na intoxicação por paracetamol, a NAC age como uma fonte de cisteínas necessárias à síntese *de novo* da GSH a qual é amplamente depletada durante a destoxificação do metabólito hepático do paracetamol (ATKURI et al., 2007). Além disso, vários estudos demonstraram que esse fármaco pode ser utilizado para outras aplicações clínicas, como a utilização em pacientes com problemas vasculares, patologias que apresentem quadros inflamatórios associados, câncer, HIV e diabetes (GREGORY & KELLY, 1998; ATKURI et al., 2007; SADOWSKA et al., 2007). Outra característica importante é o fato da NAC ser capaz de atravessar a barreira hematoencefálica e repor os níveis cerebrais de GSH (DEAN et al., 2004). Sendo assim, a NAC tem sido aplicada como medicamento em patologias que apresentem danos cognitivos como prejuízos nos processos de memória e aprendizagem (BERK et al., 2011a e b; JAIN et al., 2011).

Embora algumas ações benéficas da NAC já estejam bem estabelecidas, poucos trabalhos têm avaliado o seu efeito sobre a resposta a intoxicações por metais pesados. Alguns autores já reportaram que a NAC apresenta resultados benéficos na terapia contra esses agentes. Isto se deve ao seu grupamento sulfidrila o qual se liga aos metais pesados promovendo complexos, e assim, evitando que estes xenobióticos se acumulem no organismo e atuem diretamente nos sistemas celulares causando danos ao organismo (BENITE et al., 2007; PENUGONDA & ERCAL, 2011). Sabe-se que a NAC atua como antioxidante contra as ações dos metais pesados e que, o chumbo e o mercúrio complexados com a NAC causaram diminuição da concentração destes agentes tóxicos na circulação e nos tecidos (BANNER et al., 1986; ROONEY, 2007; PENUGONDA & ERCAL, 2011). Entretanto, raros trabalhos versam sobre a interação entre a NAC e a intoxicação por cádmio. SHAIKH et al. (1999) relataram que a NAC ou a vitamina E preveniram a lipoperoxidação induzida pelo Cd e protegeram os animas contra hepato e nefrotoxicidade. Levando em conta os fatos acima citados e a literatura escassa que indique um potencial benéfico da NAC sobre as intoxicações causadas pelo Cd, torna-se

pertinente a avaliação dos efeitos da NAC em modelos experimentais cujos animais sejam expostas a esse metal.

1.4. Sistema colinérgico

O sistema colinérgico é uma das mais importantes vias modulatórias dos sistemas nervoso central (SNC) e periférico (SNP) (DESCARRIES et al., 1997; SOREQ & SEIDMAN, 2001; PRADO et al., 2002). A acetilcolina (ACh) foi a primeira molécula a ser definida como neurotransmissor e passou a ser amplamente estudada nas sinapses do SNC e do SNP (DESCARRIES et al., 1997). A ACh é sintetizada pela enzima colina acetiltransferase (ChAT; EC 2.3.1.6) a partir da colina, um importante produto do metabolismo dos lipídios da dieta, e acetil-CoA, um produto do metabolismo celular (SOREQ & SEIDMAN, 2001; PRADO et al., 2002). Após sua síntese é carregada até as vesículas sinápticas pelo transportador vesicular da ACh (VAChT) onde fica armazenada até a sua liberação (RAND, 2007). Depois de ser liberada, a ACh se difunde na fenda sináptica e ativa os receptores específicos de ACh (AChRs), posicionados nas células pós-sinápticas. Esses receptores são designados como receptores colinérgicos e, subdivididos em dois grandes grupos: muscarínicos e nicotínicos, que transmitem os sinais por mecanismos diferentes (RANG et al., 2004). Até o momento cinco subtipos de receptores muscarínicos foram identificados (M1-M5) e agem via ativação de proteínas G, sendo que os receptores M1 e M2 estão presentes em neurônios do SNC e SNP além de outros tecidos ganglionares (VAN DER ZEE & LUITEN, 1999). Os receptores nicotínicos são compostos por cinco subunidades conhecidas por α_1, α_2, β, γ e δ e atuam como canais iônicos regulados por ligante e localizam-se, predominantemente, nas sinapses ganglionares (ARIAS, 1998). A ação da ACh é finalizada pela sua hidrólise enzimática na fenda sináptica pela enzima acetilcolinesterase (AChE). A maioria da colina resultante é recaptada pelo terminal pré-sináptico, através de um mecanismo de recaptação de alta afinidade onde poderá ser reutilizada para a síntese de novas moléculas de ACh (SOREQ & SEIDMAN, 2001; MESULAM et al., 2002) (Figura 5).

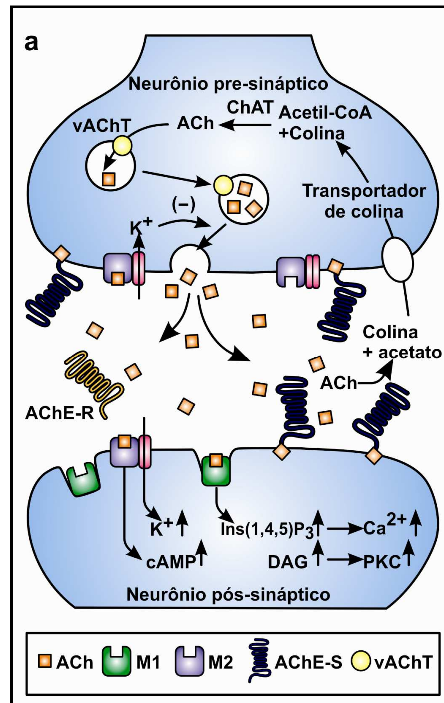


Figura 5. Sinapse colinérgica. ACh = acetilcolina; M1 = receptor muscarínico tipo 1; M2 = receptor muscarínico tipo 2; VAChT = transportador de ACh vesicular; Adaptado de Soreq & Seidman (2001).

1.4.1. Colinesterases

Existem dois tipos de colinesterases: a acetilcolinesterase (AChE; E.C. 3.1.1.7) ou colinesterase verdadeira, que hidrolisa preferencialmente ésteres com grupamento acetil como a ACh e, a butirilcolinesterase (BChE; E.C. 3.1.1.8) ou pseudocolinesterase que hidrolisa outros tipos de ésteres como a butirilcolina (NUNES-TAVARES et al., 2002; NICOLET et al., 2003; WHITTAKER, 2010). A BChE apresenta ampla semelhança estrutural com a AChE. Porém, a AChE é predominantemente encontrada no cérebro (10 vezes mais abundante que a BChE), junção neuromuscular e eritrócitos enquanto, a BChE é principalmente encontrada no plasma, rins, fígado, intestino, coração, pulmão e tem uma distribuição neuronal muito mais restrita em relação a AChE (MESULAM et al., 2002; COKUGRAS, 2003). Além disso, as duas enzimas são também diferenciadas de acordo com seu comportamento frente a diversos tipos inibidores (COKUGRAS, 2003).

1.4.1.1. Acetilcolinesterase

A enzima AChE ocorre nas formas globular e assimétrica. A forma globular é composta por monômeros (G1), dímeros (G2) e tetrâmeros (G4) da subunidade catalítica. A forma G1 é citosólica e a G4 é ligada à membrana, sendo essa última a mais encontrada no tecido nervoso (DAS et al., 2001; ALDUNATE et al., 2004). Em sangue humano, a AChE é encontrada tanto nos eritrócitos quanto no plasma, onde predominam as formas G2 e G4, respectivamente (RAKONCZAY et al., 2005). A forma assimétrica consiste de um (A4), dois (A8) e três (A12), tetrâmeros catalíticos ligados covalentemente a uma subunidade estrutural colagênica chamada Q (ColQ). Essas formas estão associadas à lâmina basal e são abundantes na junção neuromuscular (ALDUNATE et al., 2004) (Figura 6).

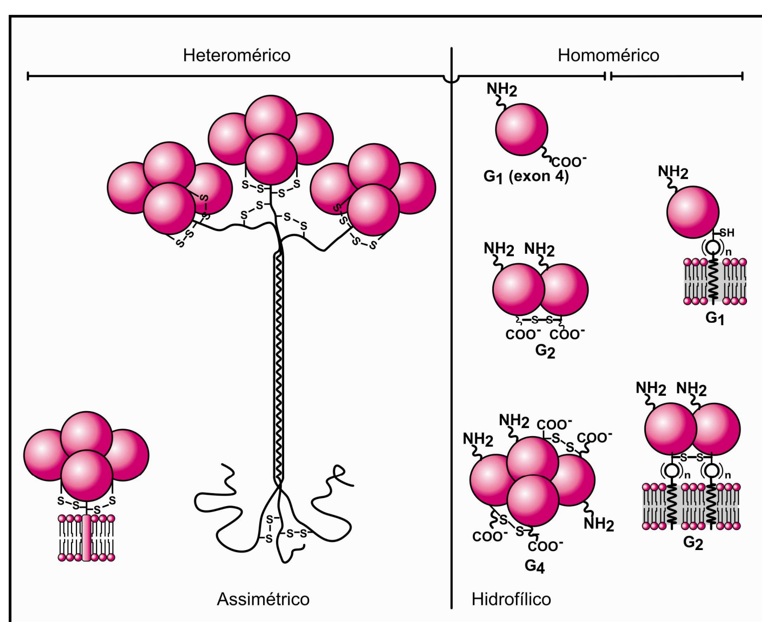
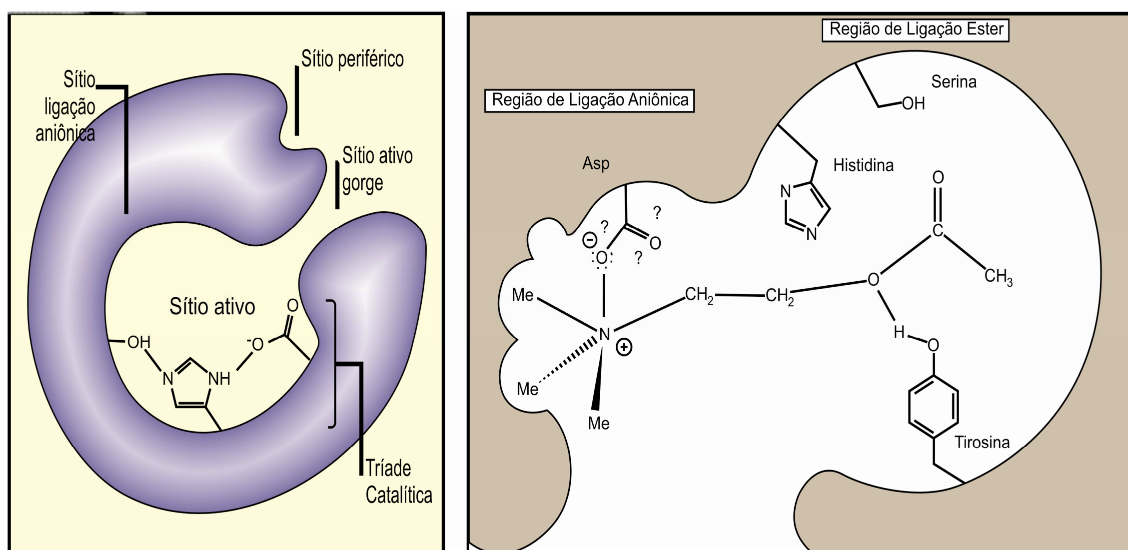


Figura 6. Isoformas da enzima AChE. Estrutura assimétrica (A12) e molecular (G1, G2 e G4) da AChE (http://www.chemistry.emory.edu/ach_inactivation.htm).

O sítio ativo da AChE situa-se na parte inferior de um estreitamento semelhante a uma garganta (*gorge*) a 20 Aº de profundidade, alinhado com resíduos hidrofóbicos, os quais parecem ser importantes na orientação do substrato ao sítio ativo (JOHNSON & MOORE, 1999). Este sítio ativo é formado por um sítio esterásico que contém uma tríade catalítica formada pela serina, histidina e glutamato ou aspartato e um sítio aniônico ao qual a cadeia de nitrogênio quaternário da ACh, carregada positivamente, se liga (SILMAN et al., 1994; DVIR et al., 2010) (Figura 7A). Na borda ou superficie do

gorge, cerca de 14 Aº do sítio ativo, situa-se um segundo sítio aniônico que se tornou conhecido como sítio aniônico periférico (“peripheral anionic site” - PAS), o qual foi proposto com base na ligação de compostos bis quaternários (BOURNE et al., 2005; SILMAN & SUSSMAN, 2008).

Para a catálise enzimática existem duas importantes áreas a serem consideradas: a região de ligação e acomodação do substrato onde a ACh se liga a AChE por ligação iônica a um resíduo de aspartato ou glutamato e a ligação do hidrogênio ao resíduo de tirosina (Figura 7B). A outra região é responsável pelo mecanismo de hidrólise do substrato ACh, composta pelos aminoácidos serina e histidina. O processo enzimático é extremamente eficiente por causa da proximidade do nucleófilo serina e a catálise ácido/básica da histidina, sendo que uma molécula da ACh é hidrolisada em 100 microssegundos (PATRICK, 2001; SILMAN & SUSSMAN, 2005).



(A) **(B)**

Figura 7. A) Sítio esterásico contendo a tríade catalítica, externamente o sítio aniônico periférico (PAS). Adaptado de Soreq & Seidman (2001). B) Interação do substrato (ACh) com o sítio esterásico da AChE. Adaptado de Patrick (2001).

1.4.1.2. Butirilcolinesterase

A BChE é uma enzima sérica, produzida no fígado e adipócitos, com ampla distribuição no organismo (KUTTY, 1980; CHATONNET e LOCKDRIDE, 1989; ORESKOVIC e KUNEC-VAJIC, 1992). A BChE encontrada no cérebro não deriva do

soro, mas é produzida no próprio tecido cerebral (PRODY et al., 1987; McTIERNAN et al., 1987). A BChE, além da ACh, também hidrolisa outros ésteres de colina como a butirilcolina, os relaxantes musculares succinilcolina, mivacúrio, ésteres alifáticos que não contêm colina, como o ácido acetilsalicílico e muitas drogas como a cocaína e a procaína (BOECK et al., 2002).

Após eletroforese em gel de amido, observaram-se cinco bandas para a BChE plasmática que foram chamadas de C1 a C5 em ordem decrescente de mobilidade: monômero (C1), dímero (C3), tetrâmero (C4), monômero ligado à albumina sérica (C2) e tetrâmero ligado a uma substância ainda não identificada (C5) (HARRIS et al., 1962; MASSON, 1989). A forma predominante e de maior atividade da BChE no plasma é o tetrâmero, constituído por dois dímeros de subunidades idênticas. As subunidades de cada dímero estão unidas por uma ligação dissulfeto e os dímeros unem-se através de forças não covalentes. Cada monômero é uma cadeia de 574 resíduos de aminoácidos, com 9 cadeias de carboidratos ligadas a 9 asparaginas (ALTAMIRANO & LOCKRIDGE, 2000). As cadeias de carboidratos parecem não interagir com moléculas do substrato, pois a glicosilação seria importante para o transporte da enzima e não para sua atividade catalítica (DREYFUS et al., 1989; MILLARD & BROOMFIELD, 1992).

Cada tetrâmero contém quatro sítios ativos. A BChE possui seu sítio ativo em forma de garganta composto por aproximadamente 55 aminoácidos (Figura 8). O volume da garganta na molécula da BChE é maior que na AChE devido ao fato de alguns resíduos de aminoácidos situados na entrada da garganta serem alifáticos na BChE e aromáticos na AChE e esse é um dos aspectos responsáveis pelas diferenças na seletividade das enzimas por substratos (SAXENA et al., 1997). O sítio aniônico periférico, localizado na boca da garganta, é formado pelos aminoácidos D70 (ácido aspártico) e Y332 (tirosina) os quais estão envolvidos na ligação inicial de substratos carregados positivamente. O aminoácido D70 forma uma alça ômega (Ω) com W82 (triptofano), componente do subsítio de ligação do sítio ativo (MASSON et al., 2001). A cavidade oxianiônica localizada próxima ao sub-sítio de ligação, inclui os aminoácidos G116 (glicina), G117 e A199 (alanina). O sítio esterásico da BChE é formado pela tríade catalítica dos aminoácidos S198 (serina), H438 (histidina) e E325 (ácido glutâmico), responsáveis pela hidrólise do substrato (SHAFFERMAN et al., 1992; ÇOKUĞRAŞ, 2003; NICOLET et al, 2003).

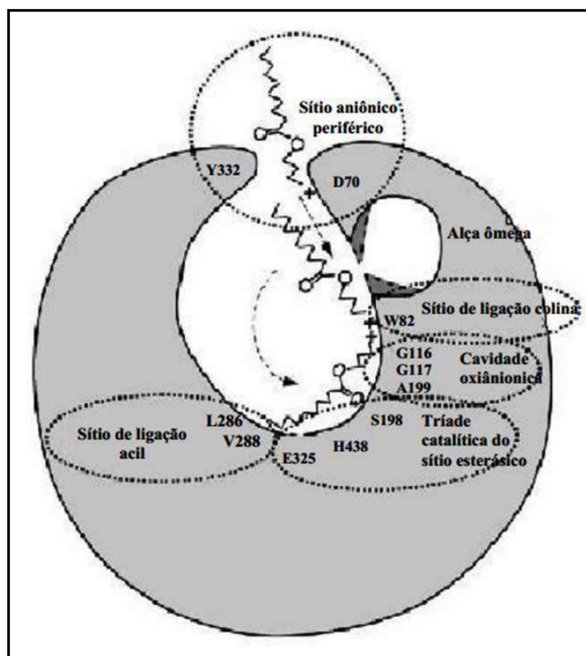


Figura 8. Estrutura esquemática do sítio ativo do monômero da butirilcolinesterase. Adaptado de Cokugras (2003).

1.4.2. Funções das colinesterases

A AChE é uma enzima muito importante no controle da transmissão dos impulsos nervosos através das sinapses, sendo por isso considerada um bom indicador da atividade colinérgica (SZEGLETES et al.,1999). Além disso, alterações na atividade da AChE tem sido associadas a danos nos processos de memória e aprendizagem (SCHMATZ et al., 2009a). Sabe-se que inibidores da AChE são frequentemente utilizados na prática clínica no tratamento contra a doença de Alzheimer e, que a intoxicação por organofosforados induz classicamente a inibição irreversível da atividade dessa enzima (SIKORA et al., 2011; MEHTA et al., 2012). Embora a maioria dos estudos relacionados à AChE versem sobre o sistema nervoso, nos últimos anos, verificou-se que essa enzima está presente em outras células além dos neurônios, tais como linfócitos, eritrócitos e células endoteliais (KAWASHIMA et al., 1990; KAWASHIMA et al., 2007; NEUMAN et al., 2007). É possível que a ACh proveniente dessas fontes esteja envolvida na modulação do processo inflamatório local (ROSAS-BALLINA & TRACEY, 2009). Esta via "colinérgica anti-inflamatória" mediada pela ACh, atua inibindo a produção de fator de necrose tumoral (TNF), interleucina-1 (IL-1) e suprime a ativação da expressão do fator nuclear kappa-B (NF-kB) (BOROVIKOVA

et al., 2000; RAO et al., 2007; ROSAS-BALLINA & TRACEY, 2009). Assim, um aumento na atividade das enzimas AChE e BChE poderia levar a diminuição dos níveis de ACh, reduzindo seus efeitos anti-inflamatórios, devido a ausência do controle de feedback negativo exercido por essa molécula (RAO et al., 2007). Dessa forma, é concebível que a atividade dessas enzimas seja reguladora intrínseca da inflamação (DAS, 2007; ROSAS-BALLINA & TRACEY, 2009).

Embora a função biológica da BChE ainda não esteja claramente estabelecida, observou-se que diversos fatores e patologias podem influenciar na atividade da BChE. MESULAM et al. (2002), em estudo realizado em ratos nulizigotos onde o gene da AChE foi desativado, observaram que a BChE passou a modular a transmissão colinérgica, substituindo a AChE nas junções neuromusculares hidrolisando a acetilcolina. Os autores sugerem que a BChE não apenas funciona como um recurso de segurança, evitando a morte do animal por hiperativação das vias colinérgicas, mas também pode estar normalmente contribuindo para o processo de transmissão nessas vias. Alguns estudos revelaram que a BChE confere uma proteção natural importante contra os organofosforados impedindo que este atinja o sítio alvo, que é a AChE nas vias sinápticas nervosas (BROOMFIELD et al., 1991). Entre fatores que ocasionam a diminuição da sua atividade encontram-se os carcinomas, tuberculose, hepatite e infarto do miocárdio e, entre os que se relacionam com o aumento encontram-se a hiperlipoproteinemia, a obesidade e o diabetes (SIDELL & KAMINSKIS, 1975; KUTTY, 1980; LUNKES et al., 2006). Como não se chegou ainda a um consenso quanto a sua função fisiológica, o principal interesse das medidas laboratoriais da BChE tem sido sua relação com relaxantes musculares e com quadros de intoxicações. Considerando-se as funções expostas acima, o estudo da atividade das enzimas colinesterases em diferentes tipos celulares de animais intoxicados por cádmio torna-se relevante e, faz-se necessário associar a atividade dessas enzimas com outras respostas bioquímicas e comportamentais relacionadas à intoxicação por este metal.

1.5. Sistema purinérgico

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

extracelulares de adenina, ATP, ADP e AMP, e o nucleosídeo adenosina são considerados, atualmente, importantes moléculas sinalizadoras devido ao seu papel em modular uma variedade de processos biológicos mediando os seus efeitos através de receptores purinérgicos localizados na superfície celular (ILLES & RIBEIRO, 2004; SCHETINGER et al., 2007) (Figura 9). Cada nucleotídeo, uma vez presente no meio extracelular, desempenha sua ação pela ligação a um tipo diferente de receptor localizado na superfície de vários tipos de células (YEGUTKIN, 2008). Esses receptores dividem-se em dois grupos: P2X e P2Y. Os receptores P2X acoplados a canais iônicos com seus domínios carboxi e aminoterminal voltados para o meio intracelular e compreendem sete subtipos nomeados de P2X1-7 (DI VIRGILIO et al., 2001). Já os receptores P2Y são acoplados a proteína G apresentando sete regiões transmembrana com a porção aminoterminal voltada para meio extracelular e a porção carboxiterminal voltada para o meio citoplasmático compreendendo 14 subtipos os quais foram nomeados de P2Y1-14 (YEGUTKIN, 2008). Os receptores para adenosina incluem quatro tipos: A1, A2A, A2B e A3, os quais são proteínas transmembrana acoplados a proteína G (HASKO et al., 2008; YEGUTKIN, 2008).

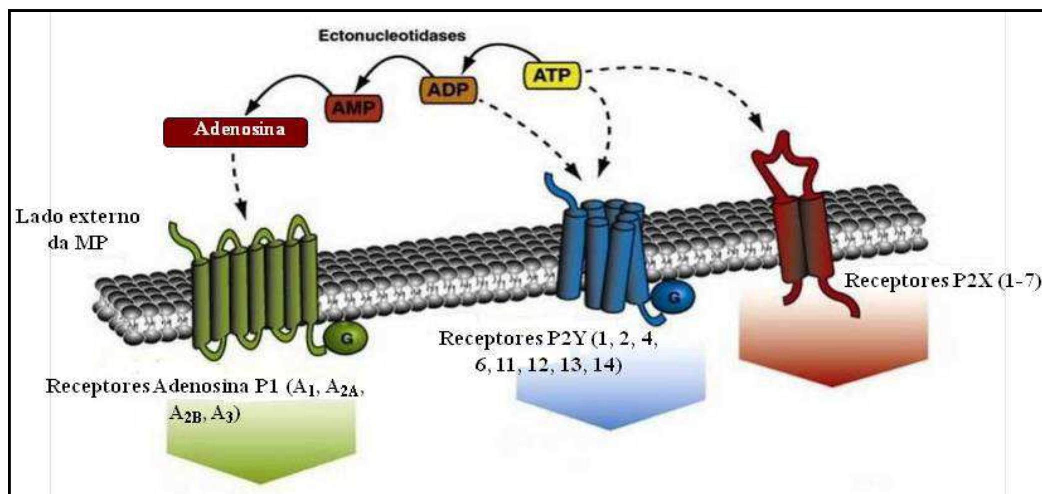


Figura 9. Receptores envolvidos na resposta fisiológica dos nucleotídeos e nucleosídeos extracelulares de adenina (http://ars.sciencedirect.com/content/image/1-s2.0-S0005273612000065-gr1.jpg).

As concentrações dessas moléculas nos fluidos extracelulares dependem de vários fatores como a quantidade liberada, mecanismos de recaptação, situações de lise celular e a presença de enzimas como as ectonucleotidases (RATHBONE et al., 1999).

1.5.1. Enzimas que degradam nucleotídeos e nucleosídeos de adenina

Uma vez exercidos seus efeitos, as moléculas de nucleotídeos e nucleosídeos devem ser degradadas de modo a manter seus níveis em concentrações fisiológicas. Para isso, existe uma ampla classe de proteínas catalíticas (ectonucleotidases) ancoradas à membrana as quais contêm seu sítio ativo voltado para o meio extracelular e efetuam a degradação destes nucleotídeos (GODING & HOWARD, 1998; ZIMMERMANN, 2001; ZIMMERMANN et al., 2007). Dentre estas enzimas pode-se destacar as E-NTPDases (Ecto-Nucleosídeo Trifosfato Difosfoidrolase), a família das E-NPPs (Ecto-Nucleotídeo Pirofosfatase/Fosfodiesterases), 5'-nucleotidase e a adenosina desaminase (ADA) (ROBSON et al., 2006; YEGUTKIN, 2008). Estas enzimas atuam em conjunto, formando uma cadeia enzimática que tem inicio com a ação da E-NTPDase e da E-NPP, as quais catalisam a hidrólise do ATP e do ADP formando AMP (ZIMMERMANN et al., 2007). A seguir a enzima 5'- nucleotidase hidrolisa a molécula de AMP formando adenosina, a qual posteriormente é degradada pela ação da ADA gerando inosina (YEGUTKIN, 2008) (Figura 10).

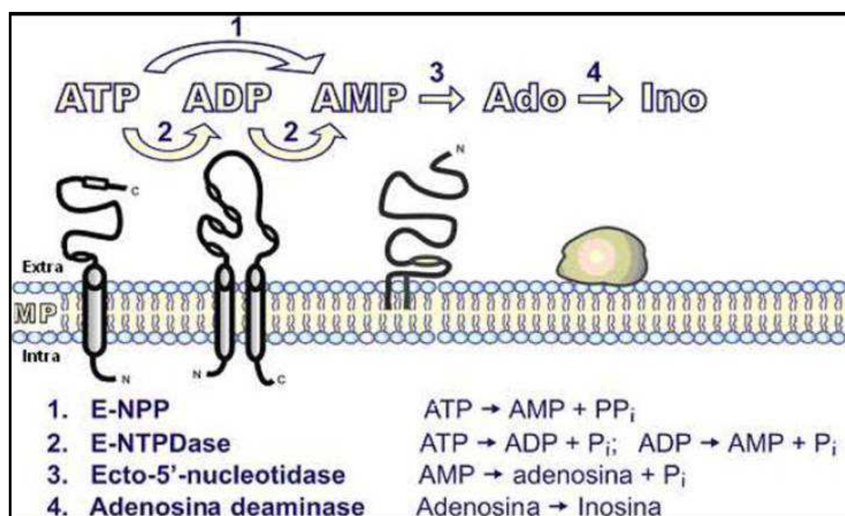


Figura 10. Enzimas envolvidas na degradação extracelular de nucleotídeos e nucleosídeos de adenina. Adaptado de Yegutkin (2008).

1.5.1.1. NTPDase

E-NTPDases é um termo genérico para designar uma família de enzimas responsáveis pela hidrólise de nucleotídeos tri e difosfatados (ZIMMERMANN et al., 2007). Essa classe de enzimas inclui oito membros nomeados de NTPDase 1-8 que diferem quanto a especificidade por substratos, distribuição tecidual e localização celular. Quatro destas enzimas estão localizadas na membrana celular com seu sítio catalítico voltado para o meio extracelular (NTPDase 1, 2, 3 e 8) e quatro delas exibem localização intracelular (NTPDase 4, 5, 6 e 7) (ROBSON et al., 2006).

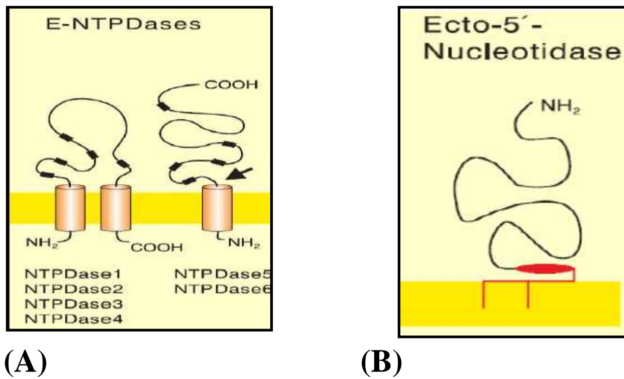
Todos os membros da família das NTPDases apresentam 5 regiões denominadas regiões conservadas da apirase (ACRs), o que torna essa característica uma "marca" desse conjunto de enzimas. Essas ACRs são de muita relevância para sua atividade catalítica (ZIMMERMANN, 2001; ROBSON et al., 2006).

A NTPDase-1 (E.C 3.6.1.5, CD39) foi a primeira enzima da família NTPDase a ser descrita, e está ancorada na superfície celular através de duas regiões transmembranas próximas ao grupamento amino e carboxiterminal, com o seu sítio catalítico voltado para o meio extracelular (ZIMMERMANN, 2001; ROBSON et al., 2006). Esta enzima hidrolisa tanto ATP como ADP formando AMP na presença de íons Ca^{2+} e Mg^{2+} (ZIGANSHIN et al., 1994) (Figura 11A).

1.5.1.2. Ecto- 5'-nucleotidase

Até o momento, sete sub-tipos da enzima 5'-nucleotidase foram isoladas e caracterizadas em humanos. Essas enzimas variam na localização subcelular, sendo cinco delas localizadas no citosol, uma na matriz mitocondrial e uma anexada à membrana plasmática externa. Enquanto essas enzimas compartilham a habilidade em hidrolisar 5'- nucleosídeos monofosfatos e se sobrepõem na especificidade ao substrato, elas variam na afinidade por 5'- monofosfatos (HUNSUCKER et al., 2005).

A ecto-5'-nucleotidase (E.C. 3.1.3.5, CD73) é uma glicoproteína ligada à membrana via um glicosil fosfatidilinositol (GPI) com seu sítio catalítico voltado para o meio extracelular que catalisa a hidrólise éster fosfórica do 5'- ribonucleotídeo para o correspondente ribonucleosídeo e fosfato. Ocorre na forma de dímero e seu peso molecular esta na faixa de 62 a 74 Kd. (ZIMMERMANN, 2001; HUNSUCKER et al., 2005) (Figura 11B).



(A) **(B)**

Figura 11. Estrutura da NTPDase (A) e ecto-5'-nucleotidase (B) ancorada à membrana. Adaptado de Zimmermann (2001).

1.5.2. Funções da NTPDase e 5'-nucleotidase

Os nucleotídeos extracelulares de adenina e o seu respectivo nucleosídeo adenosina modulam uma variedade de efeitos teciduais (CUNHA & RIBEIRO, 2000; BURNSTOCK et al., 2004). Sendo assim, as ecto-enzimas envolvidas na conversão dessas moléculas apresentam importantes funções fisiológicas.

Em relação ao sistema nervoso, as enzimas NTPDase e 5'-nucleotidase, amplamente distribuídas no cérebro de ratos (neurônios de córtex cerebral, hipocampo, cerebelo, células gliais e células endoteliais), desempenham funções importantes na neurotransmissão e neuromodulação (WANG & GUIDOTTI, 1998; CUNHA & RIBEIRO, 2000; DUNWIDDIE & MASINO, 2001). O ATP é um neurotransmissor excitatório essencial nas sinapses nervosas purinérgicas e também age como um neuromodulador. Esse nucleotídeo pode ser armazenado e liberado no meio extracelular juntamente com os outros neurotransmissores, tais como a ACh, glutamato e noradrenalina através das vesículas pré-sinápticas dependentes de Ca^{2+} (ILLES & RIBEIRO, 2004). A adenosina é capaz de regular a liberação de vários neurotransmissores, agindo tanto pré quanto pós-sinapticamente (CUNHA, 2001; DUNWIDDIE & MASINO, 2001; RIBEIRO et al., 2003). Essa atuação da adenosina tem se tornado relevante em vários processos patológicos, pois ela pode limitar o dano causado pela excitotoxicidade desses neurotransmissores, exercendo assim uma ação protetora no SNC (ZIMMERMANN et al., 1998; DUNWIDDIE & MASINO, 2001; PURVES et al., 2005).

Além das propriedades neurotransmissoras e neuromoduladoras, estudos colocaram em evidência muitas funções dos nucleotídeos e do nucleosídeo de adenina nos sistemas vascular e imunológico. A NTPDase desempenha um papel importante no

sistema hemostático, uma vez que ela controla os efeitos pró-trombóticos e pró-inflamatórios de nucleotídeos como o ATP e o ADP (YEGUTKIN, 2008). A NTPDase, presente nas células endoteliais e membrana de plaquetas, converte o ATP em ADP e subsequentemente o ADP, um nucleotídeo promotor da agregação, em AMP. A enzima 5'-nucleotidase converte o AMP formado em adenosina. A adenosina, por sua vez, tem propriedades vasodilatadoras sendo um modulador do tônus vascular e inibidor da agregação plaquetária (ZIMMERMANN, 2001).

Além disso, as enzimas NTPDase e 5'-nucleotidase também são encontradas em células do sistema imunológico estando envolvidas na regulação de muitas respostas imunes e inflamatórias (BOURS et al., 2006; COLGAN et al., 2006). O ATP é uma molécula que possui funções pró-inflamatórias como a estimulação e a proliferação de linfócitos sendo essencial para a liberação de citocinas como a interleucina 2 (IL-2) e o interferon γ (IFN-γ) (LANGSTON et al., 2003; BOURS et al., 2006). Por outro lado, a adenosina tem potentes atividades anti-inflamatórias e imunossupressoras por inibir a proliferação de células T através da ativação de receptores A2A e a liberação de citocinas pró-inflamatórias (GESSI et al., 2007).

Levando-se em consideração as funções descritas acima, a NTPDase em combinação com a 5'-nucleotidase tem um papel crucial na desfosforilação sequencial destes nucleotídeos e, consequentemente, em processos que incluem a neurotransmissão e neuromoduação do SNC, a inflamação, o tônus vascular e a função plaquetária (CUNHA & RIBEIRO, 2000; BURNSTOCK, et al., 2004). De fato, nos últimos anos o papel dessas enzimas tem sido avaliado em várias condições patológicas e toxicológicas, como no diabetes (LUNKES et al., 2003; SCHMATZ et al., 2009b); na síndrome da imunodeficiência adquirida (AIDS) (LEAL et al., 2005), no câncer (ARAÚJO et al., 2005; MALDONADO et al., 2010), na esclerose múltipla (SPANEVELLO et al., 2010a, b) e na intoxicação por alumínio (KAIZER et al., 2007). Neste sentido alguns trabalhos também já foram realizados tanto *in vitro* quanto *in vivo* visando esclarecer os efeitos do Cd sobre estes nucleotídeos e sobre as enzimas relacionadas ao seu catabolismo (DAHM et al., 2006; SENGER et al., 2006). Sendo assim, o estudo da atividade das ecto-enzimas do sistema purinérgico em diferentes tipos celulares de animais expostos ao Cd torna-se relevante e, faz-se necessário associar a atividade dessas enzimas com outras respostas bioquímicas e comportamentais relacionadas à intoxicação por esse metal.

2. OBJETIVOS

2.1. Objetivo Geral

O objetivo da presente tese foi investigar os efeitos da intoxicação por cádmio (Cd) sobre parâmetros comportamentais e memória, bioquímicos, hematológicos e a bioacumulação desse metal em diferentes tipos celulares de ratos expostos ao cloreto de Cd ($CdCl_2$) ou ao Cd proveniente de batatas contaminadas, e ainda utilizar o antioxidante N-acetilcisteína (NAC) no tratamento deste tipo de intoxicação buscando-se avaliar o seu possível efeito protetor nesses animais.

2.2. Objetivos Específicos

A. Investigar os efeitos da NAC sobre parâmetros comportamentais e memória, atividade da enzima acetilcolinesterase, níveis de peroxidação lipídica e determinação de Cd em diferentes estruturas cerebrais de ratos expostos ao $CdCl_2$ e tratados com NAC;

B. Determinar o possível efeito protetor da NAC em relação à intoxicação por $CdCl_2$ através da verificação da concentração de Cd no plasma, baço e timo, de dados hematológicos e também, da atividade de enzimas periféricas NTPDase, acetilcolinesterase e butirilcolinesterase em ratos expostos ao $CdCl_2$ e tratados com NAC;

C. Verificar a atividade das enzimas NTPDase e 5'-nucleotidase em sinaptossomas de córtex cerebral e de hipocampo e, em plaquetas de ratos expostos ao $CdCl_2$ e tratados com NAC;

D. Avaliar os efeitos do $CdCl_2$ bem como do Cd proveniente de batatas contaminadas sobre parâmetros comportamentais e memória, atividade das enzimas

acetilcolinesterase e Na^+,K^+-ATPase e determinação de Cd em diferentes estruturas cerebrais de ratos.

3. CAPÍTULO 1

Artigo: N-acetylcysteine prevents memory deficits, the decrease in acetylcholinesterase activity and oxidative stress in rats exposed to cadmium – Publicado no Periódico *Chemico-Biological Interactions*

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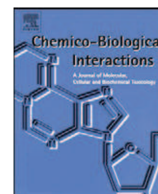

N-acetylcysteine prevents memory deficits, the decrease in acetylcholinesterase activity and oxidative stress in rats exposed to cadmium

Jamile F. Gonçalves [a], Amanda M. Fiorenza [b], Roselia M. Spanevello [b], Cinthia M. Mazzanti [c], Guilherme V. Bochi [b], Fabiane G. Antes [b], Naiara Stefanello [b], Maribel A. Rubin [b], Valderi L. Dressler [b], Vera M. Morsch [b], Maria Rosa C. Schetinger [a,b,*]

[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcellos, 2600-Anexo, 90035-003 Porto Alegre, RS, Brazil
[b] Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil
[c] Departamento de Clínica de Pequenos Animais, Setor de Patologia Clínica Veterinária, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil

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ABSTRACT

The present study investigated the effect of the administration of N-acetylcysteine (NAC), on memory, on acetylcholinesterase (AChE) activity and on lipid peroxidation in different brain structures in cadmium (Cd)-exposed rats. The rats received Cd (2 mg/kg) and NAC (150 mg/kg) by gavage every other day for 30 days. The animals were divided into four groups (n = 12–13): control/saline, NAC, Cd, and Cd/NAC. The results showed a decrease in step-down latency in the Cd-group, but NAC reversed the impairment of memory induced by Cd intoxication. Rats exposed to Cd and/or treated with NAC did not demonstrate altered shock sensitivity. Decreased AChE activity was found in hippocampus, cerebellum and hypothalamus in the Cd-group but NAC reversed this effect totally or partially while in cortex synaptosomes and striatum there was no alteration in AChE activity. An increase in TBARS levels was found in hippocampus, cerebellum and hypothalamus in the Cd-group and NAC abolished this effect while in striatum there was no alteration in TBARS levels. Urea and creatinine levels were increased in serum of Cd-intoxicated rats, but NAC was able to abolish these undesirable effects. The present findings show that treatment with NAC prevented the Cd-mediated decrease in AChE activity, as well as oxidative stress and consequent memory impairment in Cd-exposed rats, demonstrating that this compound may modulate cholinergic neurotransmission and consequently improve cognition. However, it is necessary to note that the mild renal failure may be a contributor to the behavioral impairment found in this investigation.

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1. Introduction

Cadmium (Cd) is a ubiquitous environmental toxicant, which adversely affects biological systems in various ways. It is present as an industrial pollutant, a food contaminant and as one of the major constituents of cigarette smoke [1]. Accordingly, as the environment continues to be contaminated with this metal, there is an increasing risk of humans and other mammals being exposed to Cd [2]. As of yet, Cd has not been shown to have any physiological function within the human body [1].

Cd exerts its toxic effects not only on the kidneys, liver and testis but also on the central nervous system (CNS) [3], due to a number of factors, namely, its toxicity at low levels, its long biological half-life (15–30 years in humans) and its low rate of body excretion [2]. The nervous system is protected from many potential toxicants through an anatomically defined barrier, called the blood–brain barrier (BBB) [4]. Cd can penetrate the BBB and accumulate into the brain [4,5]. Acute Cd toxicity led to brain intracellular accumulation, cellular dysfunction and lethal cerebral oedema [6,7]. Recent reports have investigated the influence of Cd on synaptic neurotransmission and neurotransmitter and antioxidant levels in animal brain [3,8,9].

Cholinergic neurons and their projections are widely distributed throughout the CNS with an essential role in regulating many vital functions, such as learning, memory, cortical organization of movement and cerebral blood flow control [10]. One of the most important mechanisms responsible for correct cholinergic activ-

* Corresponding author at: Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Av. Roraima, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil. Tel.: +55 55 3220 9557; fax: +55 55 3220 9557.
E-mail addresses: mariaschetinger@gmail.com, mariarosa@smail.ufsm.br (M.R.C. Schetinger).

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ity is performed by acetylcholinesterase (AChE) [11]. This enzyme hydrolyses the neurotransmitter acetylcholine (ACh) in the synaptic cleft of cholinergic synapses and neuromuscular junctions [12]. Some studies on Cd toxicity have found an increase or a decrease in AChE activity which may indicate alterations in cholinergic neurotransmission and consequently an association to behavioral impairments observed in both animal models and humans exposed to Cd [3]. Although there are scarce epidemiological studies in the occupational setting, it is known that Cd exposure can generate neurobehavioral disturbances such as altered attention, psychomotor, speed, memory and visuomotor functioning in workers [13,14] as well as impaired social memory process and changes in the development of the visual system in rats [15,16].

In addition, AChE responds to various insults including oxidative stress, an important event that has been related to Cd toxicity [4,17]. The literature data have demonstrated that the brain is susceptible to lipid peroxidation (LPO) because of its high rate of oxygen utilization, abundant supply of polyunsaturated fatty acids, deficient antioxidant defense and high content of transition metals such as copper and iron in several regions [18]. In fact, increasing evidence suggests that the excessive production of reactive oxygen species (ROS) in the brain, and the imbalance between oxidative stress and antioxidant defenses is related to Cd exposure [4].

Antioxidant drugs are becoming increasingly popular in oxidative stress-related disorders and hold promise as therapeutic agents [3]. N-acetylcysteine (NAC) acts as an antioxidant by restoring the pool of intracellular reduced glutathione, which is often depleted as a consequence of increased status of oxidative stress and inflammation [19]. Furthermore, NAC also has reducing and antioxidant properties, acting as a direct scavenger of ROS [20].

Therefore, considering that Cd toxicity is associated with behavioral impairments and that NAC has important antioxidant actions, the aim of this study was to investigate the effects of this compound on learning and memory, AChE activity as well as oxidative stress in brain structures of Cd-exposed rats.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), tris-(hydroxymethyl)-aminomethane GR and Coomassie brilliant blue G were obtained from Sigma Chemical Co. (St. Louis, MO, USA). N-acetylcysteine was obtained from Beg (São Paulo, SP, Brazil) with 99.1% of purity. All other reagents used in the experiments were of analytical grade and of the highest purity.

2.2. Animals

Adult male Wistar rats (80 days; 315.7 ± 14 g) from the Central Animal House of the Federal University of Santa Maria (UFSM) were used in this experiment. The animals were maintained at a constant temperature (23 ± 1 °C) on a 12 h light/dark cycle with free access to food and water. All animal procedures were approved by the Animal Ethics Committee from UFSM.

2.3. Experimental procedure

The body burden of Cd is derived primarily from the ingestion of food and water contaminated with Cd and $CdCl_2$ is the principal form of Cd associated with oral exposure, as it is highly soluble in water [2]. Thus, in the present study the rats received cadmium as $CdCl_2 \cdot H_2O$ (Cd; 2 mg/kg) [21,22] and N-acetylcysteine (NAC; 150 mg/kg) [20] by gavage every other day for 30 days. The animals were randomly divided into four groups ($n = 12$ per group): control/saline, NAC, Cd and Cd/NAC. The last group received NAC 30 min after Cd. The solutions were freshly prepared in saline and were administered (1 mL/kg) between 9 and 11 a.m.

2.4. Behavioral procedure

2.4.1. Inhibitory avoidance

One day after the end of the treatment, animals were subjected to training in a step-down inhibitory avoidance apparatus according to Guerra et al. [23]. Briefly, the rats were subjected to a single training session in a step-down inhibitory avoidance apparatus, which consisted of a 25 cm × 25 cm × 35 cm box with a grid floor whose left portion was covered by a 7 cm × 25 cm platform, 2.5 cm hight. 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 2-s 0.4-mA shock was applied to the grid. The retention test took place in the same apparatus 24 h later. Test step-down latency was taken as a measure of retention, and a cut-off time of 600 s was established.

2.4.2. Foot shock sensitivity test

Reactivity to shock was evaluated in the same apparatus used for inhibitory avoidance, except that the platform was removed. The modified "up and down" method by Rubin et al. [24] was used to determine the flinch, jump and vocalization thresholds in experimentally naïve animals. Animals were placed on the grid and allowed a 3 min habituation period before the start of a series of shocks (1 s) delivered at 10 s intervals. Shock intensities ranged from 0.1 to 0.6 mA in 0.1 mA increments. The adjustments in shock intensity were made in accordance with each animal's response. The intensity was raised by one unit when no response occurred and lowered by one unit when there was a response. A flinch response was defined as withdrawal of one paw from the grid floor, and a jump response was defined as withdrawal of three or four paws. Two measurements of each threshold (flinch, jump and vocalization) were taken and the mean of each score was calculated for each animal.

2.5. Brain tissue preparation

After behavioral tests, the animals were anesthetized and submitted to euthanasia. The cranium was opened and the structures were gently removed and separated into cerebral cortex (CO), striatum (ST), hippocampus (HC), cerebellum (CE) and hypothalamus (HT). To verify the cadmium concentration in brain structures, six or seven animals per group were randomly chosen. For the other animals, synaptosomes from the cerebral cortex were obtained and all the other brain structures were homogenized in a glass potter in a solution of 10 mM Tris–HCl, pH 7.4, on ice, at a proportion of 1:10 (w/v). The homogenate was centrifuged at 1800 rpm for 10 min and the resulting supernatant was stored at −30 °C until utilization. Protein was determined previously and adjusted for each structure: CO (0.6–0.8 mg/mL), ST (0.4 mg/mL), HC (0.8 mg/mL), CE (0.5–0.6 mg/mL), and HT (0.6 mg/mL) according to the Bradford method [25] using bovine serum albumin as standard solution.

2.6. Cadmium concentration in brain and kidney tissues

Brain structures and kidney were weighted in glass vessels and 3–8 mL of HNO_3 was added for digestion. Digestion was performed using a block (Velp Scientifica, Milano, Italy) heated at 130 °C during 3 h. After this time, 2 mL of H_2O_2 was added and the samples were heated for 1 h. Digested samples were then transferred to polypropylene flasks for Cd determination. Cd determination was

performed by inductively coupled plasma mass spectrometry (ICP-MS). An ICP-MS equipment (PerkinElmer Sciex, model ELAN DRC II, Thornhill, Canada), equipped with a concentric nebulizer (Meinhard Associates, Golden, USA), a cyclonic spray chamber (Glass Expansion, Inc., West Melbourne, Australia) and a quartz torch with an injector tube of 2 mm i.d. was used.

2.7. Synaptosome preparation

The cerebral cortex was homogenized in 10 volumes of an ice-cold medium (medium I), consisting of 320 mM sucrose, 0.1 mM EDTA and 5 mM HEPES, pH 7.5, in a motor driven Teflon-glass homogenizer. The synaptosomes were isolated as described by [26] using a discontinuous Percoll gradient. The pellet was suspended in an isoosmotic solution and the final protein concentration was adjusted to 0.4–0.6 mg/mL. Synaptosomes were prepared fresh daily, maintained at 0–4 °C throughout the procedure and used for enzymatic assays.

2.8. Cerebral AChE enzymatic assay

The AChE enzymatic assay was determined by a modification of the spectrophotometric method of Ellman et al. [27] as previously described by Rocha et al. [28]. The reaction mixture (2 mL final volume) contained 100 mM K^+-phosphate buffer, pH 7.5 and 1 mM 5,5′-dithio-bis-nitrobenzoic acid (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 2-min incubation at 25 °C. The enzyme 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 enzyme activity was expressed in μmol AcSCh/h/mg of protein.

2.9. Thiobarbituric acid reactive substances (TBARS) measurement

Brain TBARS levels were determined according to Ohkawa et al. [29] by measuring the concentration of malondialdehyde (MDA) as an end product of lipid peroxidation by reaction with thiobarbituric acid (TBA). Briefly, the reaction mixture, containing 200 μL of brain supernatants or standard (0.03 mM MDA), 200 μL of 8.1% sodium dodecyl sulphate (SDS), 500 μL of 0.8% TBA and 500 μL of acetic acid solution (2.5 M HCl, pH 3.4), was heated at 95 °C for 120 min. The absorbance was measured at 532 nm. TBARS tissue levels were expressed as nmol MDA/mg protein.

2.10. Urea and creatinine levels

Urea and creatinine levels were measured using standard enzymatic methods with the use of Ortho-Clinical Diagnostics—Johnson & Johnson reagents, with a fully automated analyzer (Vitros 950®—dry chemistry, Rochester, New York).

2.11. Statistical analysis

Statistical analysis of training and test step-down latencies was carried out by the Scheirer–Ray–Hare extension of the Kruskal–Wallis test (nonparametric two-way ANOVA). Foot shock sensitivity was analyzed by unpaired *t* test. All other parameters evaluated were analyzed by two-way ANOVA, followed by Duncan's multiple range tests, where $p < 0.05$ was considered to represent a significant difference in all experiments.

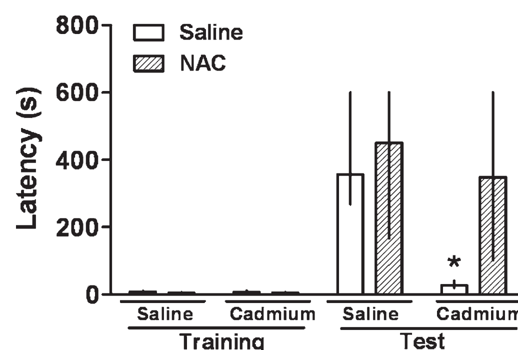

Fig. 1. N-acetylcysteine (NAC) improved memory impairment in the inhibitory avoidance test in rats exposed to cadmium (Cd). After one treatment-free day animals were tested in a step-down latency test. Data are median ± interquartile range of training and test. *$p < 0.05$ compared with the others groups at testing by the two-way ANOVA following by Duncan's test ($n = 6$).

3. Results

3.1. Behavioral tests

Fig. 1 shows the effect of NAC *per se* and in Cd-exposed rats on step-down latencies. Statistical analysis of testing (nonparametric two-way ANOVA) showed a significant Cd or saline vs NAC or saline interaction ($F_{1,20} = 4.43$; $p < 0.05$), revealing that treatment with NAC reversed the impairment of memory induced by Cd. Statistical analysis of training showed no difference between groups.

Because motivational disparities in the training session may account for differences in inhibitory avoidance at testing, experiments were performed to assess whether Cd or NAC affected shock threshold of the animals. Statistical analysis revealed that neither Cd nor NAC altered foot shock sensitivity, as demonstrated by the similar flinch, jump and vocalization thresholds exhibited by the animals. These data suggest that neither Cd intoxication nor treatment with NAC administered before training of inhibitory avoidance altered foot shock sensitivity (Table 1).

3.2. Cd concentration in brain and kidney tissues

All the brain structures analyzed showed a similar Cd concentration within the same group of rats. Control rats (saline) and rats treated with NAC alone showed Cd concentrations of lower than 0.015 μg/g in different brain structures. Rats exposed to Cd alone or Cd plus NAC showed Cd concentrations of around 0.020 μg/g in different brain structures. A significant main effect of Cd ($p < 0.05$) and NAC ($p < 0.05$) in Cd concentrations in different brain structures and in kidney tissue was observed (Table 2). Results demonstrate that Cd exposure alone or in combination with NAC caused an increase of around 40% in Cd brain concentration. On the other hand, post hoc comparisons demonstrate that rats exposed to Cd alone and in combination with NAC presented an increase of 2800% and 2500% in Cd kidney concentration, respectively. NAC administration was ineffective in restoring Cd levels to normal values in both tissues evaluated.

Table 1
Rats exposed to cadmium (Cd) and/or treated with N-acetylcysteine (NAC) did not demonstrate altered shock sensitivity. Data are means ± S.E.M. of flinch, jump and vocalization thresholds expressed in milliamps ($n = 6$–7 animals per group).

	Saline	NAC	Cd	Cd/NAC
Flinch	0.26 ± 0.033	0.27 ± 0.033	0.27 ± 0.034	0.23 ± 0.019
Jump	0.45 ± 0.033	0.43 ± 0.021	0.41 ± 0.040	0.36 ± 0.029
Vocalization	0.47 ± 0.048	0.40 ± 0.021	0.43 ± 0.045	0.46 ± 0.040

Table 2
Effects of cadmium (Cd) and N-acetylcysteine (NAC), and their combination on Cd concentration (μg/g) in different brain structures (cortex, striatum, hippocampus, cerebellum, and hypothalamus) and kidney tissue of male rats. Data are means ± S.E.M. Two-way ANOVA–Duncan's test ($p < 0.05$). Different letters in the same line indicate differences among the treatments ($n = 6–7$ animals per group).

Tissue	Saline	NAC	Cd	Cd/NAC
Cortex	0.014 ± 0.0009[b]	0.013 ± 0.0008[b]	0.019 ± 0.0005[a]	0.020 ± 0.0005[a]
Striatum	0.012 ± 0.0004[b]	0.014 ± 0.0007[b]	0.021 ± 0.0007[a]	0.019 ± 0.0005[a]
Hippocampus	0.013 ± 0.0009[b]	0.012 ± 0.0005[b]	0.020 ± 0.0006[a]	0.020 ± 0.0006[a]
Cerebellum	0.014 ± 0.0008[b]	0.011 ± 0.0004[b]	0.019 ± 0.0005[a]	0.020 ± 0.0005[a]
Hypothalamus	0.012 ± 0.0004[b]	0.013 ± 0.0009[b]	0.021 ± 0.0006[a]	0.021 ± 0.0007[a]
Kidney	0.017 ± 0.0037[b]	0.019 ± 0.0056[b]	4.815 ± 2.1083[a]	4.2630 ± 0.9966[a]

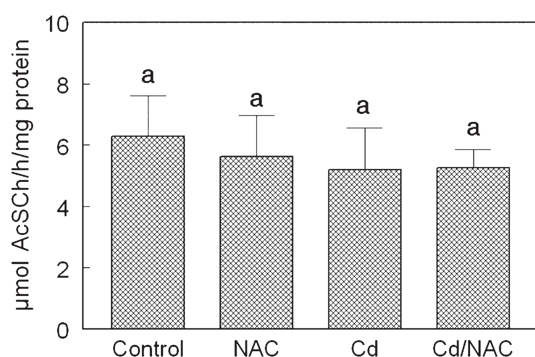

Fig. 2. Acetylcholinesterase (AChE) activity in synaptosomes of cerebral cortex of cadmium (Cd)-exposed rats and treated with N-acetylcysteine (NAC). Different lowercase letters indicate significant difference among the groups. Bars represent means ± S.E.M. Two-way ANOVA–Duncan's test ($p < 0.05$).

3.3. Activity of AChE in different brain structures

The results obtained for AChE activity in different brain structures are presented in Figs. 2 and 3.

Two-way ANOVA revealed that there were no significant alterations in AChE activity in cerebral cortex synaptosomes and striatum (Fig. 2 and Fig. 3A).

A significant main effect of Cd ($p < 0.05$) and NAC ($p < 0.05$) in hippocampus AChE activity was observed (Fig. 3B). Results demonstrated that Cd exposure caused a decrease (29%) in AChE activity. NAC administration showed a tendency to ameliorate AChE activity decreased by Cd.

A significant main effect of Cd ($p < 0.05$) and NAC ($p < 0.05$) in cerebellum AChE activity was observed (Fig. 3C). Results demonstrate that Cd exposure caused a decrease (32%) in AChE activity. NAC administration was effective in ameliorating AChE activity decreased by Cd.

A significant Cd × NAC interaction for hypothalamus AChE activity was observed ($F_{1,17} = 5.416$; $p < 0.05$) (Fig. 3D). Post hoc comparisons demonstrate that rats exposed to Cd presented a decrease (29%) in hypothalamus AChE activity. The decrease in AChE activity induced by Cd was abolished by NAC administration.

3.4. TBARS in different brain structures

The results obtained for oxidative stress in different brain structures are presented in Fig. 4.

Two-way ANOVA revealed that there were no significant alterations in TBARS levels in striatum (Fig. 4A).

A significant Cd × NAC interaction for hippocampus TBARS levels was observed ($F_{1,8} = 25.694$; $p < 0.01$) (Fig. 4B). Post hoc comparisons demonstrate that rats exposed to Cd presented an increase (21%) in hippocampus TBARS levels. The increase in TBARS levels induced by Cd was abolished by NAC administration.

A significant Cd × NAC interaction for cerebellum TBARS levels was observed ($F_{1,18} = 5.519$; $p < 0.05$) (Fig. 4C). Post hoc comparisons demonstrate that rats exposed to Cd presented an increase (59%) in TBARS levels of cerebellum. The increase in TBARS levels induced by Cd was abolished by NAC administration.

A significant Cd × NAC interaction for hypothalamus TBARS levels was observed ($F_{1,13} = 5.082$; $p < 0.05$) (Fig. 4D). Post hoc comparisons demonstrate that rats exposed to Cd presented an increase (42%) in hypothalamus TBARS levels. The increase in TBARS levels induced by Cd was abolished by NAC administration.

3.5. Urea and creatinine levels

The results obtained for renal markers damage are presented in Fig. 5.

A significant main effect of Cd ($p < 0.05$) and NAC ($p < 0.05$) in urea plasma levels was observed (Fig. 5A). Results demonstrate that Cd exposure caused an increase (13%) in urea plasma levels. NAC administration was effective in reverse urea plasma levels increased by Cd.

A significant Cd × NAC interaction for creatinine plasma levels was observed ($F_{1,19} = 0.173$; $p < 0.01$) (Fig. 5B). Post hoc comparisons demonstrate that rats exposed to Cd presented an increase (25%) in creatinine plasma levels. The increase in creatinine plasma levels induced by Cd was abolished by NAC administration.

4. Discussion

The diet is the main source of environmental cadmium exposure in non-smokers in most parts of the world [30]. Atmospheric deposition of airborne Cd, mining activities and the application of Cd containing fertilizers and sewage sludge on farm land may lead to the contamination of soils and increased Cd uptake by crops and vegetables grown for human consumption [30]. Moreover, Wätjen and Beyersmann [31] reported that low Cd doses can initiate apoptosis process leading to a selective cell death in distinct brain regions via generation of oxidative stress which is thought to play an important role in human neurodegenerative diseases. Clearly, understanding the mechanism(s) by which Cd causes neurotoxicity in experimental animals will provide insight into its diverse effects in humans. Identifying environmental factors such as Cd that can contribute to increased neurobehavioral disturbances in humans is an important undertaking and a first step to prevention. In view of this, in our present investigation the rats were orally intoxicated with Cd and/or treated with NAC in order to evaluate the possible effects of these compounds in the central nervous system.

The present study demonstrates that treatment with NAC ameliorated neurotoxicity in rat brain after sub-chronic exposure to Cd.

In adult rats, small amounts of Cd reach the brain because of the selective permeability of the BBB [32]. In fact, Yargiçoglu et al. [16] and Antonio et al. [33] reported that Cd is more toxic to newborn and young rats than to adult rats probably due to differences in the BBB integrity varying with the age of the animal. In agreement with the literature data, in the present study, we found a small amount of Cd in brain of Cd-exposed rats but significantly higher than control

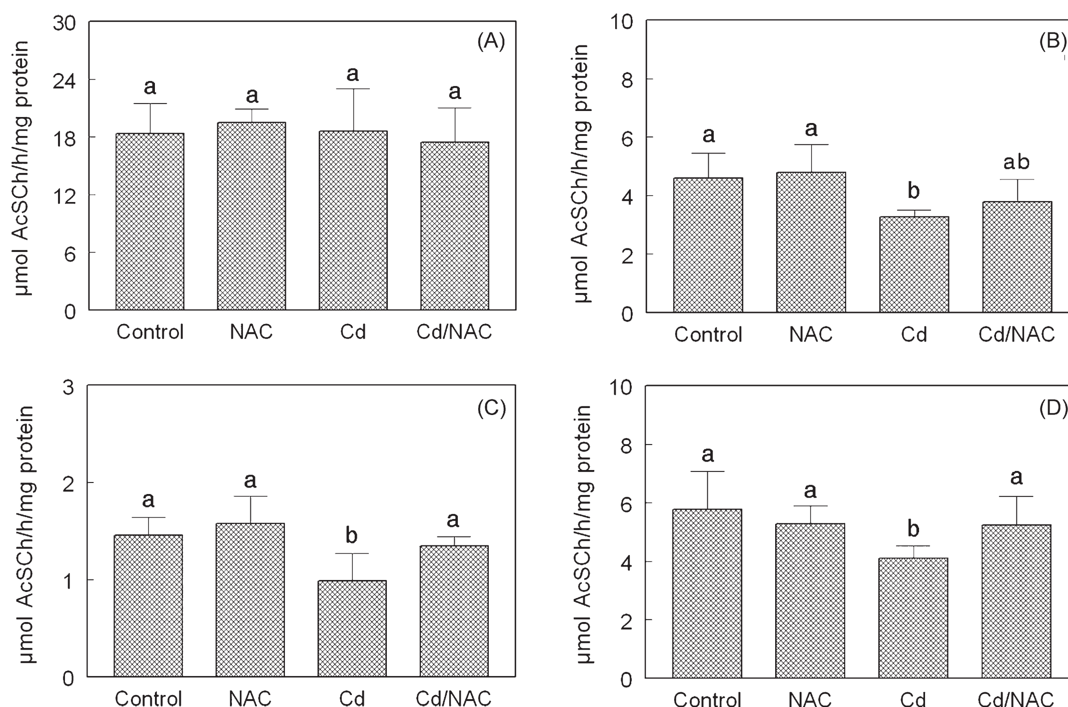

Fig. 3. Acetylcholinesterase (AChE) activity in supernatant of striatum (A), hippocampus (B), cerebellum (C) and hypothalamus (D) of cadmium (Cd)-exposed rats and treated with N-acetylcysteine (NAC). Different lowercase letters indicate significant difference among the groups. Bars represent means ± S.E.M. Two-way ANOVA–Duncan's test ($p < 0.05$).

values and enough to cause brain injury. For all the brain structures analyzed, a similar Cd concentration was found within the same group of rats and co-administration of NAC was ineffective in decreasing this level. These results suggest that the harmful effects of Cd observed in this investigation were ameliorated mainly by the antioxidant activity of NAC rather than by Cd removal from tissues. Similar findings were reported by Bludovská et al. [34] for alpha-lipoic acid treatment against Cd toxicity. Nevertheless, it is interesting to point out that although Cd does not accumulate in significant quantities in the adult rat brain following exposure [8,35], it severely disturbs the metabolism of trace and essential elements such as copper and zinc [4]. Some studies have demonstrated that Cd is able to induce neurotoxicity in animals with a wide spectrum of clinical entities including neurological and behav-

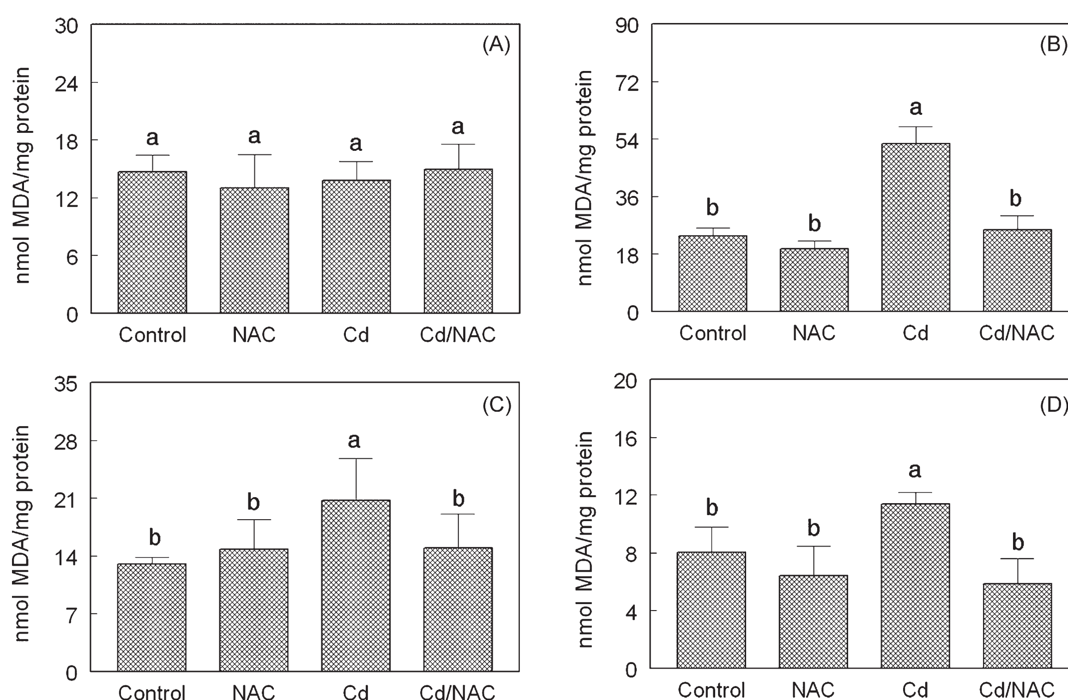

Fig. 4. Oxidative stress measured by thiobarbituric acid reactive substances (TBARS) in striatum (A), hippocampus (B), cerebellum (C) and hypothalamus (D) of cadmium (Cd)-exposed rats and treated with N-acetylcysteine (NAC). Different lowercase letters indicate significant difference among the groups. Bars represent means ± S.E.M. Two-way ANOVA–Duncan's test ($p < 0.05$).

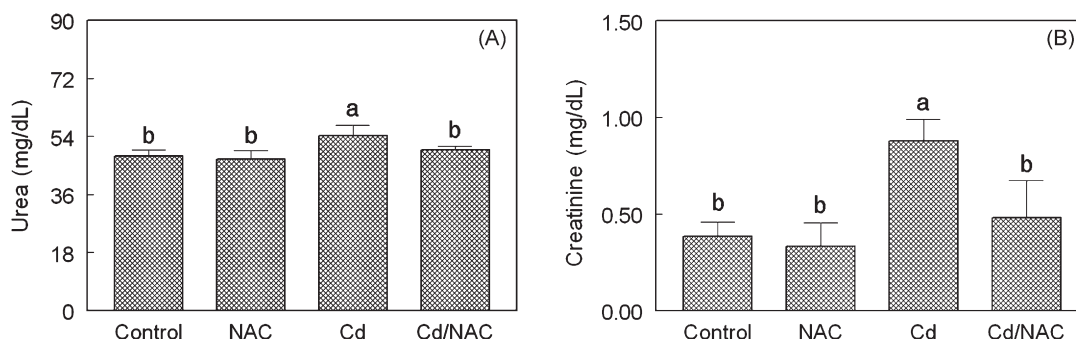

Fig. 5. Renal markers measured by urea (A) and creatinine (B) levels in serum of cadmium (Cd)-exposed rats and treated with N-acetylcysteine (NAC). Different lowercase letters indicate significant difference among the groups. Bars represent means ± S.E.M. Two-way ANOVA–Duncan's test ($p < 0.05$).

ioral disturbances, changes in the normal neurochemistry of the brain, cerebral hemorrhage and oedema [6,7,8,14,36]. Alteration in the gene expressions of RC3 (neurogranin regulated by thyroid hormones that may play roles in memory and learning) and sex hormone receptors in the brain induced by perinatal Cd and methimazole (anti-thyroid drug) exposure might be one mechanism of developmental toxicity of Cd [37]. Moreover, Lukawski et al. [38] showed that the tendency for Cd-induced memory impairments in the passive avoidance task (long-term memory) and spontaneous alternation deficits (spatial working memory) may be potentiated by cerebral oligemic hypoxia.

According to Cahill et al. [39] the inhibitory avoidance test is a classic model behavioral test, with a strongly aversive component, utilized for evaluating learning and memory in rats and mice. In the present study, we observed a significant decrease in step-down latency in Cd-intoxicated rats in the inhibitory avoidance test, suggesting learning and memory impairment in these animals. However, when the Cd-exposed rats were treated with NAC the step-down latency was similar to that found for rats from the control group. These findings indicate that treatment with NAC was able to prevent learning and memory impairment induced by Cd toxicity.

As reported by Schmatz et al. [11], a major concern in shock-motivated learning tests, particularly in those that investigate the effect of drugs given before the acquisition of a given test, is whether pharmacological treatment affects motivational aspects of learning, such as shock sensitivity. In our study, we demonstrated that rats exposed to Cd and/or treated with NAC did not demonstrate altered shock sensitivity. These data exclude the possibility that shock sensitivity may have contributed to the alteration in step-down latencies.

AChE is a very important enzyme for cholinergic neurotransmission and there are strong indications of its essential role in regulating many vital functions and its association with neurobehavioral processes [3,10]. Both *in vitro* and *in vivo* effects of Cd on AChE activity have been described in the literature for different animal models [3,9,40,41,42], yet results are controversial and activation as well as inhibition of AChE activity has been reported. The activity of AChE in rat brain synaptosomal plasma membranes showed a considerable decrease after 6 h of Cd exposure, followed by a progressive increase up to 24 h [43]. In fact, these controversies could be explained by the different routes and doses of Cd administration, differences in the biological samples assayed and differing periods of exposure (short-term and long-term) [40]. Controversial findings have been observed by our group when studying other metals such as aluminum and mercury (Hg) [44,45].

There are some studies reporting interactions between the ACh system and a variety of heavy metals. AChE activity was enhanced in the cerebral cortex and hippocampus of rats exposed to 30 doses of Hg subcutaneous injections (0.1 mg/kg) [44]. However, inhibition of brain AChE activity was also observed in different models of Hg intoxication [46,47]. Exposure to both Hg and Cd produced significant decreases in cortical ACh, whereas exposure to lead (Pb) resulted in enhanced cerebrocortical ACh levels indicating that chronic exposure (45 days) to low doses of heavy metals produces differential ACh response [48]. In addition, Antonio et al. [33] observed a decreased AChE activity in Cd-group at postnatal day 21, whereas only the Pb-group was affected at birthday probably due to the fact that Pb crosses the BBB quite readily, whereas there is a low permeability of BBB to Cd.

In the present study, hippocampus, cerebellum and hypothalamus of rats exposed to Cd showed a decrease in AChE activity but when NAC was co-administered this effect was totally or partially prevented. In cortex synaptosomes and striatum there was no alteration in AChE activity among the groups. Inactivation of AChE enzyme as a result of the occupation of its active sites by heavy metals was suggested by Shaw and Panigrahi [49] who investigated Hg-exposed fish. In fact, the mechanism of action of Cd has been hypothesized to be either the displacement of metal cofactors from the active site or the direct deactivation of the enzyme site [50]. As a result of AChE inhibition, the neurotransmitter ACh is less hydrolysed in synapses, leading to an abnormal amount of ACh, consequently causing overactivation of cholinergic receptors and possible toxic effects [51]. As reported by Olney et al. [52] the decreased activity of AChE may be associated with cholinergic hyperactivity, convulsion and status epilepticus. Moreover, Ecobichon [53] reported that central nervous system AChE inhibition may precipitate symptoms such as confusion, headache, sleep disturbances and memory lapses.

It is important to emphasize that since cerebral AChE is an important regulator of behavioral process, the decreased AChE activity found here may be an indicator of Cd-induced damage in brain, although it is not clear to what extent it contributes directly [3].

Furthermore, Shukla et al. [5] and Pal et al. [54] showed that Cd enhances the production of free radicals in the brain and interferes with the antioxidant defense system, which, in turn, leads to a Cd-induced alteration of the structural integrity of lipids and secondarily affects membrane-bound enzymes such as Na^+/K^+-ATPase and AChE. The latter is present in G4 (membrane bound) and G1 (cytosolic) forms in different brain regions [55]. In the mammalian brain, the G4 form represents 60–90% of total AChE, depending on the anatomical region, and the remainder is composed of G1 and G2 forms [56]. In addition, Tsakiris et al. [57] found a protective effect of L-phenylalanine on rat brain AChE inhibition induced by free radicals, showing that free radical production could, at least in part, be associated with decreased AChE activity. Thus, alterations in the lipid membrane by oxidative stress could be a decisive factor in the modification of the conformational state of the AChE molecule, which would explain changes in its activity [11,49,55].

It is well known that peroxidative damage of membrane structure and changes in associated enzymes, receptors, and physiological functions may ultimately result in disturbances in neuronal functions [16]. In fact, in the present study, the AChE activity was negatively correlated with TBARS levels in all cerebral structures analyzed ($r = 0.79$, $r = 0.99$, $r = 0.91$ and $r = 0.99$ to striatum, hippocampus, cerebellum and hypothalamus, respectively).

In our study, hippocampus, cerebellum and hypothalamus of rats exposed to Cd presented an increase in TBARS levels but when NAC was co-administered this effect was abolished. In striatum, there was no alteration in TBARS levels among the groups. According to Kumar et al. [58], the brain may be particularly vulnerable to oxidative damage and saturation of the lipid bi-layer of several areas of the brain as a result of LPO in Cd-exposed animals caused disturbances in membrane fluidity and intracellular calcium concentrations. Moreover, these authors proposed that Cd exposure caused region-specific biomembrane changes. The ability of Cd to induce oxidative stress in brain cells is related to the induction of ROS, LPO and apoptosis, after the interaction of Cd with mitochondrial sites, leading to the breakdown of the mitochondrial potentials, the consequent reduction of intracellular glutathione levels (GSH) and changes in catalase and superoxide dismutase activities [59]. Until rat pups exposed to Cd only gestationally and lactationally through mothers (20 ppm Cd in drinking water) showed changes in their brain antioxidant defense mechanisms at critical periods of development which may have serious implications in later life [60]. Corroborating with our results, other reports have shown that Cd induced an elevation of LPO in brain and also in other tissues [5,16,17,21,39,43,61]. In fact, according to Manca et al. [61] LPO is an early and sensitive consequence of Cd exposure. Also similar to our findings, some investigators found that the consumption of antioxidants such as garlic, vitamin E, carotene, alpha-lipoic acid, diphenyl diselenide, and NAC was able to revert Cd-induced oxidative stress [3,21,34,62,63,64,65]. As reported by Nemmiche et al. [65] the mechanism of Cd-induced LPO is still not fully understood. Available data indicate that the mechanism is multidirectional and may involve a decrease in the level of glutathione and the total pool of sulphydryl groups and changes in the activities of antioxidant enzymes [65]. In fact, some authors have shown a decreased GSH level associated with the increased LPO process in rats intoxicated with Cd [34,65]. These factors can consequently induce a prooxidant state in biological systems and lead to peroxidation of polyunsaturated fatty acids.

Another important aspect to be discussed here is that both aging and age-associated neurodegenerative diseases are associated with various degrees of behavioral impairments and among the primary candidates responsible for producing the neuronal changes mediating these behavioral deficits appear to be free radicals and the oxidative stress they generate [66]. A recent study published by our research group that utilized the same behavioral test as the present study demonstrated that the antioxidant resveratrol was able to modulate cerebral AChE activity and to reverse the cognitive deficits in diabetic rats [11]. Another study showed that vitamin E appears to have a close interaction with the cholinergic system and memory retention [67]. Taken together, these data allow us to suggest that antioxidants such as resveratrol, vitamin E and NAC have an important role in preventing oxidative stress, the cholinergic system impairment and learning and memory deficits.

In order to explain the possible mechanism by which Cd exert its toxicity in our study it is important to know whether the exposure protocol used in this study induced any nephrotoxicity, also in light of the fact that the behavioral deficits can be associated with mild renal failure we determine the urea and creatinine levels in serum. Urea is the first acute renal marker which increases when the kidney suffers any kind of injury otherwise, creatinine is the most trustable of them [22]. So, in the present study we verified that Cd induced moderate nephrotoxicity because Cd-exposed rats showed increased urea and creatinine levels in serum, but no renal histological damage was found (data not shown). The co-administration of NAC was able to abolish these undesirable effects. These results not decrease the relevance of the present study because we demonstrated the presence of Cd in the brain and its negative effects in both the cerebral and the kidney tissues. Thus, Cd can induce oxidative stress regulating learning/memory processes through the modulation of brain AChE activity and mild renal injury can contribute to these alterations.

In conclusion, our data have shown, for the first time, that NAC has beneficial actions against Cd-mediated toxic effects in brain, inhibiting oxidative stress and subsequently restoring cerebral AChE activity, thus modulating cholinergic neurotransmission and improving cognition (learning and memory). However, it is necessary to note that the mild renal failure may be a contributor to the behavioral impairment found in this investigation. It is proposed that NAC acted in this experimental protocol through its antioxidant properties but not its capacity to form a complex with Cd, since NAC did not alter the metal load. Therefore, we can suggest that NAC is a promising drug which should be investigated in future studies in order to improve therapeutic alternatives for brain injury associated with Cd-induced neurotoxicity.

Conflict of interest

There is not conflict of interest.

Acknowledgements

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4. CAPÍTULO 2

Manuscrito: Hematological indices and activity of NTPDase and cholinesterase enzymes in rats exposed to cadmium and treated with N-acetylcysteine – Aceito para publicação no Periódico *Biometals*

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Hematological indices and activity of NTPDase and cholinesterase enzymes in rats exposed to cadmium and treated with *N*-acetylcysteine

Jamile F. Gonçalves · Marta M. M. F. Duarte · Amanda M. Fiorenza · Roselia M. Spanevello · Cinthia M. Mazzanti · Roberta Schmatz · Margarete D. Bagatini · Fabiane G. Antes · Pauline Costa · Fátima H. Abdalla · Valderi L. Dressler · Vera M. Morsch · Maria Rosa C. Schetinger

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Abstract The present study aimed to investigate the influence of *N*-acetylcysteine (NAC) on cadmium (Cd) poisoning by evaluating Cd concentration in tissues, hematological indices as well as the activity of NTPDase, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes of rats exposed to Cd and co-treated with NAC. For this purpose, the rats received Cd (2 mg/kg) and NAC (150 mg/kg) by gavage every other day for 30 days. Animals were divided into four groups ($n = 6$–8): control/saline, NAC, Cd, and Cd/NAC. Cd exposure increased Cd concentration in plasma, spleen and thymus, and NAC co-treatment modulated this augment in both lymphoid organs. Cd exposure reduced red blood cell count, hemoglobin content and hematocrit value. Cd intoxication caused a decrease in total white blood cell count. NAC treatment *per se* caused an increase in lymphocyte and a decrease in neutrophil counts. On contrary, Cd exposure caused a decrease in lymphocyte and an increase in neutrophil and monocyte counts. NAC reversed or ameliorated the hematological impairments caused by Cd poisoning. There were no significant alterations in the NTPDase activity in lymphocytes of rats treated with Cd and/or NAC. Cd caused a decrease in the activities of lymphocyte AChE, whole blood AChE and serum BChE. However, NAC co-treatment was inefficient in counteracting the negative effect of Cd in the cholinesterase activities. The present investigation provides ex vivo evidence supporting the hypothesis that Cd induces immunotoxicity by interacting with the lymphoid organs, altering hematological parameters and inhibiting peripheral cholinesterase activity. Also, it highlights the possibility to use NAC as adjuvant against toxicological conditions.

J. F. Gonçalves (✉) · M. R. C. Schetinger (✉)
Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcellos 2600-Anexo, Porto Alegre, RS 90035-003, Brazil
e-mail: jamilefabbrin@yahoo.com.br

M. R. C. Schetinger
e-mail: mariachitolina@gmail.com

M. M. M. F. Duarte · A. M. Fiorenza · R. Schmatz · F. G. Antes · P. Costa · F. H. Abdalla · V. L. Dressler · V. M. Morsch · M. R. C. Schetinger
Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus Universitário Camobi, Santa Maria, RS 97105-900, Brazil

R. M. Spanevello
Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Setor de Bioquímica, Universidade Federal de Pelotas, Campus Universitário Capão do Leão, Pelotas, RS 96010-900, Brazil

C. M. Mazzanti
Departamento de Clínica de Pequenos Animais, Setor de Patologia Clínica Veterinária, Universidade Federal de Santa Maria, Campus Universitário, Camobi, Santa Maria, RS 97105-900, Brazil

M. D. Bagatini
Colegiado do Curso de Enfermagem, Universidade Federal da Fronteira Sul, Chapecó, SC 89812-000, Brazil

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Introduction

Cadmium (Cd) is a widespread environmental pollutant affecting numerous organs of the body (Järup and Akesson 2009; Nordberg 2009; Thévenod 2009). Besides causing multiorgan toxicity, Cd is a potent immunotoxicant (Dong et al. 2001). Thymus, an important primary lymphoid organ, is the place where successive stages of cell development and selection generate functionally competent T cells from immature precursor cells (Dong et al. 2001; Pathak and Khandelwal 2006a, 2007, 2009). Spleen is one of the principle sites for the initiation of most primary immune responses, for B lymphocyte activation and production of antibodies (Pathak and Khadelwal Pathak and Khandelwal 2006b, 2007, 2009). Both, thymus and spleen are target organs of Cd-induced immunotoxicity. In fact, based on experimental evidence mostly from in vivo animal models, Cd is able to cause damage to humoral immune response as well as cell mediated immunity (Dong et al. 2001; Lafuente et al. 2004; Pathak and Khandelwal 2006a, b, 2007, 2009). Many studies have demonstrated that exposure to Cd resulted in induction of apoptosis in thymus and splenic cells as well as in immune system cells (Dong et al. 2001; Pathak and Khandelwal 2006a, b). However, conflicting results have been reported on the effects of Cd exposure on the immune system development and function (Pathak and Khandelwal 2006b; Viau et al. 2007) demonstrating the necessity to study the effects of Cd on important systems involved in immunological response such as purinergic and cholinergic pathways.

It has already been established that purinergic signaling contributes to the fine-tuning of inflammatory and immune responses (Bours et al. 2006). Extracellular ATP acts in proinflammatory functions such as stimulation and proliferation of lymphocytes and cytokine release (Bours et al. 2006). On the other hand, its breakdown product, adenosine, exhibits potent anti-inflammatory and immunosuppressive action by inhibiting proliferation of T cells and secretion of cytokines and migration of leukocytes across endothelial barriers (Linden 2006). Extracellular nucleotides can be hydrolyzed by a variety of ectonucleotidases such as nucleoside triphosphate diphosphohydrolases (NTPDases), ectonucleotide pyrophosphatase/phosphodiesterase (E-NPPS) and alkaline phosphatases (Schetinger et al. 2007). The enzyme NTPDase (E.C. 3.6.1.5) is expressed in numerous types of immune cells (Schetinger et al. 2007; Spanevello et al. 2010; Leal et al. 2005, 2011) and is important in the control of lymphocyte function, including antigen recognition and/or the activation of cytotoxic T cells (Dwyer et al. 2007).

In addition, mammals have two types of cholinesterase enzymes, acetylcholinesterase (AChE, E.C. 3.1.1.7) and butyrylcholinesterase (BChE, E.C. 3.1.1.8). Although AChE is known as an enzyme responsible for the rapid hydrolysis of the neurotransmitter acetylcholine (ACh) at brain cholinergic synapses and neuromuscular junctions, AChE was also found in adult non-cholinergic neurons and in hematopoietic, osteogenic and immune cells as well as blood and other body fluids (Soreq and Seidman 2001). The existence of cholinergic receptors in the immune system cells is well documented (Kawashima and Fujii 2003). According to Das (2007), the "cholinergic anti-inflammatory pathway" mediated by ACh acts inhibiting the production of the tumor necrosis factor, interleukin-1, and suppresses the activation of nuclear factor-kappa B (NF-kB) expression. Macrophages and other cytokine-producing cells express acetylcholine receptors (AChRs), which transduce an intracellular signal that inhibits cytokine synthesis. The most characterized of these cholinergic receptors that suppress cytokines is the $\alpha7$ subunit of the nicotinic AChR ($\alpha7$nAChR) (Tracey 2007). Thus, although ACh is a classical neurotransmitter, it is also involved in the regulation of immune function. Moreover, ACh hydrolysis can also be catalyzed by BChE that is a related and less specific enzyme, which is abundant in rat serum. BChE can act as a molecular decoy for anti-AChE compounds by reacting with these toxins before they reach AChE (Soreq and Seidman 2001). According to Boeck et al. (2002), the enzyme components of the blood have a major influence on the potency of toxic agents. Of toxicological and pharmacological significance, AChE and BChE are targets for various cholinergic toxins such as Cd and also for therapeutically active compounds including antioxidants (Gonçalves et al. 2010; Jaques et al. 2011).

Because the health problems induced by many environmental pollutants such as Cd, much effort has been expended in evaluating the relative antioxidant

potency of various drugs including *N*-acetylcysteine (NAC). NAC is a derivative of the naturally occurring amino acid cysteine and is an excellent source of sulfhydryl (–SH) groups (Aremu et al. 2008). Sulfur-containing nutrients play an important role as detoxification and protection of cells and cellular components against oxidative stress (Grinberg et al. 2005; Gonçalves et al. 2010). Moreover, although the molecular mechanisms of Cd toxicity have not been yet well defined, it is known that the oxidative effect of Cd is indirect and based mainly on the depletion of -SH-group-containing compounds (Järup and Akesson 2009; Nordberg 2009). Interestingly, we recently demonstrated that NAC treatment was able to ameliorate Cd-induced neurotoxicity and consequently to improve the memory and learning processes of Cd-intoxicated rats (Gonçalves et al. 2010).

In the light of the above information, the present study was carried out to investigate the possible protective influence of NAC on Cd poisoning by evaluating Cd concentration in plasma, spleen and thymus, hematological data as well as the activity of peripheral NTPDase, AChE and BChE of Cd-intoxicated rats and treated with NAC.

Materials and methods

Reagents

Acetylthiocholine iodide, butyrylthiocholine iodide, 5,5′-dithio-bis-2-nitrobenzoic acid, tris-(hydroxymethyl)-aminomethane GR, Coomassie brilliant blue G, nucleotides, Trizma base and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ethopropazine hydrochloride from Aldrich (Steinheim, Germany). Ficoll Hypaque (Lymphoprep^{TM}) was purchased from Nycomed Pharma (Oslo, Norway). *N*-acetylcysteine was obtained from Beg (São Paulo, SP, Brazil) with 99.1 % of purity. All other reagents used in the experiments were of analytical grade and of the highest purity.

Animals and experimental design

Adult male Wistar rats (80 days; 315.7 ± 14 g) from the Central Animal House of the Universidade Federal de Santa Maria (UFSM) were used in this experiment. The animals were maintained at a constant temperature (23 ± 1 °C) on a 12/12-h light/dark cycle with free access to food and water. All animal procedures were approved by the Animal Ethics Committee from UFSM.

The body burden of Cd is derived primarily from the ingestion of food and water contaminated with Cd and $CdCl_2$ is the principal form of Cd associated with oral exposure, as it is highly soluble in water (Zalups and Ahmad 2003). Thus, in the present study the rats received cadmium as $CdCl_2{\cdot}H_2O$ (Cd; 2 mg/kg) (El-Demerdash et al. 2004; Borges et al. 2008) and *N*-acetylcysteine (NAC; 150 mg/kg) (Sadowska et al. 2007) by gavage every other day for 30 days. The animals were randomly divided into four groups ($n = 6$–8 per treatment): control/saline, NAC, Cd and Cd/NAC; the Cd/NAC group received NAC 30 min after Cd. The solutions were freshly prepared in saline and were administered (1 ml/kg) between 9 and 11 a.m.

Cadmium concentration in plasma, spleen and thymus

Plasma, spleen and thymus were weighed in glass vessels and 3–8 ml of HNO_3 was added for digestion. Digestion was performed using a block (Velp Scientifica, Milano, Italy) heated at 130 °C for 3 h. After this time, 2 ml of H_2O_2 was added and the samples were heated for 1 h. Digested samples were then transferred to polypropylene flasks for Cd determination. Cd determination was performed by inductively coupled plasma mass spectrometry (ICP-MS). An ICP-MS equipment (PerkinElmer Sciex, model ELAN DRC II, Thornhill, Canada), equipped with a concentric nebulizer (Meinhard Associates, Golden, USA), a cyclonic spray chamber (Glass Expansion, Inc., West Melbourne, Australia) and a quartz torch with an injector tube of 2 mm i.d. was used.

Hematological determinations

Quantitative determinations were measured in whole blood collected in EDTA tubes using standard methods with the fully automated PENTRA 120® (ABX Diagnostics, Montpellier, France).

Isolation of the peripheral lymphocytes

The blood was collected in tubes containing EDTA as anticoagulant. Peripheral lymphocytes were isolated

using Ficoll–Histopaque density gradient as described by Böyum (1968).

Lymphocyte NTPDase activity

After lymphocyte isolation, the NTPDase activity was determined immediately as described by Leal et al. (2005) where the reaction medium contained 0.5 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 6 mM glucose and 50 mM Tris Hcl buffer pH 8.0, at a final volume of 200 µl. The proteins of all samples were adjusted to 0.1–0.2 mg/ml. Twenty microliters of the intact mononuclear cells suspended in saline solution was added to the reaction medium (2–4 µg of protein) and pre-incubated for 10 min at 37 °C and incubation proceeded for 70 min. The reaction was initiated by the addition of the substrate (ATP or ADP) at a final concentration of 2.0 mM and stopped with 200 µl of 10 % trichloracetic acid (TCA). The released inorganic phosphate (Pi) was assayed by the method of Chan et al. (1986) using malachite green as colorimetric reagent and KH_2PO_4 as standard. Controls were carried out by adding the enzyme preparation after TCA addition to correct for non-enzymatic nucleotide hydrolysis. All samples were run in triplicate and the specific activity is reported as nmol Pi released/min/mg of protein.

Lymphocyte AChE activity

After the isolation of the lymphocytes, the AChE activity was determined immediately according to the method described by Ellman et al. (1961) modified by Fitzgerald and Costa (1993). The proteins of all samples were adjusted to 0.1–0.2 mg/ml. Two hundred microliters of intact cells was added to a solution containing 1.0 mM acetylthiocholine iodide (AcSCh), 0.1 mM 5,5′dithiobis-2-nitrobenzoic acid (DTNB) and 0.1 M phosphate buffer (pH 8.0). Immediately before and after incubation for 30 min at 27 °C the absorbance was read on a spectrophotometer at 412 nm. AChE was calculated from the quotient between lymphocyte AChE activity and protein content. Results are expressed as µmol AcSCh/h/mg of protein.

Whole blood AChE activit

For AChE activity, the blood was collected in tubes containing EDTA as anticoagulant and the samples were hemolyzed with phosphate buffer 0.1 M, pH 7.4 containing Triton X-100 (0.03 %) and stored at −30 °C for 2 weeks. Whole blood AChE activity was determined by the method of Ellman et al. (1961) modified by Worek et al. (1999). To achieve temperature equilibration and the complete reaction of sample matrix sulfhydryl groups with DTNB, the mixture was incubated at 37 °C for 10 min prior to the addition of 0.44 mM AcSCh. The enzyme activity was corrected for spontaneous hydrolysis of the substrate and DTNB degradation. Butyrylcholinesterase (BChE) was inhibited by ethopropazine. The AChE activity was measured at 436 nm and calculated from the quotient between the AChE activity and hemoglobin content (Hb). Hb was determined using the Zijlstra modified solution. Results are expressed as mU/µmol Hb.

Serum BChE activity

For BChE activity, the blood was collected in tubes without any anticoagulant to obtain the serum. The BChE enzymatic assay was determined by a modification of the spectrophotometric method of Ellman et al. (1961) as previously described by Rocha et al. (1993). The reaction mixture (2 ml final volume) contained 100 mM K^+-phosphate buffer, pH 7.5 and 1.0 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 for 2 min incubation at 25 °C. The enzyme was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM butyrylthiocholine iodide (BuSCh). All samples were run in duplicate or triplicate and enzyme activity was expressed in µmol BuSCh/h/mg of protein.

Protein determination

Protein was measured by the method of Bradford (1976) using bovine serum albumin as standard.

Statistical analysis

Statistical analysis was performed using two-way ANOVA, followed by Duncan's multiple range tests. $p < 0.05$ was considered to represent a significant difference in all experiments. All data were expressed as mean ± SEM.

Results

The Cd concentration in plasma, spleen and thymus are presented in Table 1. A significant main effect of Cd ($p < 0.05$) and NAC ($p < 0.05$) on Cd concentrations in plasma was observed. Results demonstrate that rats exposed to Cd alone and in combination with NAC presented an increase of 207 and 175 % in plasma Cd concentration, respectively. Thus, NAC administration was ineffective in restoring Cd levels to normal values in plasma. On the other hand, a significant Cd × NAC interaction was observed for spleen ($F_{1.20} = 0.0358$; $p < 0.05$) and thymus ($F_{1,20} = 0.0661$; $p < 0.05$) Cd concentration. Post hoc comparisons demonstrate that rats exposed to Cd presented an increase of 1,900 and 2,400 %, respectively in spleen and thymus Cd concentration. However, when NAC was co-administrated with Cd, the concentration of Cd in spleen and thymus increased about 1,100 and 1,300 %, respectively. Thus, NAC co-treatment was able to modulate Cd concentration in both immune organs.

The hematological data are showed in Table 2. A significant Cd × NAC interaction for total red blood cell (RBC) count ($F_{1,22} = 0.917$; $p < 0.05$), hemoglobin (Hb) content ($F_{1,22} = 4.213$; $p < 0.05$) as well as hematocrit (Hct) value ($F_{1,22} = 4.213$; $p < 0.05$) was observed. Post hoc comparisons demonstrate that rats exposed to Cd presented a decrease of 13, 13 and 14 % in RBC, Hb and Hct, respectively. The decrease in RBC, Hb and Hct induced by Cd was abolished by NAC administration. A significant main effect of Cd ($p < 0.05$) and NAC ($p < 0.05$) in total white blood cell (WBC) count was observed. Results demonstrate that Cd or Cd/NAC exposure caused a decrease of 28 and 11 %, respectively in WBC. NAC treatment was partially efficient in preventing the increase in WBC induced by Cd.

The differential count of peripheral blood leucocytes (lymphocytes, neutrophils and monocytes) is also differentially affected by Cd intoxication (Table 2). A significant main effect of Cd ($p < 0.05$) and NAC ($p < 0.05$) in lymphocyte count was observed. Results demonstrate that NAC treatment *per se* caused an increase (8 %) in lymphocyte count while Cd exposure decreased it by 17 %. The group Cd/NAC presented values similar to the control rats. In other words, NAC abolished this undesirable Cd effect. On the contrary, although a significant main effect of Cd ($p < 0.05$) and NAC ($p < 0.05$) was

Table 1 Cadmium (Cd) concentration in plasma, spleen and thymus of male rats treated with Cd, *N*-acetylcysteine (NAC) or their combination

Groups	Plasma (ng/l)	Spleen (μg/g)	Thymus (μg/g)
Saline	0.508 ± 0.07^b	0.020 ± 0.00001^c	0.020 ± 0.00001^c
NAC	0.464 ± 0.06^b	0.020 ± 0.00001^c	0.020 ± 0.00001^c
Cd	1.560 ± 0.13^a	0.400 ± 0.03^a	0.499 ± 0.03^a
Cd/NAC	1.400 ± 0.24^a	0.241 ± 0.04^b	0.283 ± 0.05^b

Data are mean ± SEM. Two-way ANOVA–Duncan's Test ($p < 0.05$). Different letters in the same column indicate significant difference among the treatments. ($n = 4$–8 animals per group)

Table 2 Effects of cadmium (Cd), *N*-acetylcysteine (NAC) or their combination on total red blood cell (RBC) count, hemoglobin (Hb) content, hematocrit (Ht) value, total white blood cell (WBC) count and on differential count of peripheral blood leucocytes (lymphocytes, neutrophils and monocytes) of male rats

Groups	RBC (10^3/mm^3)	Hb (g/dl)	Ht (%)	WBC (10^3/mm^3)	Lymphocyte (%)	Neutrophil (%)	Monocyte (%)
Saline	8.84 ± 0.05^a	14.73 ± 0.3^a	44.33 ± 1.0^a	8.40 ± 0.3^a	84.83 ± 1.2^b	11.17 ± 1.4^b	4.00 ± 0.8^b
NAC	8.91 ± 0.05^a	14.52 ± 0.4^a	43.62 ± 1.0^a	8.43 ± 0.3^a	91.87 ± 1.3^a	5.25 ± 1.0^c	2.87 ± 0.5^b
Cd	7.65 ± 0.19^b	12.86 ± 0.2^b	38.25 ± 0.6^b	6.01 ± 0.4^c	70.50 ± 1.3^c	19.50 ± 1.4^a	10.00 ± 0.9^a
Cd/NAC	8.50 ± 0.21^a	14.33 ± 0.2^a	43.25 ± 0.8^a	7.48 ± 0.3^b	80.75 ± 1.1^b	13.75 ± 1.0^b	5.50 ± 1.0^b

Data are mean ± SEM. Two-way ANOVA–Duncan's Test ($p < 0.05$). Different letters in the same column indicate significant difference among the treatments. ($n = 4$–8 animals per treatment)

observed in neutrophil count, the results demonstrated that NAC treatment *per se* caused a decrease (53 %) in neutrophil count while Cd exposure increased it (75 %). The Cd/NAC group presented values similar to the control group abolishing this Cd effect. A significant main effect of Cd ($p < 0.05$) and NAC ($p < 0.05$) in monocyte count was observed. Results demonstrated that Cd exposure caused an increase of 150 % in monocyte count. NAC treatment was totally efficient in reversing this Cd-induced impairment.

The results obtained for NTPDase activity in lymphocytes are presented in Fig. 1. Two-way ANOVA revealed that there were no significant alterations in the hydrolysis of ATP and ADP (NTPDase activity) in lymphocytes of rats treated with Cd and/or NAC.

The cholinesterase activities are showed in Figs. 2, 3 and 4. A significant main effect of Cd ($p < 0.05$) was observed in the AChE activity from whole blood (Fig. 2) and lymphocytes (Fig. 3) as well as serum BChE activity (Fig. 4). Results demostrated that Cd exposure caused a decrease of 25, 50 and 37 % in the activities of whole blood AChE, lymphocyte AChE and serum BChE, respectively. Moreover, Cd/NAC group decreased about 25, 49 and 35 % the activities of whole blood AChE, lymphocyte AChE and serum BChE, respectively. Thus, the NAC co-treatment was inefficient in counteracting the negative effect of Cd in cholinesterase activities.

Analyzing linear regression between the whole blood AChE activity and red blood cell count ($r = 0.93$) (Fig. 5a) and between the lymphocyte

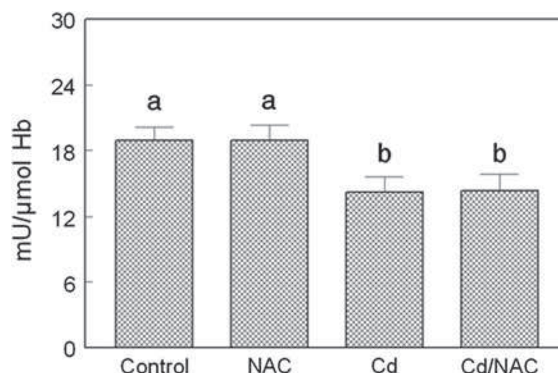

Fig. 2 Effects of cadmium (Cd), *N*-acetylcysteine (NAC) or their combination on the acetylcholinesterase (AChE) activity from whole blood of male rats. *Bars* represent mean ± SEM, two-way ANOVA–Duncan's Test ($p < 0.05$). *Different lowercase letters* indicate significant difference among the treatments. ($n = 4$–7 animals per treatment)

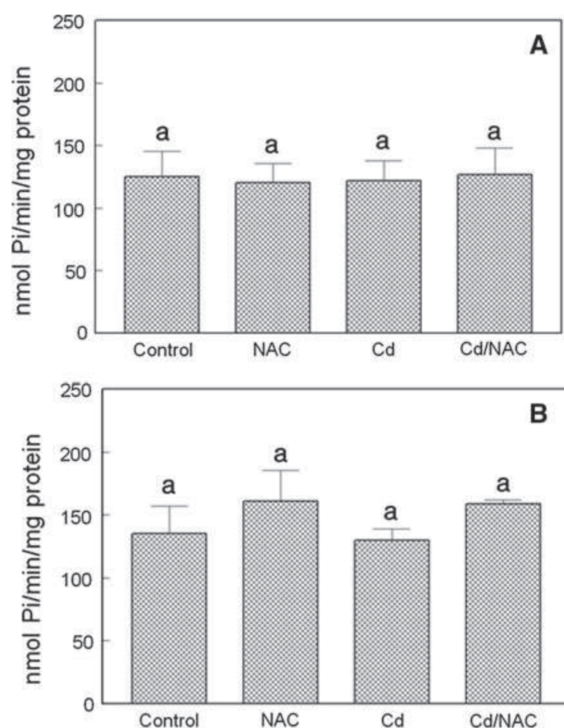

Fig. 1 Effects of cadmium (Cd), *N*-acetylcysteine (NAC) or their combination on NTPDase activity from lymphocytes of male rats using ATP (**a**) and ADP (**b**) as substrate. *Bars* represents mean ± SEM, two-way ANOVA–Duncan's Test ($p < 0.05$). *Different lowercase letters* indicate significant difference among the treatments. ($n = 4$–6 animals per treatment)

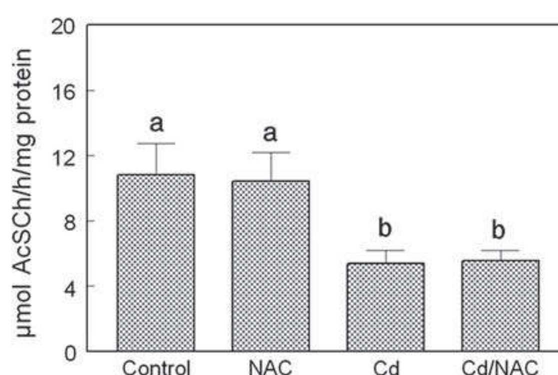

Fig. 3 Effects of cadmium (Cd), *N*-acetylcysteine (NAC) or their combination on the acetylcholinesterase (AChE) activity from lymphocytes of male rats. *Bars* represent mean ± SEM, two-way ANOVA–Duncan's Test ($p < 0.05$). *Different lowercase letters* indicate significant difference among the treatments. ($n = 4$–7 animals per treatment)

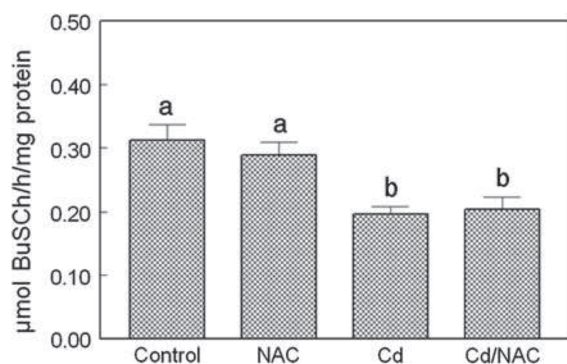

Fig. 4 Effects of cadmium (Cd), *N*-acetylcysteine (NAC) or their combination on the butyrylcholinesterase (BChE) activity from serum of male rats. *Bars* represent mean ± SEM, two-way ANOVA–Duncan's Test ($p < 0.05$). *Different lowercase letters* indicate significant difference among the treatments. ($n = 4$–6 animals per treatment)

AChE activity and lymphocyte count ($r = 0.87$) (Fig. 5b) there were significantly positive correlations in Cd-exposed rats ($p < 0.05$).

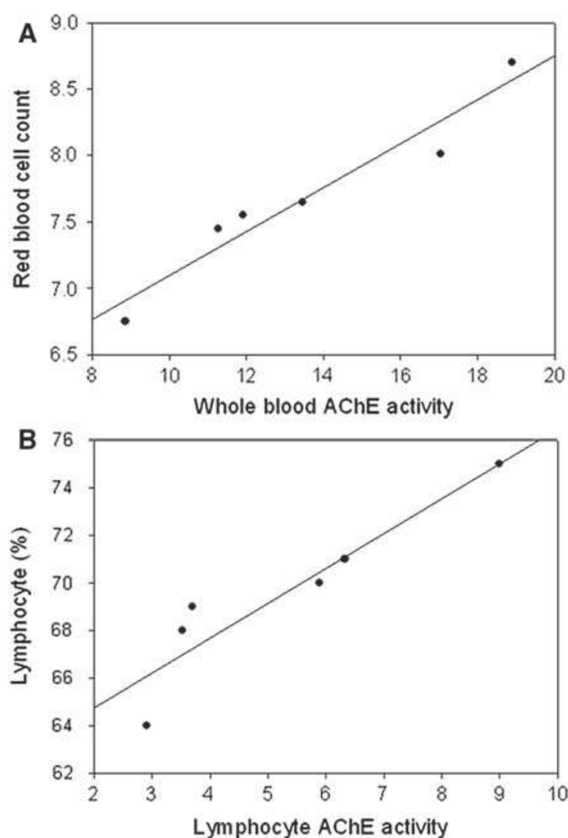

Fig. 5 Correlations between the whole blood AChE activity and red blood cell count ($r = 0.93$) (**a**) and between the lymphocyte AChE activity and lymphocyte count ($r = 0.87$) (**b**) in Cd-exposed rats ($p < 0.05$)

Discussion

To our knowledge, the current study is the first one evaluating the role of NAC in protecting Cd poisoning by measuring Cd concentration in plasma, spleen and thymus, hematological data as well as the activity of peripheral NTPDase, AChE and BChE of Cd-intoxicated rats and co-treated with NAC.

According to Arrieta et al. (2005) and Mesdaghinia et al. (2010) experimental studies with heavy metal intoxication can be extrapolated to human population with caution. Interestingly, in our experimental animals, Cd concentration in blood (0.00156 µg/l) was smaller or similar to those observed in adult humans from some urban centers with levels of exposure to this metal (Järup et al. 1998; Järup and Akesson 2009). This fact could be a result of the long biological half-life of Cd in humans (15–30 years) mainly due to its low rate of excretion from the body. In the present study the Cd concentration in rat tissues increased after Cd exposure as reported by several investigations (Karmakar et al. 2000; Pari and Murugavel 2005; Gonçalves et al. 2010). Corroborating our results, Viau et al. (2007) reported that Cd could also reach lymphoid organs, thymus and spleen. They also found that spleen accumulates about two times more Cd than the thymus. In the present study, a similar Cd accumulation in the lymphoid organs was observed. Pathak and Khandelwal (2007, 2009) demonstrated that splenic cells appeared more susceptible than thymus cells to the adverse effects of Cd which promoted a spleen enlargement. According to them, the gain in spleen weight may be associated with the expansion of red pulp (damaged RBC) area. The RBC damage-hemolysis-splenomegaly sequence was considered to be associated with the etiology of Cd-induced anemia. In fact, in the present investigation we found a decrease in the hematological parameters (RBC, Hct and Hb) of Cd-exposed rats; however, NAC was able not only to reverse these negative effects, but also to diminish the Cd level in thymus and spleen of rats co-treated with the metal and the antioxidant. Pathak and Khandelwal (2007, 2009) also demonstrated that Cd induces splenomegaly and

thymic atrophy in mice possibly due to the potentiation of oxidative stress, i.e., over production of ROS and GSH depletion, followed by mitochondrial-caspase dependent apoptotic pathway. However, it has already been reported that NAC in vitro inhibited ROS generation and raised GSH levels in thymocytes and splenocytes (Pathak and Khandelwal 2006a, b). Thus, based on our results and considering the antioxidant properties described here for NAC, it is reasonable to believe that this compound may produce positive effects on Cd-induced lymphoid damage.

In addition, it is known that after exposure, Cd enters the blood, binds the erythrocytic membrane and stimulates the formation of ROS, leading to alterations in the antioxidant system of erythrocytes and imposing oxidative damage upon the membrane (Ognjanovic et al. 2003; Sinha et al. 2008; Messaoudi et al. 2010). In fact, anemia is one of the characteristic clinical manifestations of Cd intoxication as Cd is known to reduce hematological parameters (RBC, Hct and Hb). In the present study we demonstrated a decrease in these parameters by Cd which is in agreement with Karmakar et al. (2000), El-Demerdash et al. (2004), Nemmiche et al. (2007) and Simsek et al. (2009) that have shown that cadmium chloride caused changes in the blood indices of rats. The significant reduction in RBC indicated the fact that Cd adversely affected the erythropoiesis in time-dependent fashion (Nemmiche et al. 2007). Furthermore, the reduction in the blood parameters may be attributed to the hyperactivity of bone marrow that leads to the production of RBC with impaired integrity easily destructed in the circulation (Tung et al. 1975; El-Demerdash et al. 2004). This premise is supported by Pathak and Khandelwal (2009) who reported that Cd induced-splenomegaly was due to excessive deposition of damaged erythrocytes. The reduction in Hb content may be due to the increased rate of destruction or reduction in the rate of erythrocyte formation (El-Demerdash et al. 2004). In fact, it is well known the inhibitory effect of Cd on the activity of δ-aminolevulinate dehydratase (δ-ALAD) enzyme that catalyses the asymmetric condensation of two molecules of δ-aminolevulinic acid to porphobilinogen in the initial steps of heme biosynthesis, an essential structure present in Hb molecule (Luchese et al. 2007). Moreover, a decrease in blood iron may be also harmful to erythrocyte formation (Simsek et al. 2009). Thus, we can also suggest that Cd could be decreasing the blood iron level and/or inhibiting the ALA-D activity of rats resulting in impaired Hb formation. Moreover, Pari and Murugavel (2005) when working with rats exposed to 3.0 mg/kg Cd, s.c., for 3 weeks attributed the decreased hemoglobin content to a possible decrease in the levels of erythrocyte adenosine triphosphate (ATP) and 2,3-diphospho-glycerate (2,3-DPG) as well as an increased oxidative stress. These events initially alter membrane skeleton of erythrocyte followed by the deformation of the cell promoting hemolysis and resulting in decreased levels of hemoglobin and anemia (Hamada et al. 1998; Ye et al. 1999; Klapcinska et al. 2000).

In relation to leukocyte values, the results of the present investigation corroborate with the data presented by Karmakar et al. (2000) who reported a decrease in WBC count associated with low lymphocyte and high neutrophil values in rats exposed to 2.0 mg/kg Cd, s.c., for 14 and 21 days. Moreover, Simsek et al. (2009) demonstrated that 50 mg/l Cd dissolved in water induced leucopenia in rats after 30 days of exposure. On the other hand, El-Demerdash et al. (2004) reported an increased WBC count in rats that received 5 mg/kg Cd every other day for 30 days, a higher dose than ours. These authors attributed the increase in WBC count to an activation of the animal immune system. Lafuente et al. (2004) showed a biphasic effect of Cd on blood lymphocyte subsets (B and T lymphocyte and its subpopulations, $CD4^{+}$ and $CD8^{+}$) because lower doses of the metal (5 and 10 ppm Cd) inhibited the humoral and cellular response; however, an opposite effect was detected with higher doses (25, 50 and 100 ppm Cd). Moreover, Karmakar et al. (2000) showed that monocytes were increased after 21 days of Cd exposure while 7 and 14 administrations of Cd caused a decrease in the monocytes count corroborating our results. The alterations in differential count of leucocytes induced by Cd may be due to alterations of the lymphoid/myeloid ratio (Karmakar et al. 2000). It is interesting to note that activated neutrophils produced ROS during inflammatory reaction, and if neutrophils accumulate due to Cd toxicity, the tissue could be exposed to large quantities of potentially injurious neutrophil content (Karmakar et al. 2000).

The treatment with antioxidant compounds such as ascorbic acid, β-carotene, diallyl tetrasulfide, α-tocopherol, and NAC was effective to reverse the negative effects of Cd and other metals on immune cells, RBC as well as hematological parameters (El-

Demerdash et al. 2004; Pari and Murugavel 2005; Pathak and Khandelwal 2006a, b; Nemmiche et al. 2007). In the present study, NAC treatment *per se* caused an increase in lymphocyte and a decrease in neutrophil counts and also, reversed or ameliorated the hematological impairments caused by Cd exposure. The sulfur-containing antioxidant NAC has been widely used as an antioxidant in several in vitro and in vivo experiments (Carvalho et al. 2004). In addition, NAC has some other mechanisms of action, such as reestablishment of the normal erythrocyte morphology in diabetic patients, inhibition of neutrophil activation and decreased microbial attachment (Straface et al. 2002; Aito 2005). In fact, NAC can act attenuating, alleviating, adding resistance, healing and strengthening the immune response in many diseases such as Leishmania infection, influenza and cancer (Mantovani et al. 2003; Aito 2005). According to Blackwell et al. (1996), NAC reduces the inflammatory process through the inhibition of ROS production and decreasing both NF-kB activation and the expression of proinflammatory cytocines. To our knowledge, this investigation is the first one showing the role of NAC in reducing the harmful effects of Cd poisoning on hematological parameters as well as leukocyte cells.

In addition, several studies from our laboratory have demonstrated that ectonucleotidases and cholinesterases have an important role in immunomodulation, thromboregulation and neurotransmission, and alterations in their activities have been observed in various diseases and intoxication processes suggesting that they could be important physiological and pathological parameters (Schetinger et al. 2007; Mazzanti et al. 2009; Schmatz et al. 2009; Gonçalves et al. 2010; Kaizer et al. 2010; Spanevello et al. 2010; Da Silva et al. 2011; Jaques et al. 2011; Leal et al. 2011).

NTPDase plays a pivotal role in lymphocytes as an activation marker essential for certain effector functions, since extracellular nucleotides are mediators of immune and non-immune cell functions (Dombrowski et al. 1998). The participation of this enzyme in the progress of diseases and intoxications involving several tissues and cell types has been widely discussed (Schetinger et al. 2007; Jaques et al. 2011). Kaizer et al. (2010) and Spanevello et al. (2010) suggested that the up-regulation of NTPDase activity in lymphocytes from rats intoxicated with aluminium and from patients with relapsing–remitting form of multiple sclerosis (RRMS), respectively, represents a compensatory response to the inflammatory process. Recently, our group postulated that NTPDase could be used as a peripheral enzyme marker in patients treated with antiretroviral drugs once therapeutic doses of these anti-HIV drugs did not influence the NTPDase activity in lymphocytes obtained from healthy subjects (Leal et al. 2011). In the present study, unfortunately, Cd intoxication did not alter the NTPDase activity in rat lymphocytes. However, we must take into account that different results have been reported on the effects of Cd exposure on immune system development and function, depending on the route of administration, the Cd chemical form and the duration and dose exposure of the model studied. Thus, an alteration in the NTPDase activity could be noted in other circumstances of Cd intoxication.

In relation to the AChE activity in the whole blood and in the lymphocytes, the results showed that an inhibition of this enzyme occurred in Cd-exposed rats. These data corroborate with studies of El-Demerdash et al. (2004) and Pari and Murugavel (2005) who showed an inhibition in both plasma and brain AChE activity from rats exposed to Cd. The inactivation of the AChE enzyme as a result of the occupation of its active sites by heavy metals was suggested by Shaw and Panigrahi (1990) who investigated Hg-exposed fish. In fact, Cd mechanism of action has been hypothesized to be either the displacement of metal cofactors from the active site or the direct deactivation of the enzyme site (Casalino et al. 1997). The results of the AChE inhibition were similar to the results recently published by our research team in the cerebral regions of the same animal group studied here (Gonçalves et al. 2010). In fact, several studies have demonstrated the importance of cholinergic peripheral markers in diseases with neurological symptoms to evaluate biochemical processes that occur in the central nervous system (CNS) since blood and lymphocyte AChE activity is easily obtained and show functional and structural properties similar to AChE of the synaptic cleft, which may reflect its status (Mazzanti et al. 2009; Schmatz et al. 2009). Thus, in agreement with previous findings of our laboratory we can suggest that the whole blood and lymphocyte AChE activity could be a good peripheral marker for Cd toxicity because it permits the evaluation through a more accessible method than the direct evaluation of this enzyme in CNS (Mazzanti et al. 2009; Schmatz et al. 2009).

In addition, it is interesting to note that the decreased AChE lymphocyte activity found in Cd-intoxicated rats may lead to a slow degradation of ACh, which is considered a molecule with anti-inflammatory action because it binds to nicotinic receptors in lymphocyte surfaces. Thus, it inhibits the proliferation of cytokines, serotonin, histamine, nitric oxide, lysosomal enzymes, prostaglandins and leukotrienes which are among the mediators of the inflammatory process (Kawashima and Fujii 2003; Czura and Tracey 2005; Das 2007). Therefore, the decrease of AChE may have an anti-inflammatory action in order to have more ACh free, to bind to lymphocytes and to inhibit inflammation. In this scenario, we can infer that this inhibition of enzyme activity can be explained as a compensatory effect of the organism to prevent or attenuate inflammation as reported by us recently in rats infected with *Trypanosoma evansi* (Da Silva et al. 2011).

It is interesting to note that analysis between the whole blood AChE activity and RBC count and between lymphocyte AChE activity and lymphocyte count showed positive correlations. Therefore, the activity of this enzyme in whole blood (erythrocytes) and lymphocytes may be reduced due to a decrease in these circulating cells (Wolkmer et al. 2007, 2010; Da Silva et al. 2011).

It is generally agreed that BChE acts scavenging naturally and man-made occurring poisons to protect AChE from inhibition (Masson and Lockridge 2010). In the present study, Cd inhibited serum BChE activity, and this enzyme was not efficient in protecting AChE since Cd also inhibited the AChE activity in whole blood, lymphocytes and cerebral structures. The mechanisms by which Cd toxicity decreases serum BChE activity have not been investigated; however, this inhibitory effect appears to be due to increased enzyme degradation and/or structural alterations of protein molecules caused by ROS action since the oxidation of target amino acid residues such as Met and Trp in the active site of both BChE and AChE by hydrogen peroxide (H_2O_2) lead to an inhibition/deactivation of both ACh degrading enzymes (Garcia et al. 2008). Other possibility is its decreased synthesis in the liver, as serum BChE is produced and secreted into blood by the liver being used as an indicator of hepatic disorders (Randell et al. 2005). In fact, the main target organs of Cd are liver and kidney. In our study, Cd induced moderate hepatic toxicity because Cd-exposed rats showed increased serum AST and ALT activities, classical indicators of liver damage (data not shown), but no hepatic histological damage was found. These results not decrease the relevance of the present study because we demonstrated the presence of Cd in the plasma, spleen and thymus and its negative effects in the circulating cells.

Taken together, the results of the present study provide ex vivo evidence supporting the hypothesis that Cd induces immunotoxicity by interacting with the lymphoid organs, altering hematological parameters, and inhibiting peripheral cholinesterases activity. Another interesting finding here is that although NAC co-treatment was inefficient in counteracting the negative effect of Cd in the peripheral cholinesterase activities, this compound showed ability in decreasing Cd levels in thymus and spleen and in reversing or ameliorating the hematological damages caused by Cd poisoning. In addition, further investigations are essential to elucidate the precise mechanism of NAC protection against Cd-immunotoxicology and tests against other immunotoxicants are needed before evaluating it as an immunoprotective agent.

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Conflict of interest There are no conflicts of interest for any of the authors.

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5. CAPÍTULO 3

Manuscrito: NTPDase and 5'-nucleotidase activities from synaptosomes and platelets of rats exposed to Cd and treated with N-acetylcysteine – Submetido ao Periódico *International Journal of Developmental Neuroscience*

NTPDase and 5'-nucleotidase activities from synaptosomes and platelets of rats exposed to Cd and treated with N-acetylcysteine

Jamile F. Gonçalves[a*], Roselia M. Spanevello[b], Amanda M. Fiorenza[c], Cinthia M. Mazzanti[d], Margarete D. Bagatini[e], Cíntia S. da Rosa[c], Lara V. Becker[c], Pauline da Costa[c], Fátima H. Abdalla[c], Vera M. Morsch[c], Maria Rosa C. Schetinger[a,c*]

[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcellos, 2600-Anexo, 90035-003 Porto Alegre, RS, Brazil.

[b] Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil.

[c] Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Setor de Bioquímica, Universidade Federal de Pelotas, Campus Universitário Capão do Leão, 96010-900, Pelotas, RS, Brazil.

[d] Departamento de Clínica de Pequenos Animais, Setor de Patologia Clínica Veterinária, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil.

[e] Colegiado do Curso de Enfermagem, Universidade Federal da Fronteira Sul, 89812-000, Chapecó, SC, Brazil.

[*]Corresponding authors

Address correspondence and reprint requests to: Maria Rosa Chitolina Schetinger (mariachitolina@gmail.com) and Jamile Fabbrin Gonçalves (jamilefabbrin@yahoo.com.br). Tel/Fax: + 55-55-3220-9557

ABSTRACT

The purpose of the present investigation was to evaluate the hydrolysis of adenine nucleotides on synaptosomes and platelets obtained from rats exposed to cadmium (Cd) and treated with N-acetylcysteine (NAC). Rats received Cd (2 mg/kg) and NAC (150 mg/kg) by gavage every other day for 30 days. Animals were divided into four groups (n = 4-6): control/saline, NAC, Cd, and Cd/NAC. The results of this study demonstrated that NTPDase and 5′-nucleotidase activities were increased in the cerebral cortex synaptosomes of Cd-poisoned rats, and NAC co-treatment reversed these activities to the control levels. In relation to hippocampus synaptosomes, no differences on the NTPDase and 5’-nucleotidase activities of Cd-poisoned rats were observed and only the 5’-nucleotidase activity was increased by the administration of NAC *per se*. In platelets, Cd-intoxicated rats showed a decreased NTPDase activity and no difference in the 5′-nucleotidase activity; NAC co-treatment was inefficient in counteracting this undesirable effect. Our findings reveal that adenine nucleotide hydrolysis in synaptosomes and platelets of rats were altered after Cd exposure leading to a compensatory response in the central nervous system and acting as a modulator of the platelet activity. NAC was able to modulate the purinergic system which is interesting since the regulation of these enzymes could have potential therapeutic importance. Thus, our results reinforce the importance of the study of the ecto-nucleotidases pathway in poisoning conditions and highlight the possibility of using antioxidants such as NAC as adjuvant against toxicological conditions.

Keywords: Cadmium, NAC, Purinergic system

1. Introduction

Neurons and platelets express a multienzymatic complex on their surface including the enzymes NTPDases and 5′-nucleotidase. NTPDases hydrolyzes ATP and ADP to AMP, which is subsequently hydrolyzed to adenosine by 5′-nucleotidase (Zimmermann, 2001; Colgan et al., 2006; Robson et al., 2006). Both ecto-enzymes are able to regulate the extracellular concentrations of adenine nucleotides (ATP, ADP and AMP) and nucleosides (adenosine) playing an important role in vascular tone, thromboregulation (Zimmermann, 1999; Remijin et al., 2002), neurotransmission (Burnstock, 2006) neuroprotection (Cunha, 2001) and proliferation of glial cells (Fields and Burnstock, 2006). Alterations in their activities have been demonstrated by our research group in various diseases and poisonings suggesting that these enzymes could be important physiological and pathological parameters (Kaizer et al., 2007; Spanevello et al., 2007; Mazzanti et al., 2007; Schetinger et al., 2007; Bagatini et al., 2008; Schmatz et al., 2009).

Cadmium (Cd) is one of the most toxic pollutants in the environment causing many toxic effects in various organs and systems such as cardiovascular, immune/haemopoietic and nervous (Nakagawa and Nishijo, 1996; Gonçalves et al., 2010). Although small amounts of this metal reach the brain because of the blood–brain barrier (BBB), Cd induces neurotoxicity with a wide spectrum of clinical entities including oxidative stress, changes in normal neurochemistry of the brain, memory impairment, cerebral hemorrhage and edema (Gutierrez-Reyes et al., 1998; Viaene et al., 2000; Minami et al., 2001; Méndez-Armenta and Ríos, 2007; Gonçalves et al., 2010).

75 Moreover, the vascular effect of Cd leading to cardiovascular diseases is of
76 toxicological significance in populations exposed to higher Cd levels. Both vascular
77 endothelial cells and blood platelets play important roles in the maintenance of vascular
78 integrity (Mukhopadhyay et al., 1988). Platelets are important to prevent blood loss by a
79 process of aggregation and to elicit the responses of the blood to injury or even
80 xenobiotics (Kumar and Bhattacharya, 2000). The platelet model has been used since
81 the peripheral tissues are easily measured in humans as a surrogate marker of damages,
82 dysfunctions or interactions involving neural targets of toxicants (Kaizer et al., 2007;
83 Mazzanti et al. 2007; Schmatz et al., 2009; Spanevello et al., 2009).

84 Although the molecular mechanisms of Cd toxicity are not well defined, it has
85 been reported that its oxidative effect is indirect and based mainly on the depletion of
86 sulphydryl (-SH)-group-containing compounds. Therefore, the high affinity of Cd to
87 thiol groups can be an important mechanism in Cd-induced toxicity, and compounds
88 that restore this pool could be beneficial against Cd intoxication. N-acetylcysteine
89 (NAC) is an antioxidant and a thiol donor acting as an intracellular cysteine precursor
90 leading to increased level of -SH content (Pinho et al., 2005). NAC is considered a
91 therapeutic agent commonly used in clinical practice, and it is able to ameliorate Cd-
92 induced neurotoxicity (Sadowska et al., 2007; Gonçalves et al., 2010).

93 By the prerogatives outlined above, the purpose of the present investigation was
94 to evaluate the hydrolysis of adenine nucleotides on synaptosomes and platelets
95 obtained from rats exposed to Cd and treated with NAC.

96

97 **2. Material and Methods**

98

99 *2.1. Chemicals*

100 Nucleotides, Trizma Base, Percoll, HEPES and Comassie brilhant blue G were
101 obtained from Sigma–Aldrich (St. Louis, MO, USA). N-acetylcysteine was obtained
102 from Beg (São Paulo, SP, Brazil) with 99.1% of purity. All other reagents used in the
103 experiments were of analytical grade and of the highest purity.

104

105 *2.2. Animals*

106 Adult male Wistar rats (80 days; 315.7±14 g) from the Central Animal House of
107 the Universidade Federal de Santa Maria (UFSM) were used in this experiment. The
108 animals were maintained at a constant temperature (23±1 ºC) on a 12 h light/dark cycle
109 with free access to food and water. All animal procedures were approved by the Animal
110 Ethics Committee from UFSM.

111

112 *2.3. Experimental procedure*

113 The body burden of Cd is derived primarily from the ingestion of food and water
114 contaminated with Cd. $CdCl_2$ is the principal form of Cd associated with oral exposure,
115 as it is highly soluble in water (Zalups and Ahmad, 2003). Thus, in the present study the
116 rats received cadmium as $CdCl_2 \cdot H_2O$ (Cd; 2mg/kg) (El-Demerdash et al., 2004; Borges
117 et al., 2008) and N-acetylcysteine (NAC; 150mg/kg) (Sadowska et al., 2007) by gavage
118 every other day for 30 days. The animals were randomly divided into four groups (n =
119 12-13 per group): control/saline, NAC, Cd and Cd/NAC. The last group received NAC
120 30 min after Cd. The solutions were freshly prepared in saline and administered (1
121 mL/kg) between 9 and 11 a.m. After the exposure period, the animals were anesthetized
122 and submitted to euthanasia. The total blood was collected by cardiac puncture to
123 separate the platelets. The cranium was carefully opened for cerebral cortex and
124 hippocampus removal to obtain the synaptosomes.

125 *2.4. Synaptosome preparation*

126 The cerebral cortex and hippocampus were homogenized in 10 volumes of an
127 ice cold medium (medium I), consisting of 320 mM sucrose, 0.1 mM EDTA and 5 mM
128 HEPES, pH 7.5, in a motor driven Teflon-glass homogenizer. The synaptosomes were
129 isolated as described by Nagy and Delgado-Escueta (1984) using a discontinuous
130 Percoll gradient. The pellet was suspended in an isoosmotic solution and the final
131 protein concentration was adjusted to 0.4–0.6 mg/mL. Synaptosomes were prepared
132 fresh daily, maintained at 0–4 ºC throughout the procedure and used for enzymatic
133 assays.

134

135 *2.5. Platelet separation*

136 The platelet-rich plasma preparation (PRP) was prepared by the method of Pilla
137 et al. (1996) and modified by Lunkes et al. (2004). Total blood was collected by cardiac
138 puncture and placed into a flask with sodium citrate as anticoagulant. The total blood–
139 citrate system was centrifuged at 1,000 rpm for 15 min to remove residual blood cells.
140 The PRP was centrifuged at 3,500 rpm for 25 min and washed twice by centrifugation
141 at 3,500 rpm for 10 min with 3.5 mM HEPES isosmolar buffer, pH 7.2. The washed
142 platelets were resuspended in HEPES isosmolar buffer and the protein was adjusted to
143 0.4–0.6 mg/ml.

144

145 *2.6. Assay of NTPDase (EC 3.6.1.5) and 5'-nucleotidase (EC 3.1.3.5) activities*

146 The NTPDase enzymatic assay of the synaptosomes was carried out in a reaction
147 medium containing 5 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225
148 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 µl as
149 described in a previous study from our laboratory (Schetinger et al., 2000). The 5'-

150 nucleotidase activity was determined essentially by the method of Heymann et al.
151 (1984) in a reaction medium containing 10 mM $MgSO_4$ and 100 mM Tris–HCl buffer,
152 pH 7.5, in a final volume of 200 µl.

153 In platelets, the reaction medium for the NTPDase activity contained 5 mM
154 $CaCl_2$, 120 mM NaCl, 5 mM KCl, 6 mM glucose and 50 mM Tris–HCl buffer, pH 7.4,
155 at a final volume of 200 µl as described by Lunkes et al. (2004). For AMP hydrolysis,
156 the chemical reagents used were the same described for the NTPDase activity, except
157 that 5 mM $CaCl_2$ was replaced by 10 mM $MgCl_2$. In both synaptosomes and platelets,
158 20 µl of enzyme preparation (8–12 µg of protein) was added to the reaction mixture and
159 pre-incubated at 37 ºC for 10 min.

160 The reaction was initiated by the addition of ATP or ADP to obtain a final
161 concentration of 1.0 mM and the incubation proceeded for 20 min (synaptosomes) and
162 60 min (platelets). For AMP hydrolysis, the 5'-nucleotidase activity was carried out as
163 previously described and the final concentration of the nucleotide AMP added was 2
164 mM.

165 In all cases, reactions were stopped by the addition of 200 µl of 10%
166 trichloroacetic acid (TCA) to provide a final concentration of 5%. Released inorganic
167 phosphate (Pi) was assayed by the method of Chan et al. (1986) using malachite green
168 as the colorimetric reagent and KH_2PO_4 as standard. Controls were carried out to correct
169 for non-enzymatic hydrolyses of nucleotides by adding platelets after TCA addition. All
170 samples were run in triplicate. Enzyme specific activities are reported as nmol Pi
171 released/min/mg of protein.

172

173 *2.7. Protein determination*

174 Protein was measured by the method of Bradford (1976) using bovine serum
175 albumin as standard.
176
177 *2.8. Statistical analysis*
178 The statistical analysis was performed using two-way ANOVA, followed by
179 Duncan's multiple range tests. $p<0.05$ was considered to represent a significant
180 difference in all experiments. All data were expressed as mean ± SEM.
181
182 **3. Results**
183
184 *3.1. NTPDase and 5'-nucleotidase activities in synaptosomes from cerebral cortex and*
185 *hippocampus*
186 The results obtained for the NTPDase and 5'-nucleotidase activities in cerebral
187 cortex synaptosomes (CCS) are presented in Fig. 1. A significant Cd×NAC interaction
188 for the NTPDase activity of CCS was observed ($F_{1,18} = 6.054$; $p < 0.05$) when ATP was
189 used as substrate (Fig. 1A). Post hoc comparisons demonstrated that rats exposed to Cd
190 presented an increase (48%) in the NTPDase activity (substrate ATP) of CCS. A
191 significant Cd×NAC interaction for the NTPDase activity of CCS was observed ($F_{1,17} =$
192 6.170; $p < 0.05$) when ADP was used as substrate (Fig. 1B). Post hoc comparisons
193 demonstrated that rats exposed to Cd presented an increase (60%) in the NTPDase
194 activity (substrate ADP) of CCS. A significant Cd×NAC interaction for the 5'-
195 nucleotidase activity of CCS was observed ($F_{1,16} = 20.359$; $p < 0.05$) using AMP as
196 substrate (Fig. 1C). Post hoc comparisons demonstrated that rats exposed to Cd
197 presented an increase (39%) in the 5'-nucleotidase activity of CCS. The increase in the

198 NTPDase and 5´-nucleotidase activities induced by Cd was abolished by NAC
199 administration.

200 The results obtained for the NTPDase and 5'-nucleotidase activities in
201 hippocampus synaptosomes (HS) are presented in Fig. 2. Two-way ANOVA revealed
202 that there were no significant alterations in the NTPDase activity (substrate ATP or
203 ADP) in HS (Figs. 2A and 2B). A significant Cd×NAC interaction for the 5'-
204 nucleotidase activity (substrate AMP) of HS was observed ($F_{1,8} = 8.145$; $p < 0.05$) (Fig.
205 2C). Post hoc comparisons demonstrated that rats exposed to NAC presented an
206 increase (74%) in the 5'-nucleotidase activity of HS.

207

208 *3.2. NTPDase and 5'-nucleotidase activities in platelets*

209 The results obtained for the NTPDase and 5'-nucleotidase activities in platelets
210 are presented in Fig. 3. A significant main effect of Cd ($p < 0.05$) and NAC ($p < 0.05$) in
211 platelet NTPDase activity was observed when ATP was used as substrate (Fig. 3A).
212 Results demonstrated that Cd or Cd/NAC exposure caused a decrease of 48% and 36%,
213 respectively, in the NTPDase activity (substrate ATP). A significant main effect of Cd
214 ($p < 0.05$) and NAC ($p < 0.05$) in platelet NTPDase activity was observed when ADP
215 was used as substrate (Fig. 3B). Results demonstrated that Cd or Cd/NAC exposure
216 caused a decrease of 38% and 29%, respectively, in the NTPDase activity (substrate
217 ADP). NAC administration did not show a tendency to ameliorate the NTPDase activity
218 (substrate ATP or ADP) decreased by Cd. Two-way ANOVA revealed that there were
219 no significant alterations in the 5'-nucleotidase activity (substrate AMP) in platelets
220 (Fig. 3C).

221

222

223 **4. Discussion**

224

225 The current study is, to our knowledge, the first which evaluates the *ex vivo*
226 effects of oral administration of cadmium or N-acetylcysteine and their combination on
227 the NTPDase and 5'-nucleotidase activities from synaptosomes and platelets of adult
228 rats.

229 As reported previously by our research group, Cd concentration in rat tissues
230 was increased after Cd exposure demonstrating the efficacy of this Cd poisoning model
231 (Gonçalves et al., 2010). Although a small amount of Cd reaches the brain regions of
232 these animals, it was enough to cause brain injury. The co-administration of NAC was
233 ineffective in decreasing these Cd levels in brain and also in plasma, suggesting that the
234 harmful effects of Cd observed in these animals were ameliorated mainly by the
235 antioxidant or –SH supplier activity of NAC rather than by the Cd removal from tissues
236 (Gonçalves et al., 2010; Gonçalves et al. *in press*).

237 The results of the present study demonstrated that the NTPDase and 5′-
238 nucleotidase activities were increased in the cortex synaptosomes of Cd-poisoned rats.
239 Previous studies carried out in our laboratory also demonstrated the up-regulation in the
240 NTPDase and 5′-nucleotidase activities in synaptosomes from cerebral cortex of rats
241 submitted to other experimental conditions such as demyelination, diabetes and
242 intoxication with aluminium (Spanevello et al., 2006, 2009; Kaizer et al., 2007;
243 Schmatz et al., 2009). The increased NTPDase and 5′-nucleotidase activities found in
244 these situations have been related to a compensatory organic response. One explanation
245 may be that the rapid ATP and ADP hydrolysis favors adenosine production, a
246 neuroprotector molecule.

247 ATP molecule has a diverse array of functions in the central nervous system
248 (CNS) acting as a fast excitatory neurotransmitter (Sperlágh et al., 1995) and as a
249 presynaptic neuromodulator (Cunha and Ribeiro, 2000). Moreover, ADP molecule is
250 also involved in neuron–glial interactions (Fields and Stevens, 2000) with a role in
251 neuronal development and plasticity (Burnstock, 2006). On the other hand, it has also
252 been demonstrated that in brain injury, ATP released in large amounts could cause
253 significant tissue damage (Feuvre et al., 2002). Its breakdown product, adenosine, plays
254 an important role in neuromodulation, homeostatic regulation and neuroprotection of
255 the CNS (Cunha, 2001; Dumwiddie and Masino, 2001). According to Schmatz et al.
256 (2009) and Spanevello et al. (2009) the increase in the NTPDase and 5′-nucleotidase
257 activities in the cortex synaptosomes may be related to an important adaptive plasticity
258 of ectonucleotidases pathway that could occur in order to terminate the function of
259 extracellular ATP, including its cytotoxic effects, and in order to increase adenosine
260 levels, a known endogenous neuromodulator and neuroprotective. In this context and
261 considering our results, we can suggest that the organism could be avoiding
262 neurotoxicity processes by depleting ATP and enhancing adenosine production during
263 Cd intoxication.

264 In relation to hippocampus synaptosomes, no differences on the NTPDase and
265 5'-nucleotidase activities of Cd-poisoned rats were observed. However, only the 5'-
266 nucleotidase activity was increased by the administration of NAC *per se*. These data
267 demonstrated the need to consider that the modulation of ectonucleotidases by NAC
268 may also be a protective function, by which extracellular ATP/ADP is kept within
269 physiological levels and adenosine production is increased.

270 It is interesting to note that our research team recently published that brain
271 structures of the same animal group studied here showed a decreased

272 acetylcholinesterase (AChE) activity leading to an abnormal amount of the
273 neurotransmitter acetylcholine which is less hydrolyzed in synapses, causing
274 consequently an over activation of cholinergic receptors and possible toxic effects.
275 However, when NAC was co-administered, the AChE activity was totally or partially
276 reverted improving cognition (Gonçalves et al., 2010). At the same manner, in the
277 present investigation, NAC co-treatment reversed to control levels the NTPDase and 5'-
278 nucleotidase activities in brain of rats exposed to Cd demonstrating that this compound
279 is able to modulate not only the cholinergic, but also the purinergic neurotransmission.

280 In addition, Cd-intoxicated rats showed a decreased NTPDase activity and no
281 difference in the 5′-nucleotidase activity in platelets; NAC treatment was inefficient in
282 counteracting this undesirable effect. This reduction in ATP and ADP may allow
283 circulating ATP and ADP concentrations to be significantly elevated in Cd-poisoned
284 rats whereas a tendency in the reduction of adenosine production could occur in these
285 animals. ATP, ADP and adenosine regulate the vascular response to endothelial damage
286 by exerting a variety of effects on platelets. It is known that ATP promotes
287 vasoconstriction in the vascular endothelium and ADP activates platelet aggregation
288 (Birk et al., 2002a, b; Rozalski et al., 2005), whereas adenosine induces vasodilatation
289 and the inhibition of platelet aggregation (Afonsi et al., 2002; Borowiec et al., 2006). In
290 this scenario, our results suggest, for the first time that the inhibition of the NTPDase
291 activity associated with a tendency in the reduction of the 5'-nucleotidase activity due to
292 Cd exposure may contribute to the hypertensive property and thrombogenic activity of
293 Cd described by several authors.

294 Over the decades, different researchers have turned their attention to understand
295 the effects of Cd on platelets. Caprino et al. (1979) demonstrated the enhanced
296 aggregability of Cd-treated platelets to the ADP and other aggregating agents. It is well

297 established that ADP acts as the principal agonist producing thrombogenesis in animal
298 models and in humans. Mukhopadhyay et al. (1988) postulated that the intracellular
299 accumulation of Cd may result in platelet hyperactivity due to higher intraplatelet free
300 calcium levels resulting directly through Cd action or indirectly through higher H_2O_2
301 level due to catalase inhibition. Moreover, platelets of Cd-exposed rats presented an
302 increased phospholipase A_2 (PLA_2) and ciclooxygenase (COX) activities as well as
303 thromboxane A_2 (TXA_2) and prostaglandin D_2 (PGD_2) levels, whereas aortic
304 prostacyclin (PGI_2) production was reduced in these animals (Kim et al., 2001). Their
305 results indicate that the arachidonic acid cascade was accelerated in Cd-poisoned rats
306 enhancing platelet aggregability. Dorn (1990) reported that prostaglandin/thromboxane
307 receptors in mammalian platelets have essential sulfhydryl groups and therefore it is
308 expected that Cd would bind to platelet membrane and induce changes in its function
309 including platelet aggregation. In regard to this, alterations in the membrane of platelets
310 (Remijin et al., 2002; Wagner and Burger, 2003; Keating et al., 2004; Spanevello et al.,
311 2007, 2009) could be a decisive factor in changing the conformational state of the
312 NTPDase and 5'-nucleotidase molecule, which would explain the reduced activity
313 observed in the present study in the Cd-intoxicated rats. In addition, it has been also
314 described the interference of Cd with absorption/reabsorption rate, distribution in the
315 body, and bioavailability of important divalent cations such as calcium (Ca),
316 magnesium (Mg), zinc, copper, and iron which are co-factors for essential enzymes
317 (Glauser et al., 1976; Thun et al., 1989; Pizent et al., 2001). Thus, the replacement of a
318 divalent cation, which occurs normally by Cd, might contribute to the less active or
319 inactive enzyme verified here in rat platelets since NTPDase and 5'-nucleotidase
320 optimal activities depend on Ca and Mg ions, respectively.

321 Several investigators have attempted to discern the molecular mechanism of Cd-
322 induced hypertension. To date, however, this mechanism has not been elucidated and
323 conflicting results have been reported in both animal models and population (Nakagawa
324 and Nishijo, 1996; Eum et al., 2008; Tellez-Plaza et al., 2008). The literature brings that
325 a primary mechanism for Cd toxicity is depletion of GSH and alteration of sulfhydryl
326 homeostasis, thus, indirectly increasing oxidative stress and lipid peroxidation (Valko et
327 al., 2005; Sompamit et al., 2010). Alternatively, hypertension may be caused by Cd-
328 induced impaired kidney function inducing the injury of renal proximal tubular, salt
329 retention, and volume overload (Perry Jr. and Erlanger, 1981; Satarug et al., 2005,
330 2006). Other potential mechanisms include partial agonism for calcium channels
331 (Varoni et al., 2003), increased activity of the renin-angiotensin system (Perry Jr. and
332 Erlanger, 1973), reduced acetylcholine-induced vascular relaxation, direct
333 vasoconstrictor action, activation of the sympathetic nervous system, inhibition of
334 vasodilator substances such as nitric oxide (Demontis et al., 1998; Bilgen et al., 2003;
335 Varoni et al., 2003; Mollaoglu et al., 2006; Yoopan et al., 2006, 2008) and inhibition of
336 monoamine oxidase and catechol-O-methyltranferase, two enzymes which inactivate
337 the neurotransmitters noradrenaline and adrenaline which regulate local arterial blood
338 pressure and have been associated with hypertension (Revis, 1977).

339 Taken together, these findings indicate the up- and down-regulation of ecto-
340 enzymes in cortex synaptosomes and platelets, respectively, implying that these
341 enzymes play an important role in the control of cellular responses induced by the Cd
342 intoxication. Another important factor to be addressed is that since the results obtained
343 with the platelet NTPDase and 5'-nucleotidase were not in line with the data obtained in
344 the CNS and given the differences between them, caution must be exerted when

extrapolating interpretations from peripheral models to central systems (Borges and Nogueira, 2008).

5. Conclusions

In conclusion, our findings reveal that adenine nucleotide hydrolysis in synaptosomes and platelets of rats were altered after Cd exposure leading to a compensatory response in CNS and acting as a modulator of the platelet activity. NAC was able to modulate the purinergic system which is interesting since the regulation of these enzymes could have potential therapeutic importance because ATP, ADP and adenosine have vital roles in brain and platelets in response to injury. Thus, our results reinforce the importance of the study of the ecto-nucleotidases pathway in poisoning conditions such as Cd intoxication and also highlight the possibility of using antioxidant drugs such as NAC as adjuvant against toxicological conditions.

Conflict of interest statement

No conflict of interest to be stated for any of the authors.

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372

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594 **Figures captions**

595

596 **Figure 1.** Effects of cadmium (Cd), N-acetylcysteine (NAC) or their combination on
597 NTPDase and 5'-nucleotidase activities in cerebral cortex synaptosomes of male rats
598 using ATP (A), ADP (B) and AMP (C) as substrate, respectively. Bars represent means ±
599 SEM, two-way ANOVA–Duncan's Test ($p<0.05$). Different lowercase letters indicate
600 significant difference among the treatments. (n=4-6 animals per group).

601

602 **Figure 2.** Effects of cadmium (Cd), N-acetylcysteine (NAC) or their combination on
603 NTPDase and 5'-nucleotidase activities in cerebral hippocampus synaptosomes of male
604 rats using ATP (A), ADP (B) and AMP (C) as substrate, respectively. Bars represent
605 means ± SEM, two-way ANOVA–Duncan's Test ($p<0.05$). Different lowercase letters
606 indicate significant difference among the treatments. (n=4-6 animals per group).

607

608 **Figure 3.** Effects of cadmium (Cd), N-acetylcysteine (NAC) or their combination on
609 NTPDase and 5'-nucleotidase activities in platelets of male rats using ATP (A), ADP (B)
610 and AMP (C) as substrate, respectively. Bars represent means ± SEM, two-way
611 ANOVA–Duncan's Test ($p<0.05$). Different lowercase letters indicate significant
612 difference among the treatments. (n=4-6 animals per group).

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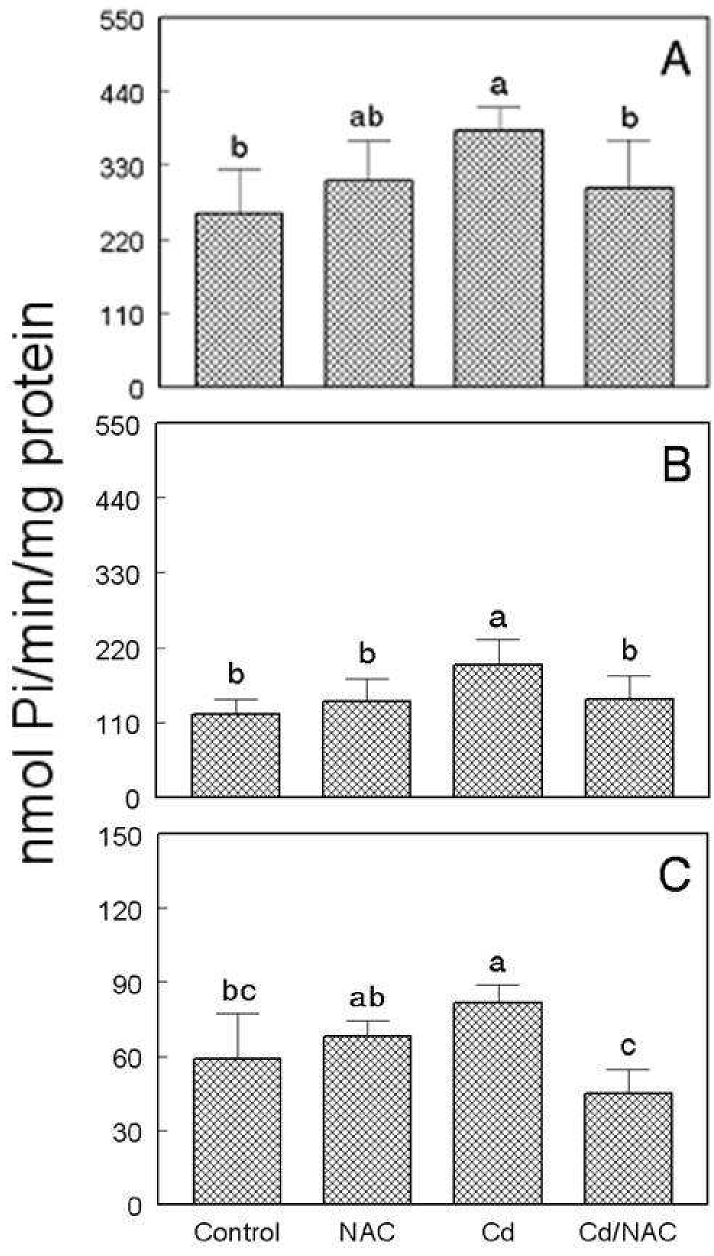

631 **Figure 1**

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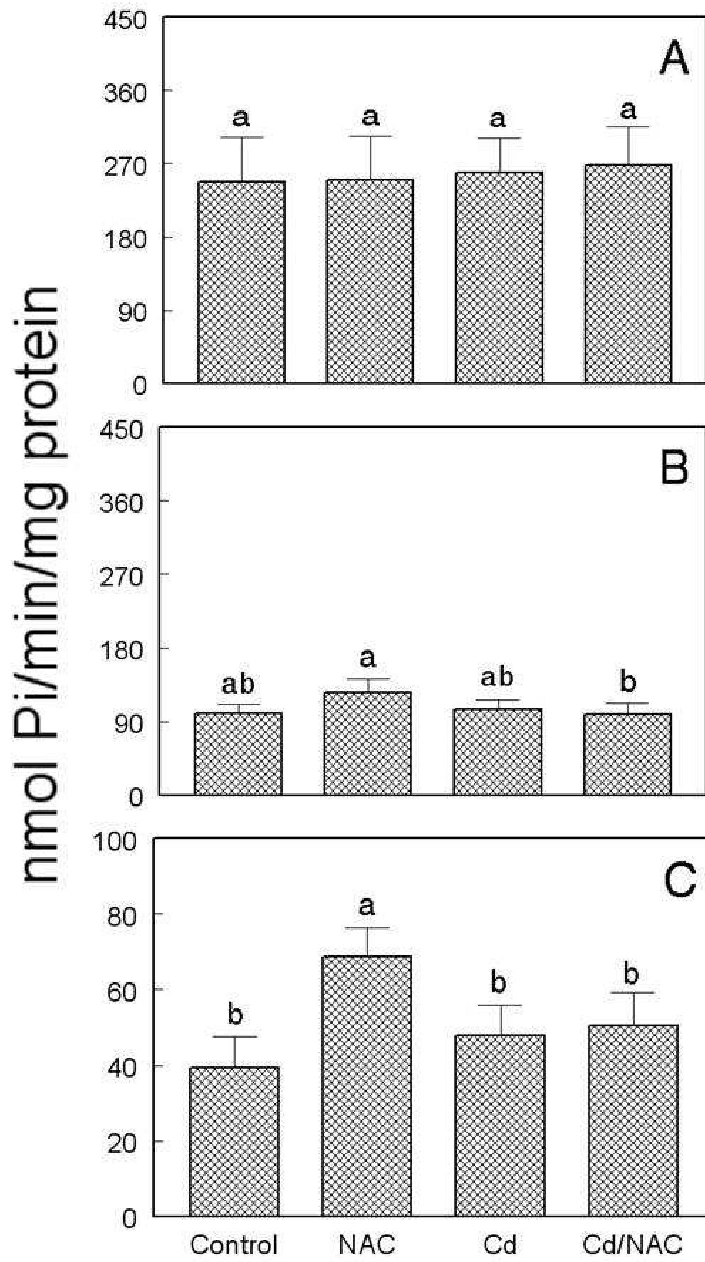

656 **Figure 2**

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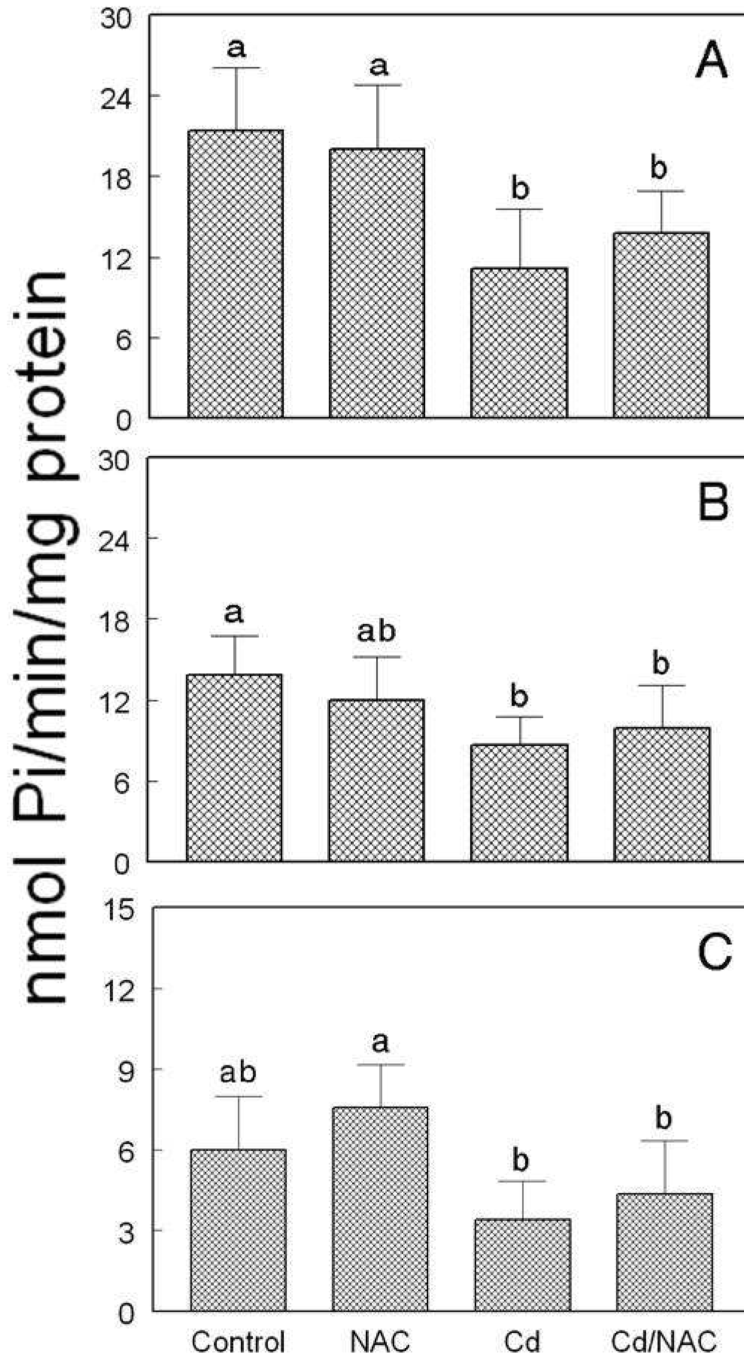

682 **Figure 3**

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6. CAPÍTULO 4

Manuscrito: Behavior and brain enzymatic changes after long-term intoxication with cadmium salt or contaminated potatoes – Publicado no Periódico *Food and Chemical Toxicology*

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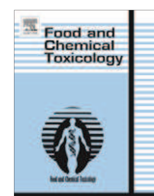

Behavior and brain enzymatic changes after long-term intoxication with cadmium salt or contaminated potatoes

Jamile F. Gonçalves [a,*], Fernando T. Nicoloso [b], Pauline da Costa [c], Júlia G. Farias [b], Fabiano B. Carvalho [c], Michelle M. da Rosa [c], Jessié M. Gutierres [c], Fátima H. Abdalla [c], Juliana S.F. Pereira [c], Glaecir R.M. Dias [c], Nilda B.V. Barbosa [c], Valderi L. Dressler [c], Maribel A. Rubin [c], Vera M. Morsch [c], Maria Rosa C. Schetinger [a,c,*]

[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcellos, 2600-Anexo, 90035-003 Porto Alegre, RS, Brazil
[b] Departamento de Biologia, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil
[c] Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil

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ABSTRACT

This study investigated the cadmium (Cd) intoxication on cognitive, motor and anxiety performance of rats subjected to long-term exposure to diet with Cd salt or with Cd from contaminated potato tubers. Potato plantlets were micropropagated in MS medium and transplanted to plastic trays containing sand. Tubers were collected, planted in sand boxes and cultivated with 0 or 10 μM Cd and, after were oven-dried, powder processed and used for diet. Rats were divided into six groups and fed different diets for 5 months: control, potato, potato + Cd, 1, 5 or 25 mg/kg $CdCl_2$. Cd exposure increased Cd concentration in brain regions. There was a significant decrease in the step-down latency in Cd-intoxicated rats and, elevated plus maze task revealed an anxiolytic effect in rats fed potato diet *per se*, and an anxiogenic effect in rats fed 25 mg/kg Cd. The brain structures of rats exposed to Cd salt or Cd from tubers showed an increased AChE activity, but Na^+,K^+-ATPase decreased in cortex, hypothalamus, and cerebellum. Therefore, we suggest an association between the long-term diet of potato tuber and a clear anxiolytic effect. Moreover, we observed an impaired cognition and enhanced anxiety-like behavior displayed by Cd-intoxicated rats coupled with a marked increase of brain Cd concentration, and increase and decrease of AChE and Na^+,K^+-ATPase activities, respectively.

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1. Introduction

Diet is the most important source of cadmium (Cd) exposure in the general population (non-occupational and nonsmoking) since Cd is not degraded in the environment and can also enter into the food chain (ATSDR, 2005). Both natural and anthropogenic sources of this heavy metal, including industrial emissions and the application of fertilizer and sewage sludge to farm land, may lead to the contamination of soils and to increased Cd uptake by crops and vegetables grown for human consumption (Järup and Akesson, 2009; Olsson et al., 2002). The uptake process of Cd from soil by plants is enhanced at low pH. Cd uptake by potatoes (*Solanum tuberosum*) and carrots (*Daucus carota*) is sensitive to changes in pH (Öborn et al., 1995). Thus, the decrease of soil pH due to environmental acidification may further increase the Cd content in human exposure pathway (Eriksson et al., 1996; Järup et al., 1998). Total human intake of Cd from food has been estimated as 2.8–4.2 μg/kg body weight/week, which equates to approximately 40–60% of the current provisional tolerable weekly intake of 7 μg/kg body weight/week (Järup et al., 1998).

Cd has a long biological half-life (15–30 years in humans) mainly due to its low rate of excretion from the body. Thus, prolonged exposure to it has been linked to toxic effects since it gets accumulated over time in a variety of structures including kidneys, liver and central nervous system (CNS) (Zadorozhnaja et al., 2000). Cd is more toxic to newborn and young rats than to adult rats probably due to differences in the blood–brain barrier (BBB) integrity (Antonio et al., 2003; Yargiçoglu et al., 1997). However, this metal can increase BBB permeability, thus penetrating and accumulating in the brain of developing and adult rats (Gonçalves et al., 2010; Méndez-Armenta and Ríos, 2007; Pari and Murugavel, 2007; Shukla et al., 1996; Takeda et al., 1999) leading to brain intracellular accumulation, cellular dysfunction, and cerebral edema. Also, it can affect the degree and balance of excitation–inhibition

* Corresponding authors. Tel./fax: +55 55 3220 9557 (J.F. Gonçalves).
E-mail addresses: jamilefabbrin@yahoo.com.br (J.F. Gonçalves), mariachitolina@gmail.com (Maria Rosa C. Schetinger).

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in synaptic neurotransmission as well as the antioxidant levels in animal brain (Méndez-Armenta and Ríos, 2007; Minami et al., 2001; Provias et al., 1994). Exposure to Cd can produce long-term impairment of neurobehavioral status such as alterations in attention and memory as well as in the psychomotor, and visuomotor functioning and speed in workers (Hart et al., 1989; Viaene et al., 2000). Moreover, in rat studies have observed increased aggressive and anxiety-like behaviours, impaired learning and memory processes, and changes in the development of the visual system (Desi et al., 1998; Gonçalves et al., 2010; Holloway and Thor, 1988; Terçariol et al., 2011; Yargiçoglu et al., 1997).

Some studies on Cd toxicity have found an association with behavioral disturbances and cholinergic neurotransmission since an increase or a decrease in the acetylcholinesterase (AChE, E.C. 3.1.1.7) activity was verified in both animal models and humans that showed behavioral impairments after exposure to Cd (Gonçalves et al., 2010; Pari and Murugavel, 2007). This enzyme hydrolyses the neurotransmitter acetylcholine (ACh) in the synaptic cleft of cholinergic synapses and neuromuscular junctions (Soreq and Seidman, 2001). Alterations in the AChE activity have been demonstrated by our research group in various diseases and poisonings suggesting that this enzyme could be an important physiological and pathological parameter (Gonçalves et al., 2010; Kaizer et al., 2005; Schmatz et al., 2009).

In addition, Na^+,K^+-ATPase (E.C. 3.6.1.3), or sodium pump, is among the enzymes particularly affected by Cd exposure (Antonio et al., 2002; Rajanna et al., 1983). This enzyme is one of the major membrane proteins responsible for generating the membrane potential through the active transport of Na^+ and K^+ ions in the CNS (Aperia, 2007; Kaplan, 2002), consuming about 40–60% of ATP produced in the brain (Erecinska et al., 2004). It is implicated in the metabolic energy production, in the uptake and release of serotonin and catecholamines as well as in the need to maintain cellular volume control and neuronal excitability (Bogdanski et al., 1968; Hernandez, 1987; Mata et al., 1980; Mobasheri et al., 2000). Thus, disturbances in its activity could have serious consequences for the CNS functioning and play a relevant role in the learning and memory mechanisms (Zhan et al., 2004).

In the present work we investigated the effect of Cd intoxication on cognitive, motor and anxiety performance of rats subjected to long-term exposure to diet with low levels of Cd salt or with Cd from contaminated potato tubers measured on inhibitory avoidance, open field and elevated plus maze tests. In addition, we also evaluated the influence of this metal on AChE and Na^+,K^+-ATPase activities in different brain structures.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide, cadmium chloride monohydrate, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), *tris*-(hydroxymethyl)-aminomethane GR, ouabain and Coomassie brilliant blue G were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Agar–agar and thiamine chloride hydrochloride were obtained from Merk KGaA (Rockland, Massachusetts, USA). Myo inositol was obtained from Vetec Chemical Co. (Duque de Caxias, Rio de Janeiro, Brazil). All other reagents used in the experiments were of analytical grade and of the highest purity.

2.2. Potato cultivation

Potato plantlets (*S. tuberosum* L.) for tissue culture were obtained from the Potato Breeding and Genetics Germplasm Program, Universidade Federal de Santa Maria, RS, Brazil. One potato genotype widely planted in Southern Brazil, Macaca, was used in this study. Nodal segments (1.0 cm long) without leaves were micropropagated in MS medium (Murashige and Skoog, 1962), supplemented with 30 g/L of sucrose, 0.1 g/L of myo-inositol and 6 g/L of agar in the absence growth regulators (Nicoloso et al., 2001). The plants were selected, comprising a homogeneous population, and used as donor explants. The apical and basal regions of shoots were not used.

After 21 days in MS medium, *in vitro*, plants were transplanted to plastic trays containing sand as substrate and cultivated for 27 days in a greenhouse with shading (50%). A second hydroponic growth period was performed in sand box with spacing of 10 cm, reaching a density of 100 per m^2 of surface seedlings. These plants received three daily irrigations (15 min each) with complete nutrient solution containing (mg/L): 155.90 N; 46.40 P; 5271.00 S; 123.00 Ca; 30.00 Mg; 253.60 K; 2622.00 B; 133.00 Na; 277.00 Mo; 2274.00 Zn; 636.00 Cu; 6501.00 Mn and 1.20 Fe.

The produced tubers were planted in sand box and cultivated during the spring growing season (September to November) with the same experimental system previously described. The Cd exposure was with the addition of the same nutrient solution in the form of cadmium chloride monohydrate ($CdCl_2{\cdot}H_2O$) at concentrations of 0 (control) or 10 μM. At all stages of the experiment the pH of the solutions used was adjusted daily to 5.5 ± 0.1 by titration with solutions of HCl or NaOH 0.1 M. The plants were cultivated for 91 days.

At the end of the cycle, the plants were collected and tubers were washed three times with distilled water and dried with tissue paper. The produced tubers were oven-dried at 65 °C to a constant mass for the determination of biomass as well as Cd accumulation. Dried plant tissues (0.01–0.1 g) were ground and digested with 4 mL of concentrated HNO_3. Sample decomposition was carried out using a heating block Velp Scientifica (Milano, Italy). Heating was set at 130 °C for 2 h. Plastic caps were fitted to the vessels to prevent losses by volatilization. The final Cd concentration in the produced tubers (powder) was 4.5 μg/g of dry weight.

2.3. Animals and dietary treatment

Three-week-old male Wistar rats weighing 85.5 ± 5.3 g from the Central Animal House of the Universidade Federal de Santa Maria (UFSM) was used in this experiment. At weaning, the pups were acclimated for 10 days before the beginning of the experiments and had free access to drinking water and food (Supra®, Brazil) during the acclimatation period or diet during the experimental period. Rats were maintained at a constant temperature (23 ± 1 °C) on a 12 h light/dark cycle. All animal procedures were approved by the Animal Ethics Committee from UFSM.

Animals (10 rats per treatment) were randomly divided into six experimental groups and fed different diets (Table 1) for 5 months. The diet was prepared in an industrial mixer to allow the uniformity of the mixture according to Barbosa et al. (2008). The choice of Cd concentrations was based on previous studies, which show that Cd diet intake causes overt signals of toxicity in rats (Callegaro et al., 2010a,b). After preparation, the diets were frozen (−20 °C) until use. The body weight of animals was obtained weekly until the end of the experiment period.

2.4. Behavior procedure

2.4.1. Inhibitory avoidance

One day after the end of the treatment, animals were subjected to training in a step-down inhibitory avoidance apparatus according to Guerra et al. (2006). Briefly, rats were subjected to a single training session in a step-down inhibitory avoidance apparatus, which consisted of a 25 × 25 × 35 cm box with a grid floor whose left portion was covered by a 7 × 25 cm platform, 2.5 cm height. 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 2-s 0.4-mA shock was applied to the grid. Memory retention was evaluated in a test session carried out 24 h after training, in which trained animals were placed on the training box platform, and the step-down latency was measured. A cut-off time of 300 s was imposed on step-down latency during testing session. The apparatus was cleaned with 30% ethanol before and after each rat occupied it.

2.4.2. Open field

Immediately after the inhibitory avoidance testing session, all animals were transferred to an open field in order to assess exploratory activity. Animals were placed on the center quadrant of a round open field (56 × 40 × 30 cm) with the floor divided into 12 squares measuring 12 × 12 cm each. An observer, who was not aware of the pharmacological treatments, manually recorded the number of crossing and rearing responses over 5 min. Crossing was defined as the total number of areas crossed with the four paws and rearing was defined as the total number of stand-up responses on two paws. This test was carried out to identify possible motor disabilities that might have influenced inhibitory avoidance performance at testing.

2.4.3. Foot shock sensitivity test

The reactivity to shock was evaluated in the same apparatus used for inhibitory avoidance, except that the platform was removed. The modified ‘‘up and down’’ method by Rubin et al. (2004) was used to determine the flinch, jump and vocalization thresholds in experimentally naive animals. Animals were placed on the grid and allowed a 3 min habituation period before the start of a series of shocks (1 s) delivered at 10 s intervals. Shock intensities ranged from 0.1 to 0.6 mA in 0.1 mA increments. Adjustments in the shock intensity were made in accordance with each animal response. The intensity was raised by 1 unit when no response occurred and lowered by 1 unit when there was a response. A flinch response was defined as the withdrawal of one paw from the grid floor, and a jump response was defined as the

Table 1
Experimental groups, diets and the concentration of Cd found in the diets.

Groups	Diets	Cd concentration (mg/kg diet)*
Control	Only diet	<0.03
Pot	10% of diet composed of tuber powder from potatoes grown without Cd	<0.03
Pot + Cd	10% of diet composed of tuber powder from potatoes grown with Cd	0.64 ± 0.04
1 mg/kg Cd	Diet mixed with 1 mg/kg of $CdCl_2$ salt dissolved in water	0.88 ± 0.02
5 mg/kg Cd	Diet mixed with 5 mg/kg of $CdCl_2$ salt dissolved in water	3.62 ± 0.23
25 mg/kg Cd	Diet mixed with 25 mg/kg of $CdCl_2$ salt dissolved in water	18.47 ± 0.41

Abbreviations: Pot (potato); Cd (cadmium).
*Values are expressed as means ± SEM ($n = 4$). Measured by ICP OES with detection limit of 0.03 mg/kg.

withdrawal of three or four paws. Two measurements of each threshold (flinch, jump and vocalization) were taken and the mean of each score was calculated for each animal.

2.4.4. Elevated plus maze task

The anxiolytic-like behavior was evaluated using the task of the elevated plus maze as previously described (Frussa-Filho et al., 1999; Rubin et al., 2000). The apparatus was made of wood and consisted of four arms, all with the same dimensions (50 × 10 cm) and elevated 50 cm above the floor. Two of these arms were enclosed by 40 cm high lateral walls with an open roof and located perpendicularly to the other two opposed open arms. The four arms delimited a central area of 100 cm^2. Rats were placed in the center of the maze facing an open arm and were allowed to explore the maze freely for 5 min. The time spent and the number of entries in open and closed-arms as well as the total number of arm entries were recorded. The apparatus was thoroughly cleaned with 30% ethanol between each session.

2.5. Brain tissue preparation

After behavioral tests, animals were anesthetized and submitted to euthanasia. The cranium was opened and the structures were gently removed and separated into cerebral cortex (CO), hippocampus (HC), hypothalamus (HT), cerebellum (CE) and striatum (ST). To verify the cadmium concentration in brain structures, three or four animals per group were randomly chosen. For the other animals, the brain structures were homogenized in a glass potter in a solution of 10 mM Tris–HCl, pH 7.4, on ice, at a proportion of 1:10 (w/v). The resulting homogenate was used to determine the Na^+,K^+-ATPase activity. A fraction of homogenate was centrifuged at 1800 rpm for 10 min and the resulting supernatant was used to determine the AChE activity.

2.6. Cadmium concentration in brain regions

Brain structures were weighted in glass vessels and 3–8 mL of HNO_3 was added for digestion. Digestion was performed using a block (Velp Scientifica, Milano, Italy) heated at 130 °C during 3 h. After this time, 2 mL of H_2O_2 was added and samples were heated again for more 1 h. Digested samples were then transferred to polypropylene flasks and the volume was completed with water to 30–50 mL. Cadmium determination was performed by graphite furnace atomic absorption spectrometry (GF AAS, contrAA 700, Analytik Jena) using a xenonium lamp set at 28.8018 nm. The heating program was performed using the temperatures of 120, 600, 1600 and 2300 °C for drying, pyrolysis, atomization and cleaning steps, respectively. In addition, Cd determination was also performed by inductively coupled plasma optical emission spectrometry (ICP OES) using a concentric nebulizer (Meinhard Associates, Golden, USA) and a cyclonic spray chamber (Glass Expansion, Inc., West Melbourne, Australia). However, in view of higher limit of detection obtained in comparison with the results obtained by GF AAS, Cd measurements were preferentially performed by GF AAS.

2.7. Cerebral AChE enzymatic assay

The AChE enzymatic assay was determined by a modification of the spectrophotometric method of Ellman et al. (1961) as previously described by Rocha et al. (1993). The reaction mixture (2 mL final volume) contained 100 mM K^+-phosphate buffer, pH 7.5 and 1 mM 5,5′-dithio-bis-nitrobenzoic acid (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 2-min incubation at 25 °C. The enzyme was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh). Protein was determined previously and adjusted for each structure: CO (0.6–0.8 mg/mL), HC (0.8 mg/mL), HT (0.6 mg/mL), CE (0.5–0.6 mg/mL), and ST (0.4 mg/mL). All samples were run in duplicate or triplicate and the enzyme activity was expressed in μmol AcSCh/h/mg of protein.

2.8. Na^+,K^+-ATPase enzymatic assay

The Na^+,K^+-ATPase activity was measured as previously described (Wyse et al., 2000) and modified by Carvalho et al. (2012). Briefly, the assay medium consisted of (in mM) 30 Tris–HCl buffer (pH 7.4), 0.1 EDTA, 50 NaCl, 5 KCl, 6 $MgCl_2$ and 50 μg of protein in the presence or absence of ouabain (1 mM), in a final volume of 350 μL. The reaction was started by the addition of adenosine triphosphate 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. Saturating substrate concentrations were used and the 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 and Subbarow, 1925), using NaH_2PO_4 as reference standard. Specific the 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.

2.9. Protein determination

The protein content was determined colorimetrically according to the Bradford method (1976) using bovine serum albumin (1 mg/mL) as standard solution.

2.10. Statistical analysis

Statistical analysis of the training and test step-down latencies was carried out by Kruskal–Wallis followed by post hoc analyses (nonparametric Dunn's test). All other parameters evaluated were analyzed by one-way ANOVA, followed by Student–Newman–Keuls (SNK) or Duncan's multiple range tests, where $p < 0.05$ was considered to represent a significant difference in all experiments.

3. Results

3.1. Cd or potato intake did not affect body weight measurements

Table 2 shows the body weight of the animals at the onset and at the end of the experiment as well as their body weight gain. As can be observed, there were no statistical differences in the body weight measurements between the groups.

3.2. Cd exposure caused memory deficits

Fig. 1 shows the effect of Cd and potato diet intake on step-down latencies. The statistical analysis of testing showed that long-term exposure to Cd salt (1, 5 and 25 mg/kg) as well as Cd accumulated in potato tubers caused memory impairment. Statistical analysis of training showed no significant differences between the groups.

Because the motivational disparities in the training session may account for differences in inhibitory avoidance at testing, experiments were performed to assess whether Cd or potato intake affected the shock threshold or the locomotor ability of the animals. Statistical analysis of open-field data revealed that neither Cd nor potato intake caused gross motor disabilities at testing as demonstrated by the similar number of crossing or rearing responses exhibited by the animals. Moreover, Cd or potato intake did not alter foot shock sensitivity as demonstrated by the similar flinch, jump and vocalization thresholds exhibited by the rats (Table 3).

Table 2
Effects of cadmium (Cd) and potato (pot) diet intake in the body weight gain (g) of experimental groups.

Groups	Initial body weight (g)	Final body weight (g)	Body weight gain (g)
Control	81.5 ± 4.3	412.6 ± 3.1	+331.1
Pot	87.8 ± 6.3	383.1 ± 0.4	+295.3
Pot + Cd	83.3 ± 6.4	438.7 ± 1.1	+355.4
1 mg/kg Cd	85.0 ± 3.9	388.3 ± 1.6	+303.3
5 mg/kg Cd	87.9 ± 2.4	424.5 ± 9.1	+336.6
25 mg/kg Cd	87.3 ± 4.3	435.7 ± 2.8	+348.4

Values are expressed as means ± SEM. n = 6–10 rats per group ($p < 0.05$).

3.3. Cd exposure or potato intake was anxiogenic and anxiolitic, respectively

Anxiety studies using an elevated plus maze showed a lower anxiety in rats fed potato diet since these rats spent more time in the open arms, spent less time in the closed arms, and entered the open arms more times compared with those of the other groups (Fig. 2A, B and, C, respectively). On the other hand, rats fed 25 mg/kg Cd diet showed a higher anxiety since these rats spent less time in the open arms, spent more time in the closed arms, and entered the open arms less times compared with those of the other groups. There were no significant alterations in the number of entries in the closed arms or in the total number of entries in arms between the experimental groups (Fig. 2D and E).

3.4. Cd exposure increased Cd concentration in brain regions

Cd exposure increased Cd concentration in brain regions of rats fed potato grown with Cd, 1, 5 and 25 mg/kg Cd diet in relation to the control (Table 4). Control rats and rats fed potato diet *per se* showed Cd concentrations lower than the detection limit (0.009 μg/g) in different brain structures. Rats fed potato grown with Cd, 1 and 5 mg/kg Cd diet showed small and similar Cd concentrations of around 0.011 μg/g in different brain structures. Rats fed 25 mg/kg Cd showed highest Cd concentrations in all brain regions when compared with the other groups.

3.5. Cd exposure increased AChE activity

The AChE activity in different brain structures is given in Fig. 3. There were no significant alterations in the AChE activity in cerebral cortex (Fig. 3A). Hippocampus AChE activity increased 25% in rats fed 25 mg/kg Cd diet in relation to the control (Fig. 3B). Hipothalamus AChE activity increased 63% and 52% in rats fed 5 and 25 mg/kg Cd diet in relation to the control, respectively (Fig. 3C). Cerebellum AChE activity increased 45%, 32% and 39% in rats fed 1, 5 and 25 mg/kg Cd diet in relation to the control, respectively (Fig. 3D). Striatum AChE activity increased 44%, 44%, 57% and 66% in rats fed potato grown with Cd, 1, 5 and 25 mg/kg Cd diet when compared with the control, respectively (Fig. 3E).

3.6. Cd exposure decreased Na^+,K^+-ATPase activity

Results obtained for Na^+,K^+-ATPase activity in different brain structures are presented in Fig. 4. There was a decrease of 32%, 25% and 39% in Na^+,K^+-ATPase activity in cerebral cortex of rats fed potato grown with Cd, 5 and 25 mg/kg Cd diet in relation to the control, respectively (Fig. 4A). There were no significant alterations in the Na^+,K^+-ATPase activity in hippocampus (Fig. 4B). A decrease of 28% in Na^+,K^+-ATPase activity in hipothalamus of rats fed 25 mg/kg Cd diet was observed in relation to the control (Fig. 4C). "There were no significant alterations in the Na^+,K^+-ATP-

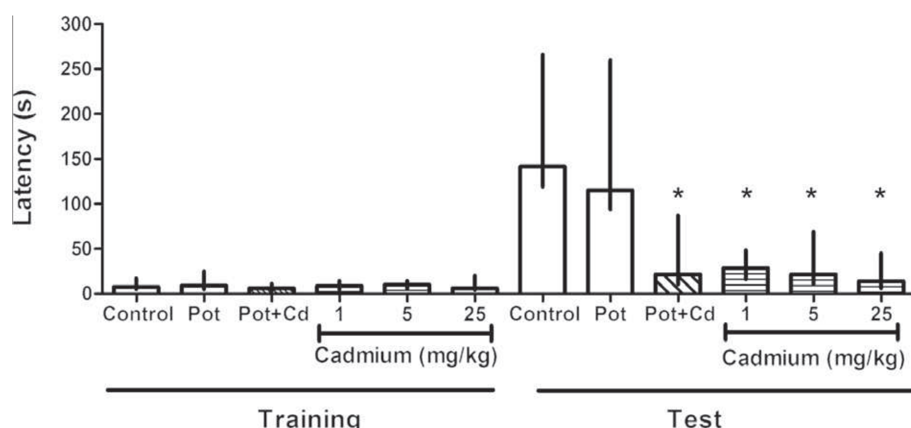

Fig. 1. Effect of cadmium (Cd) and potato (Pot) diet intake on step-down latencies. After one treatment-free day, animals were tested in a step-down latency test. Data are median ± interquartile range of training and test. $^*p < 0.05$ compared with the other groups at testing by the Dunn's nonparametric multiple comparison task (n = 8–10).

Table 3
Effects of cadmium (Cd) and potato (pot) diet intake on the behavior of rats in the open-field (number of crossing and rearing responses) immediately after the inhibitory avoidance testing session and on foot shock sensitivity (flinch, jump and vocalization).

Group	Crossing	Rearing	Flinch (mA)	Jump (mA)	Vocalization (mA)
Control	31.50 ± 4.76	25.75 ± 3.85	0.32 ± 0.03	0.36 ± 0.03	0.45 ± 0.01
Pot	33.75 ± 4.85	25.25 ± 2.84	0.31 ± 0.02	0.39 ± 0.01	0.47 ± 0.02
Pot + Cd	34.50 ± 3.55	26.40 ± 3.12	0.33 ± 0.02	0.36 ± 0.02	0.43 ± 0.01
1 mg/kg Cd	23.20 ± 3.90	18.50 ± 3.04	0.33 ± 0.02	0.33 ± 0.01	0.45 ± 0.01
5 mg/kg Cd	25.40 ± 3.46	20.80 ± 2.80	0.33 ± 0.02	0.37 ± 0.03	0.49 ± 0.01
25 mg/kg Cd	32.55 ± 2.31	23.70 ± 1.74	0.34 ± 0.02	0.37 ± 0.02	0.46 ± 0.01
Statistical analysis	$F_{(5,51)} = 0.13$ $p > 0.05$	$F_{(5,51)} = 0.33$ $p > 0.05$	$F_{(5,51)} = 0.88$ $p > 0.05$	$F_{(5,51)} = 0.61$ $p > 0.05$	$F_{(5,51)} = 0.15$ $p > 0.05$

Data are means ± SEM for 8–10 animals per group.

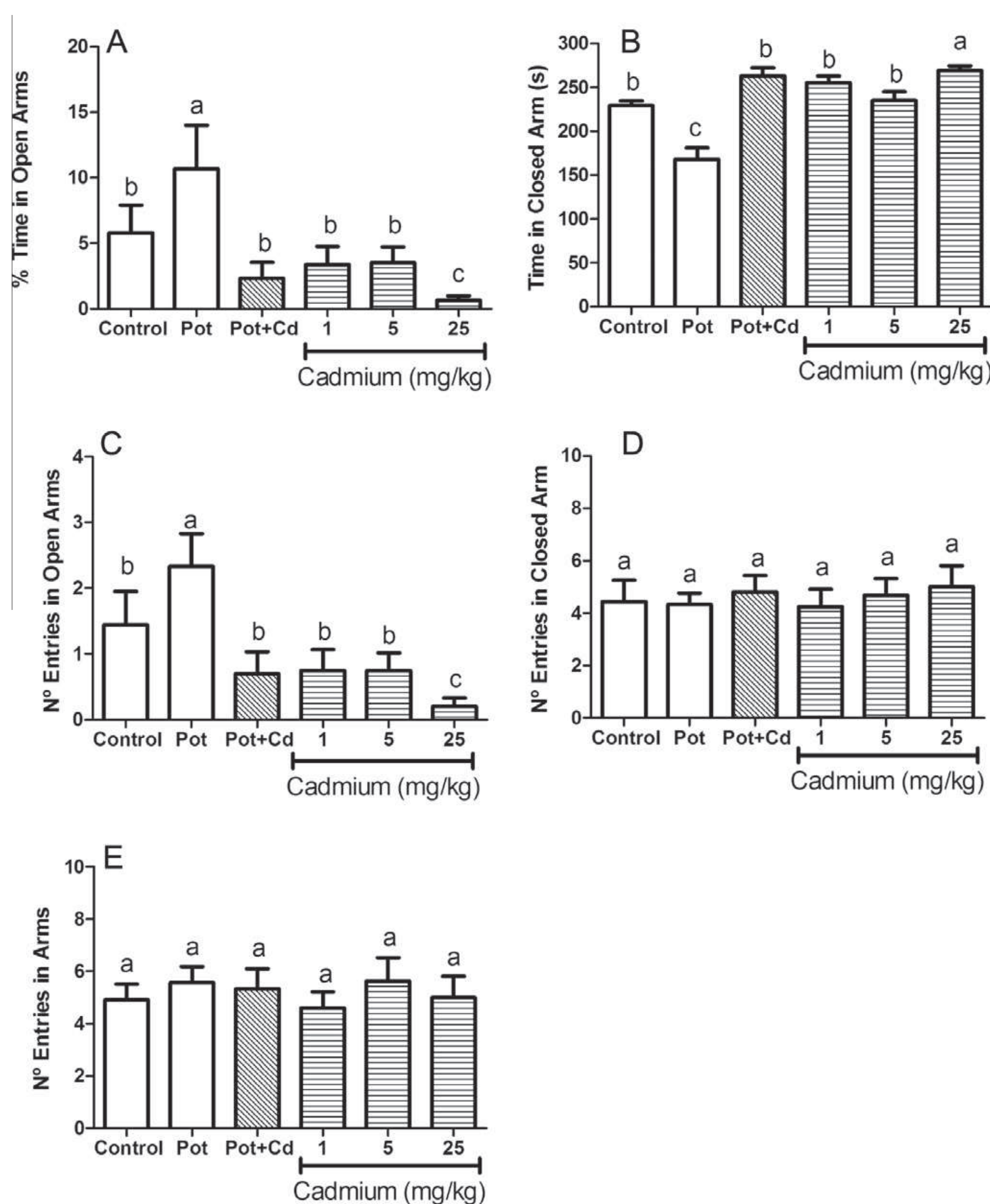

Fig. 2. Time spent in open (A) and closed (B) arms, number of entries in open (C) and closed (D) arms and number of total entries in arms (E) measured in the elevated plus maze over the 5 min test in rats fed potato (Pot) grown with and without cadmium (Cd), 1, 5 and 25 mg/kg Cd diet. Different lowercase letters indicate significant difference between the groups. Bars represent means ± SEM. One-way ANOVA–Student–Newman–Keuls (SNK) ($p < 0.05$) ($n = 6–10$).

Table 4
Effects of cadmium (Cd) and potato (pot) diet intake on Cd concentration (μg/g) in different brain structures (cortex, hippocampus, hypothalamus, cerebellum and striatum) of male rats.

Groups	Cortex	Hippocampus	Hypothalamus	Cerebellum	Striatum
Control	$<0.009^b$	$<0.009^b$	$<0.009^b$	$<0.009^b$	$<0.009^b$
Pot	$<0.009^b$	$<0.009^b$	$<0.009^b$	$<0.009^b$	$<0.009^b$
Pot + Cd	0.011 ± 0.0005^a	0.011 ± 0.0005^a	0.010 ± 0.0005^a	0.010 ± 0.0005^a	0.014 ± 0.0029^a
1 mg/kg Cd	0.011 ± 0.0005^a	0.011 ± 0.0005^a	0.010 ± 0.0005^a	0.011 ± 0.0005^a	0.016 ± 0.0035^a
5 mg/kg Cd	0.011 ± 0.0005^a	0.011 ± 0.0005^a	0.011 ± 0.0005^a	0.011 ± 0.0005^a	0.022 ± 0.0005^a
25 mg/kg Cd	0.040 ± 0.0058^a	0.065 ± 0.0014^a	0.079 ± 0.0075^a	0.042 ± 0.0017^a	0.060 ± 0.0092^a

Data are means ± SEM. One-way ANOVA–Duncan's test ($p < 0.05$). Different letters in the same column indicate differences among the treatments ($n = 3–4$ animals per group). Measured by GF AAS with detection limit of 0.009 mg/kg.

ase activity in striatum (Fig. 4D). Cerebellum Na^+,K^+-ATPase activity decreased 33% and 27% in rats fed potato grown with Cd, and 25 mg/kg Cd diet in relation to the control, respectively (Fig. 4D). There were no significant alterations in the Na^+,K^+-ATPase activity in striatum (Fig. 4E)".

4. Discussion

Since Cd exposure to general population primarily occurs through the ingestion of contaminated food, this heavy metal does not present known beneficial biological function, and its half-life in

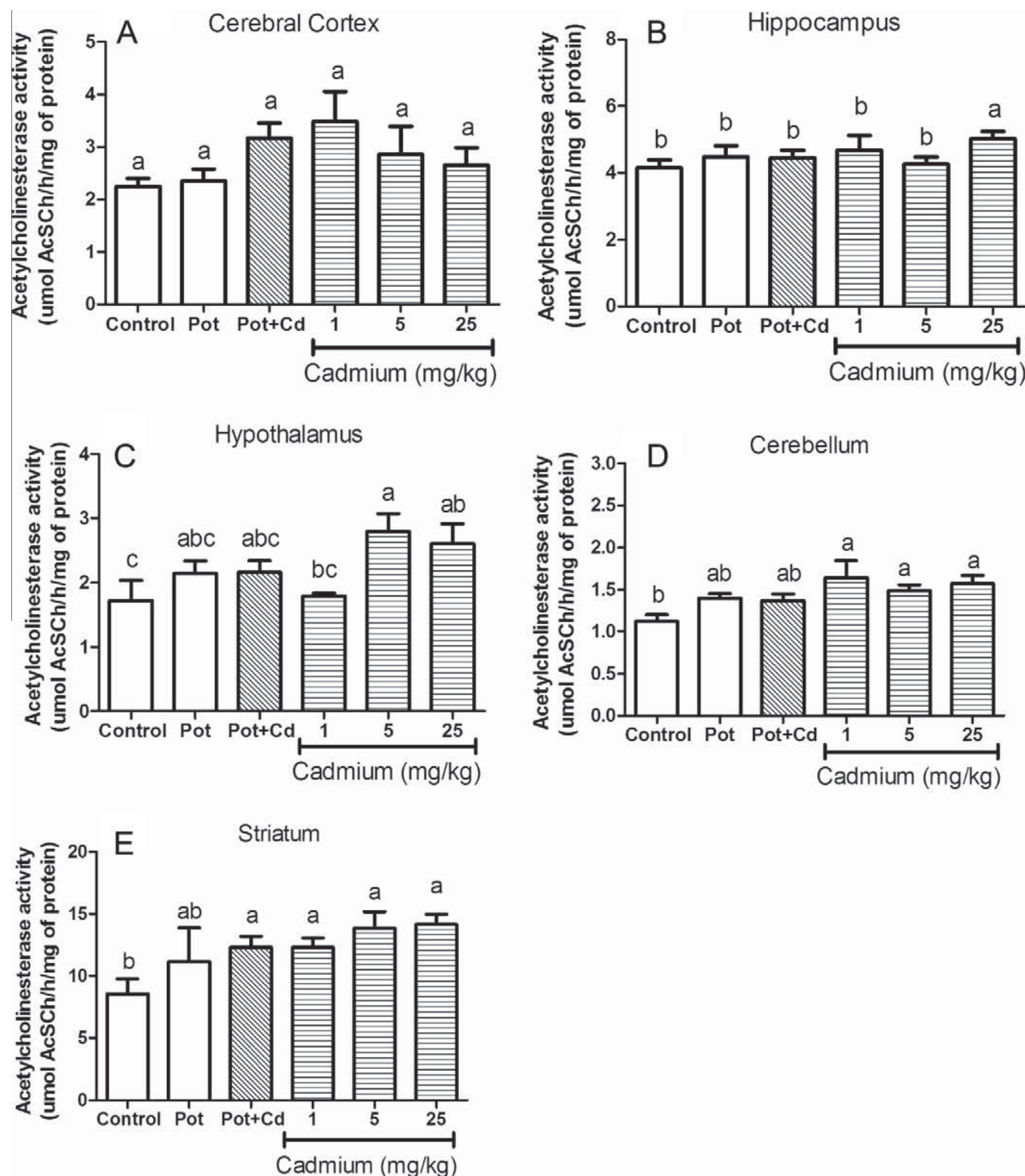

Fig. 3. Acetylcholinesterase (AChE) activity in supernatant of cerebral cortex (A), hippocampus (B), hypothalamus (C), cerebellum (D) and striatum (E) of rats fed potato grown with and without Cd, 1, 5 and 25 mg/kg Cd diet. Different lowercase letters indicate significant difference between the groups. Bars represent means ± SEM. One-way ANOVA–Duncan's multiple range test ($p < 0.05$) ($n = 4–6$).

the organisms is elevated, a long period of exposure to Cd levels via diet may represent a great risk to human health. Thus, the present investigation on the long-term exposure to diet with low levels of Cd salt or with Cd from contaminated potato tubers represents a significant contribution to better understand the Cd toxicology. In this study Cd-exposed adult rats showed small amount of Cd in brain structures but significantly higher than control values and enough to cause brain injury, corroborating with the literature data (Gonçalves et al., 2010; Takeda et al., 1999; Wong and Klaassen, 1982). Moreover, although Cd insult did not alter the body weight measurements of the rats, it was harmful to the brain functions and consequently to animal behavior.

The inhibitory avoidance test is a classic model behavioral test with a strong aversive component used to evaluate learning and memory in rats and mice (Cahill et al., 1986). In the present study, we observed a significant decrease in the step-down latency in Cd-intoxicated rats by the inhibitory avoidance test, suggesting learning and memory impairment in these animals. This result corroborates our recently published data where rats intoxicated orally with Cd showed impaired cognition (Gonçalves et al., 2010). In fact, some studies have demonstrated that Cd induces neurotoxicity in animals with a wide spectrum of clinical entities such as changes in the normal brain neurochemistry as well as neurological and behavioral disturbances (Gonçalves et al., 2010; Minami et al., 2001).

As reported by Schmatz et al. (2009), a major concern in shock-motivated learning tests, particularly in those investigating the effect of drugs given before the acquisition of a given test, is whether pharmacological treatments affect locomotor activities or motivational aspects of learning, such as shock sensitivity. To rule out this possibility, immediately after inhibitory avoidance testing sessions, the animals were subjected to an open-field test which is widely used to evaluate motor disorders (Belzung and Griebel, 2001). The open field session revealed that the pharmacological

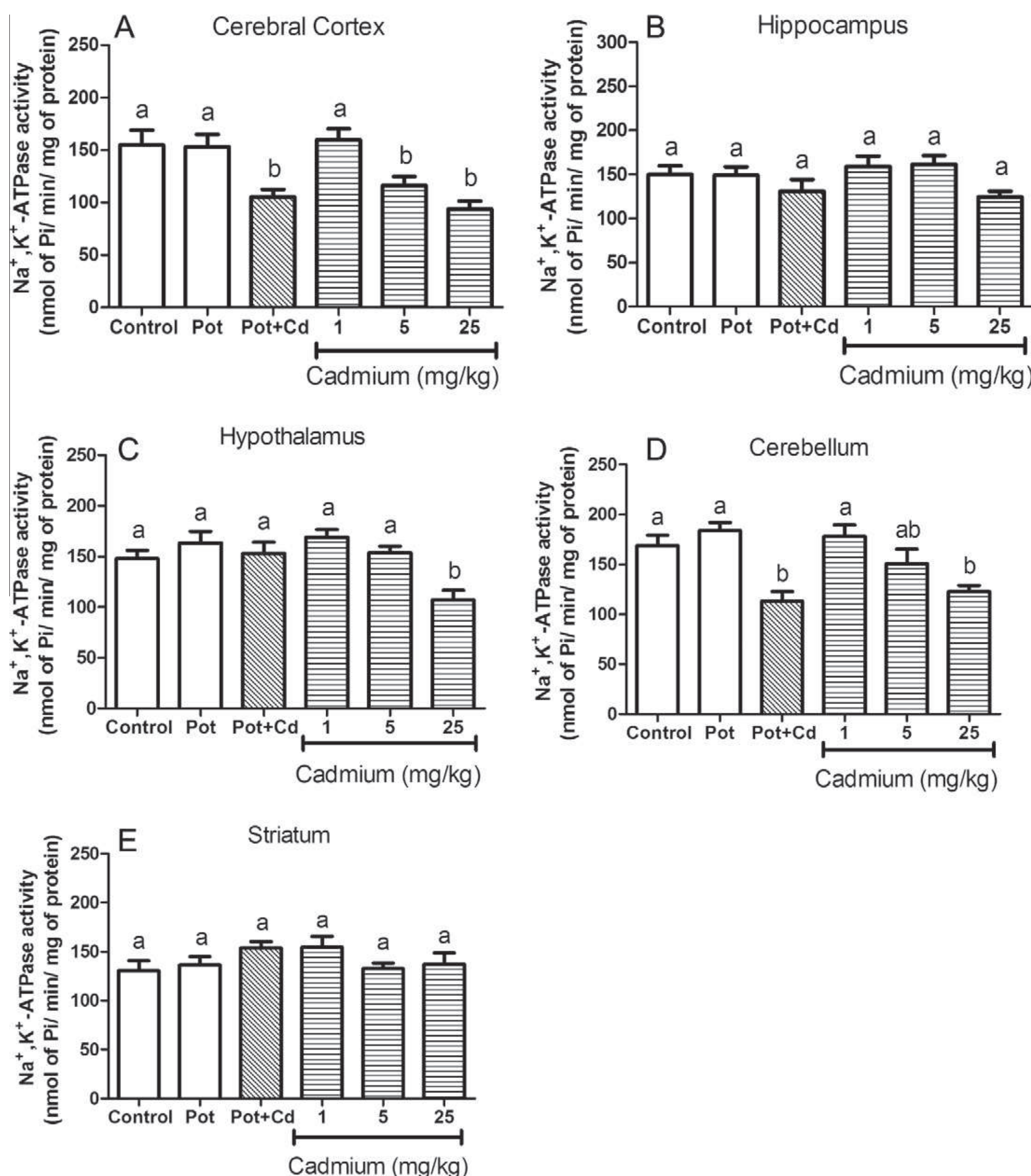

Fig. 4. Activity of Na+K+-ATPase in homogenate of cerebral cortex (A), hippocampus (B), hypothalamus (C), cerebellum (D) and striatum (E) of rats fed potato (Pot) grown with and without cadmium (Cd), 1, 5 and 25 mg/kg Cd diet. Different lowercase letters indicate significant difference between the groups. Bars represent means ± SEM. One-way ANOVA–Student–Newman–Keuls (SNK) ($p < 0.05$) ($n = 4–6$).

treatment did not alter spontaneous locomotor activity since rats fed the different diets showed a similar number of crossing or rearing responses. Moreover, in our study we demonstrated that rats of different treatments did not show altered shock sensitivity as verified by their similar flinch, jump and vocalization thresholds. Thus, these data suggest that neither Cd nor potato intake before training of inhibitory avoidance caused motor disabilities nor altered foot shock sensitivity. This way, we can exclude the possibility that these parameters may have contributed to the alteration in step-down latencies.

Besides learning and memory evaluation, we also measured the anxiolytic performance of the rats by the elevated plus maze, commonly used to study anxiety-related behavior in rodents (Belzung and Griebel, 2001). Our results showed a lower anxiety in rats fed potato diet *per se*. According to Allemão (1976), the word *Solanum* comes from the Latin word *Solari* which means “to relieve”, and the vegetable which receives this denomination has been employed for its tranquilizer property. It is well known that benzodiazepines are among the most frequently prescribed drugs which present tranquilizing, sedative-hypnotic, anxiolytic, and anticonvulsant effects mediated by binding to a specific subtype of the GABA(A) receptor, i.e., the a1-type GABA(A) receptor, which is mainly expressed in cortical areas and in the thalamus of all vertebrates (Rudolph et al., 1999). Interestingly, Kavvadias et al. (2000) reported the endogenous formation of benzodiazepines such as *N*-desmethyldiazepam (temazepam) and diazepam in amounts of about 70–450 ng/g cell tissue of *S. tuberosum*. These contents are approximately 10- to 100-fold higher than those previously found in potato tuber (2–4 ng diazepam equivalents/g) (Wildmann, 1988). Moreover, Muceniece et al. (2008) postulated that potato juice extracted from tuber marrow might contain GABA(A) receptor GABA-site active compounds since its low content of diazepam could not sustain the anticonvulsant activity of potato juice *in vivo*, suggesting that juice as well as potato taken as food may have the capacity of influencing brain GABA-ergic activity. Besides benzodiazepine derivatives, other compounds that exhibit anxiolytic effects were

identified in potato such as a variety of flavonoids that could be a subtype-selective partial agonist of GABA(A) receptors (Giorgetti and Negri, 2011). Thus, our results reinforce the anxiolytic properties of the Solanaceae family and to the best of our knowledge this is the first approach associating the long-term diet of potato tuber to a clear anxiolytic effect in rodents. With regard to Cd-based diet, the present investigation showed that the highest Cd concentration applied to the diet (25 mg/kg) caused higher anxiety in rats. Leret et al. (2003) showed that the rats co-exposed to Cd (10 mg/L) and Pb (300 mg/L) in drinking water from the beginning of pregnancy until weaning showed an increased anxiety-like behaviour and although the mechanism by which these metals are able to alter behaviour in the elevated plus-maze is yet to be established, it appears that there is a link with hippocampal serotoninergic and dopaminergic neurons. On the other hand, Minetti and Reale (2006) showed a lower anxiety in all the offspring prenatally exposed to 0.6 mg/kg Cd subcutaneously. It is important to point out that their dose of Cd is about five times less than that applied by us here and also the male offspring in all the groups studied by Minetti and Reale (2006) evidenced a clear anxious behavior with respect to the female rats. Moreover, when the total number of arm entries was evaluated, the absence of changes in this measure indicated that all groups have similar locomotor activity corroborating our findings in the open field test. This indicates that the changes observed in the plus maze are consistent with a decreased and an increased anxiety behavior produced by potato and Cd intake, respectively.

In the present study, all Cd diets increased the AChE activity in the striatum. However, the activation of AChE in the hippocampus, hypothalamus and cerebellum was observed in a dependence of the Cd concentration administered and no effect in response to Cd was detected in the cerebral cortex between the groups. Both *in vitro* and *in vivo* effects of Cd on the AChE activity have been described in the literature for different animal models (Carageorgiou et al., 2004, 2005; Luchese et al., 2007; Pari and Murugavel, 2007). The mechanism of action of Cd on AChE enzyme has been hypothesized to be either the displacement of metal cofactors from the active site or the direct deactivation of the enzyme site (Casalino et al., 1997). Moreover, Cd can leads to alteration of the structural integrity of lipids and secondarily affects membrane-bound enzymes such as AChE and Na^+,K^+-ATPase. Thus, alterations in the lipid membrane by oxidative stress could be a decisive factor in the modification of the conformational state of the AChE and Na^+,K^+-ATPase molecules, which would explain changes in their activities (Gonçalves et al., 2010; Pal et al., 1993; Schmatz et al., 2009; Srinivasan and Ramprasath, 2011). However, results are controversial and the activation as well as the inhibition of the AChE activity has been reported. The activity of AChE in rat brain synaptosomal plasma membranes showed a considerable decrease after 6 h of Cd exposure, followed by a progressive increase up to 24 h (Fasitsas et al., 1991). Brain AChE activity was decreased in rats exposed to 1, 2 or 5 mg/kg Cd for 8 h (Carageorgiou et al., 2004) as well as in mice exposed subcutaneously to 10 mmol/kg, five times/week for 4 weeks (Luchese et al., 2007). Decreased AChE activity was found in hippocampus, cerebellum and hypothalamus of rats intoxicated with 2 mg/kg Cd by gavage every other day for 30 days (Gonçalves et al., 2010). However, an increase in the AChE activity was found in brain of rats exposed to 1 mg/kg Cd intraperitoneally for 14 days or intramuscularly for 4 months (Carageorgiou et al., 2004, 2005). Interestingly, AChE activation leads to a fast ACh degradation and a subsequent down stimulation of ACh receptors causing undesirable effects on cognitive functions (Soreq and Seidman, 2001). Based on our results we can suggest that the increase in the AChE activity caused by Cd leads to a reduction of cholinergic neurotransmission efficiency due to a decrease in ACh levels in the synaptic cleft thus contributing to progressive cognitive impairment. Furthermore, we may infer that the activator effect elicited by Cd diet on the AChE activity could be one of the mechanisms involved in the memory impairment observed in the inhibitory avoidance test in this study (Schmatz et al., 2009). In fact, there is a wealth of evidence that brain cholinergic systems participate in memory processes. Our research team has already reported that cerebral AChE activity was restored by the antioxidant *N*-acetylcysteine which has beneficial actions modulating cholinergic neurotransmission and improving cognition (Gonçalves et al., 2010).

Cholinergic pathways, which fulfil a pivotal role in the control of cognitive-attentional, arousal and motivation function, ramify extensively in the hippocampus, amygdala, septum, frontal cortex and several other limbic regions. They reciprocally interact with GABAergic, monoaminergic, and glutamatergic pathways playing a role in the control of anxious states (Milan, 2003). There is emerging evidence that increased ACh levels in brain reduce anxiety. More specifically there is evidence that some of these anxiolytic effects of ACh are modulated by the hippocampus since an overall enhancement of cholinergic transmission in the hippocampus was accompanied by anxiolytic properties (Degroot et al., 2001; Degroot and Treit, 2002). Degroot et al. (2001) found that infusions of the AChE inhibitor physostigmine (10 mg/mL) into the dorsal hippocampus increased open-arm exploration in the plus-maze test and decreased burying behavior in the shock-probe test, demonstrating that general up-regulation of hippocampal cholinergic activity (increased ACh levels) resulted in a clear anxiolytic effect in two different tests of anxiety. The anxiolytic effects induced by stimulating the hippocampal cholinergic system are consistent with previous data indicating that intrahippocampal infusions of both muscarinic and nicotinic antagonists increase anxiety (File et al., 1998; Hess and Blozovski, 1987; Smythe et al., 1998). Our study produced consistent results that the cholinergic system may be involved in anxiety-like behaviour as well as memory impairment observed in rats fed with the highest Cd diet (25 mg/kg) since we found that the enzyme AChE was activated, which probably decreases ACh levels in four cerebral structures including the hippocampus, a region particularly important in anxiety and memory processes.

The activity of Na^+,K^+-ATPase was decreased in cerebral cortex, hypothalamus, and cerebellum of rats exposed to Cd salt or Cd from tubers. In hippocampus and striatum there was no alteration in the Na^+,K^+-ATPase activity between the groups. Several studies have demonstrated that certain divalent metal ions are potent inhibitors of brain Na^+,K^+-ATPase (Antonio et al., 2002; Chetty et al., 1992; Hexum, 1974; Prakash et al., 1973; Rajanna et al., 1983). In this sense, our results confirm that Cd may exert an inhibitory effect on the Na^+,K^+-ATPase activity. According to Antonio et al. (2002), this enzyme plays a vital role in linking the extracellular signals to the intracellular medium in neural tissues. Thus the inhibition of this enzyme by Cd could be damaging to the brain cells leading to an earlier stage of edema, followed by a later stage of degeneration and necrosis. Also, Na^+,K^+-ATPase inhibition by ouabain impairs learning and memory in Morris water maze and step-through passive avoidance tasks (Sato et al., 2004; Zhan et al., 2004), showing the main role of this enzyme on learning and memory processes which could be associated with Cd-induced impaired cognition observed in the present investigation. Rajanna et al. (1983) proposed that Cd may compete with ATP and Na^+ sites on Na^+,K^+-ATPase inhibiting its activity in rat brain synaptosomes. Chetty et al. (1992) have reported that Cd interferes with phosphorylation state of the enzyme which results in its inhibition. Borges et al. (2005) postulated that the inhibition of cerebral Na^+,K^+-ATPase by organochalcogens may occur through a change in the crucial thiol groups of this enzyme. In fact, Cd has high affinity for the sulphydril groups in enzymes and proteins and its binding

can alter their correct function in numerous and not related processes (Antonio et al., 2002). Moreover, the binding of Cd to the -SH groups of glutathione could implicate in the induction of oxidative stress associated with Cd poisoning (Antonio et al., 2002). In relation to this, Stefanello et al. (2011) showed that the administration of antioxidants (vitamins E plus C) partially prevented the inhibition of the Na^+,K^+-ATPase activity caused by acute and chronic hypermethioninemia which strongly suggests the oxidative damage as a possible mechanism involved in the reduction of this enzyme activity. On the other hand, Carageorgiou et al. (2004) have found a whole brain Na^+,K^+-ATPase stimulation as a dose-dependent effect of acute Cd administration in rats possibly due to Cd-induced acute oxidative stress. In fact, these controversies in AChE and Na^+,K^+-ATPase activities could be explained by the different routes and doses of Cd administration, differences in the biological samples assayed, and differing periods of exposure (short-term and long-term) (Carageorgiou et al., 2004).

5. Conclusions

Although all the proposed mechanisms by which Cd especially affects behavior continue to engender controversy, from the present study it is clear that long-term exposure to diet with low doses of Cd salt or with Cd from contaminated food increased Cd concentration in brain regions of rats, was harmful to the brain functions and consequently to animal behavior. Our data suggest that impaired cognition and enhanced anxiety-like behavior displayed by Cd-intoxicated rats is coupled with a marked increase in the AChE activity and a decrease in the Na^+,K^+-ATPase activity in the brain structures, two important enzymes that can indicate marked alterations in the synaptic transmission. Moreover, additional studies about potato tubers and Cd toxicity would be desirable to determine their influence on anxiety processes since there are scarce reports on the anxiolitic and anxiogenic properties exhibited by rats treated with potato tubers and Cd, respectively.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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7. DISCUSSÃO GERAL

A toxicologia do Cd é extremamente complexa e tem sido amplamente estudada, mas ainda não está completamente esclarecida. Estudos sobre os efeitos do $CdCl_2$ ou do Cd proveniente de batatas contaminadas relacionando a atividade das enzimas colinesterases, ectonucleotidases e Na^+,K^+-ATPase com a bioacumulação de Cd em diferentes tipos celulares bem como com parâmetros hematológicos, comportamentais e de memória ainda não foram registrados na literatura. Além disso, não é reportado o uso do antioxidante NAC no tratamento deste tipo de intoxicação interligando os parâmetros acima citados.

Claramente, uma melhor compreensão dos mecanismos pelos quais o Cd pode causar neurotoxicidade em animais experimentais fornecerá importantes achados para relacionarmos a algumas respostas similares que são encontradas em seres humanos. Sendo assim, identificar os fatores ambientais que possam contribuir para o aumento de distúrbios neurocomportamentais em humanos, tal como o Cd, é uma importante realização e um primeiro passo para a prevenção dessas complicações. Além disso, sabe-se que a utilização de fármacos antioxidantes tem tornando-se cada vez mais popular no tratamento de transtornos relacionados ao dano oxidativo, sendo assim, considerados agentes terapêuticos promissores. Em vista disso, o **Capítulo 1** dessa tese teve por objetivo investigar os efeitos do antioxidante NAC sobre parâmetros comportamentais e memória, atividade da enzima AChE, níveis de peroxidação lipídica e determinação de Cd em diferentes estruturas cerebrais de ratos expostos ao $CdCl_2$ e tratados com NAC. Para tal, os animais foram expostos oralmente a 2 mg/kg de Cd e/ou 150 mg/kg de NAC, um dia sim e outro não, durante um período experimental de 30 dias. Uma vez que, a dieta é a principal fonte de exposição ao Cd ambiental em pessoas não fumantes, todos os trabalhos realizados nessa tese tiveram a ingestão oral como a principal fonte de exposição a esse metal, seja na forma de gavagem ou na forma de ingestão de bolo.

No **capítulo 1**, corroborando com os dados presentes na literatura, foi demonstrado que o Cd é capaz de atravessar a membrana hematoencefálica, pois embora a quantidade de Cd acumulada nas diferentes estruturas cerebrais estudadas

(córtex cerebral, cerebelo, estriado, hipocampo e hipotálamo) tenha sido baixa, ainda assim, foi significativamente maior que o controle e suficiente para causar possíveis danos cerebrais. Além disso, a NAC foi ineficiente em diminuir estes níveis, sugerindo que os prejuízos ocasionados pelo Cd e que foram revertidos ou amenizados pela NAC tenham sido devido a sua atividade antioxidante e não a sua atividade como quelante uma vez que essa molécula não removeu o Cd dos tecidos.

Em adição, observou-se uma diminuição na atividade da enzima AChE em cerebelo, hipocampo e hipotálamo dos ratos, no entanto, após a co-administração da NAC esse efeito foi totalmente ou parcialmente revertido. Porém, nenhuma alteração na atividade dessa enzima foi observada em estriado e em sinaptossoma de córtex cerebral. Segundo Shaw & Panigrahi (1990), a inativação da enzima AChE pode ser uma consequência da ocupação do seu sítio ativo por metais pesados, tal como o mercúrio. De fato, tem sido proposto que o mecanismo de ação do Cd possa ser o deslocamento de co-fatores metálicos do sítio ativo ou a desativação direta da enzima (CASALINO et al., 1997). Como resultado da inibição da AChE, o neurotransmissor ACh é menos hidrolisado nas sinapses conduzindo a uma quantidade anormal de ACh, consequentemente, causando uma superativação dos receptores colinérgicos e possíveis efeitos tóxicos (WALKER, 2001). A atividade diminuída da AChE tem sido associada a hiperatividade colinérgica, convulsão, estado epiléptico, confusão, dor de cabeça, sono e distúrbios de memória (OLNEY et al., 1986; ECOBICHON, 1996).

Outro aspecto a ser discutido é que a AChE é um importante componente nas membranas biológicas, contribuindo assim para a integridade das mesmas. E, sabe-se que o Cd pode causar um aumento na peroxidação lipídica de alguns tecidos (MANCA et al., 1991; KUMAR et al., 1996; SHUKLA et al., 1996; MÉNDEZ-ARMENTA et al., 2003). No presente estudo, verificou-se uma correlação positiva entre a inibição da atividade da AChE e o aumento de peroxidação lipídica nas estruturas cerebrais dos ratos intoxicados por Cd o que pode ter sido um fator decisivo na modificação do estado conformacional da molécula de AChE e pode estar relacionado com alterações na atividade dessa enzima após exposição ao Cd. Ainda, verificou-se que a NAC foi capaz de reverter totalmente este processo danoso uma vez que os ratos intoxicados e tratados com NAC apresentaram níveis de peroxidação lipídica semelhante ao controle. Baseado nessas colocações pode-se sugerir que a inibição da AChE encontrada em ratos

intoxicados nesse estudo pode estar associada ao processo de estresse oxidativo nas diferentes regiões encefálicas desses animais e que, as propriedades antioxidantes da NAC podem ser responsáveis por reverter disfunções colinérgicas em ratos intoxicados por Cd.

É importante ressaltar que a AChE cerebral é um importante regulador do processo comportamental incluindo os processos cognitivos (PARI & MURUGAVEL, 2007). Tendo em vista os prejuízos das funções cognitivas observadas em animais experimentais e humanos expostos ao Cd, nesse trabalho também foi investigado o efeito da NAC sobre a memória e aprendizagem através da tarefa de esquiva inibitória em ratos intoxicados pelo Cd. Foi observado um decréscimo na latência dos ratos intoxicados quando submetidos a essa tarefa sugerindo, portanto, prejuízos na memória e aprendizagem, o que, também, pode ser associado a diminuição da atividade da AChE um dos mecanismos envolvidos nos processos de dano cognitivo. Porém, é importante salientar que, quando os ratos intoxicados foram submetidos ao tratamento com NAC a latência de descida da plataforma no teste de esquiva inibitória foi similar àquela encontrada em ratos controles. Finalmente, os resultados apresentados no capítulo 1 dessa tese sugerem que a NAC é capaz de diminuir os níveis de peroxidação lipídica e, subsequentemente, restaurar a atividade da AChE, modulando assim, a neurotransmissão colinérgica e melhorando os processos cognitivos.

Para obter-se uma melhor compreensão dos efeitos do $CdCl_2$ e da NAC sobre o sistema colinérgico exposto no primeiro trabalho, no **Capítulo 2** decidiu-se avaliar os efeitos do $CdCl_2$ sobre a atividade da AChE em linfócitos e sangue total bem como sobre a atividade da BChE em soro de ratos. Além disso, verificou-se os efeitos do $CdCl_2$ e da NAC sobre a atividade da NTPDase de linfócitos, parâmetros hematológicos, e concentração de Cd no plasma, baço e timo desses animais. Para tal, utilizou-se o mesmo protocolo descrito, anteriormente, para o capítulo 1. Em relação às enzimas, verificou-se que o Cd não altera a atividade da enzima NTPDase em linfócitos, e diminui a atividade da AChE em linfócitos e sangue total bem como da BChE em soro de ratos. Embora a atividade da NTPDase de linfócitos não tenha sido alterada nesse estudo, não pode-se descartar a possibilidade de que o Cd afete essa enzima, pois vários estudos do nosso laboratório têm demonstrado que a atividade e a expressão da NTPDase de linfócitos encontra-se alterada em diferentes situações fisiológicas,

patológicas e toxicológicas uma vez que essa enzima é crucial nessas células devido às funções desempenhadas pelos nucleotídeos e nucleosídeos (SCHETINGER et al., 2007; KAIZER et al., 2010; SPANEVELLO et al., 2010a, b; JAQUES et al., 2011). Portanto, acredita-se que, uma alteração na atividade dessa enzima poderia ser notada em outras circunstâncias de intoxicação por Cd, o que irá depender da via de administração, forma química, e, ainda, duração e dose de exposição de Cd no modelo estudado.

Em relação à atividade da AChE em linfócitos e no sangue total, os resultados encontrados foram similares aos obtidos nas regiões cerebrais. De acordo com Thiermann et al. (2005), a AChE periférica possui propriedades funcionais similares com a AChE do SNC e assim pode refletir seu estado com a AChE da fenda sináptica. Neste contexto, corroborando com resultados publicados pelo nosso grupo de pesquisa, pode-se sugerir que a atividade da enzima AChE em tecidos periféricos possa ser um importante marcador periférico para estudar disfunções neurológicas, pois permite avaliar através de métodos mais acessíveis a ação dessa enzima no SNC (MAZZANTI et al., 2009; SCHMATZ et al., 2009). Além disso, é interessante notar que, a diminuição na atividade da AChE de linfócito encontrada nos ratos intoxicados pode levar a uma lenta degradação de ACh a qual é considerada uma molécula anti-inflamatória, sugerindo-se que a inibição da atividade dessa enzima seja um mecanismo compensatório do organismo para prevenir ou atenuar um processo inflamatório (DA SILVA et al., 2011). Por outro lado, a diminuição encontrada na atividade da enzima BChE em ratos intoxicados por Cd pode estar relacionada a uma diminuição da síntese dessa enzima, uma vez que, a BChE é produzida no fígado, um dos principais órgãos afetados por esse metal, ou ainda, à degradação aumentada da enzima ou a alterações das moléculas proteicas causadas pela ação de espécies reativas de oxigênio (ERO) (GARCIA et al., 2008).

Os resultados mostraram que a exposição ao $CdCl_2$ promove um aumento na concentração de Cd no plasma, baço e timo. Corroborando com os nossos achados, Viau et al. (2007) reportaram que o Cd pode atingir aos órgãos linfóides mesmo que em concentrações baixas. Pathak & Khandelwal (2007, 2009) demonstraram que as células esplênicas são sensíveis ao Cd, o qual pode causar o alargamento do baço. De acordo com eles, o aumento do baço pode estar associado com a expansão da sua polpa vermelha, local onde são depositados os eritrócitos danificados. Sabe-se que a sequência

eritrócitos danificados-hemólise-esplenomegalia tem sido considerada a etiologia da anemia induzida pelo Cd. De fato, no presente estudo observou-se uma diminuição nos parâmetros hematológicos (número de eritrócitos, hematócrito e conteúdo de hemoglobina) em ratos expostos ao Cd o que pode conduzir a um estado anêmico. A redução no número de eritrócitos pode indicar que o Cd altera a hematopoiese, ou ainda, pode estar associada a uma hiperatividade da medula óssea conduzindo a uma maior produção de eritrócitos defeituosos (EL-DEMERDASH et al., 2004; NEMMICHE et al., 2007). Já a redução no conteúdo de hemoglobina pode ser devido ao aumento da destruição de eritrócitos, à redução na sua síntese, a inibição da atividade da enzima ALA-D ou, ainda, à baixa concentração de ferro no sangue (EL-DEMERDASH et al., 2004; LUCHESE et al., 2007; SIMSEK et al., 2009).

Além dos danos hematológicos, verificou-se que a exposição ao $CdCl_2$ causa diminuição do número total de células brancas e de linfócitos bem como aumenta o número de monócitos e neutrófilos, similarmente aos dados reportados por Karmakar et al. (2000) após tratarem os ratos com 2 mg/kg de Cd, s.c., durante 14 e 21 dias. Em adição, esses mesmos autores, também, relataram alterações na contagem diferencial de leucócitos, provavelmente, em função de modificações na taxa linfóide/mielóide. Além disso, é interessante ressaltar que os neutrófilos uma vez ativados produzem ERO durante as reações inflamatórias, então o acúmulo de neutrófilos em ratos intoxicados pelo Cd pode expor ainda mais os tecidos aos danos potenciais decorrentes do estresse oxidativo (KARMAKAR et al., 2000).

Interessantemente, a NAC foi capaz, não somente, de reverter ou amenizar esses efeitos negativos causados pelo Cd, mas também em diminuir os níveis de Cd no baço e no timo dos animais intoxicados, mesmo sem influenciar na atividade das enzimas colinesterases periféricas. Pathak & Khandelwal (2007, 2009) reportaram que o Cd causa esplenomegalia e atrofia do timo, possivelmente, devido à potenciação do estresse oxidativo. E, sabe-se que a NAC *in vitro* pode diminuir a geração de ERO e aumentar os níveis de GSH em timócitos e esplenócitos (PATHAK & KHANDELWAL 2006a, b). Assim, baseado nesses resultados e considerando as propriedades antioxidantes da NAC, é plausível que este composto possa ocasionar efeitos benéficos em relação aos órgãos linfóides e, assim contribuir para a melhoria dos parâmetros hematológicos e relacionados aos leucócitos em animais intoxicados pelo Cd.

No **capítulo 3** objetivou-se continuar a investigação sobre o efeito de $CdCl_2$ e da NAC sobre a atividade das enzimas ectonucleotidases. Assim, verificou-se a atividade das enzimas NTPDase e 5'-nucleotidase em sinaptossomas de córtex cerebral e de hipocampo e, em plaquetas de ratos expostos ao $CdCl_2$ e tratados com NAC. Para tal, utilizou-se o mesmo protocolo descrito anteriormente para os capítulos 1 e 2. Em relação às ectonucleotidases em sinaptossomas de córtex cerebral, foi observado um aumento na atividade da NTPDase e da 5'-nucleotidase e, o tratamento com NAC foi eficiente em conter esses efeitos indesejáveis. De fato, estudos prévios realizados no nosso laboratório também têm demonstrado uma ativação dessas enzimas em doenças como esclerose múltipla e diabetes bem como em intoxicação por alumínio (SPANEVELLO et al., 2006, 2009; KAIZER et al., 2007; SCHMATZ et al., 2009). Sabe-se que, em caso de dano cerebral, o ATP liberado em excesso pode causar significativos danos teciduais (FEUVRE et al., 2002). Por outro lado, a adenosina, produto da hidrólise de ATP, atua na neuromodulação e neuroproteção do SNC (CUNHA, 2001). Nesse contexto, pode-se sugerir que o aumento na atividade da NTPDase e da 5'-nucleotidase em sinaptossomas de córtex cerebral de ratos devido à intoxicação pelo Cd, possa estar relacionado com uma importante capacidade adaptativa da via das ectonucleotidases, que ocorre com o objetivo de terminar a função do ATP extracelular, incluindo seus efeitos tóxicos, e produzir adenosina, um composto neuroprotetor. Por outro lado, nenhuma diferença significativa foi encontrada nas enzimas NTPDase e 5'-nucleotidase de sinaptossomas de hipocampo de ratos intoxicados pelo Cd.

Além disso, as plaquetas dos ratos intoxicados por Cd apresentaram uma diminuição na atividade da NTPDase e nenhuma alteração na atividade da enzima 5′-nucleotidase e, o tratamento com NAC foi ineficiente em conter estes efeitos indesejáveis. Embora vários estudos tenham sido realizados na tentativa de entender os mecanismos pelos quais o Cd causa hipertensão (PERRY Jr. & ERLANGER, 1981; SATARUG et al., 2005, 2006; YOOPAN et al., 2006, 2008), estes mecanismos não estão completamente elucidados e, resultados conflitantes têm sido verificados tanto em modelos experimentais quanto na população (NAKAGAWA & NISHIJO, 1996; EUM et al., 2008; TELLEZ-PLAZA et al., 2008). Algumas pesquisas, assim como essa, têm sido realizadas visando entender os efeitos do Cd sobre as plaquetas uma vez que essas

estruturas também poderiam estar envolvidas nesses mecanismos (CAPRINO et al., 1979; MUKHOPADHYAY et al., 1988; KIM et al., 2001). A redução na hidrólise de ATP e ADP podem levar a uma concentração maior dessas moléculas na circulação enquanto que pode existir uma tendência à redução da concentração de adenosina circulante nos animais intoxicados por esse metal. Em adição, o ATP promove vasoconstrição, o ADP é um ativador da agregação de plaquetas, enquanto a adenosina induz vasodilatação e inibição da agregação plaquetária (ANFOSSI et al., 2002; BIRK et al., 2002a, b; BOROWIEC et al., 2006; ROZALSKI et al., 2005). Portanto, os resultados apresentados nessa tese sugerem, pela primeira vez, que a inibição da atividade da NTPDase associada com a tendência de redução na atividade da 5'-nucleotidase possa contribuir para as propriedades trombogênicas e hipertensivas do Cd descrita por alguns autores.

Analisando os dados obtidos, um fator importante a ser discutido é o fato dos presentes resultados para a atividade da NTPDase e 5'-nucleotidase de plaquetas serem diferentes dos dados obtidos para as ectonucleotidases no SNC, portanto, uma certa cautela deve ser tomada quando extrapola-se interpretações de modelos periféricos para dados do SNC (BORGES & NOGUEIRA, 2008). Além disso, do capítulo 1 ao 3 dessa tese, verificou-se que a NAC pode interferir não somente o sistema colinérgico, mas também, o sistema purinérgico, o que é extremamente interessante uma vez que as enzimas integrantes desses sistemas apresentam uma potencial importância terapêutica.

Até o capítulo 3, avaliou-se os efeitos do $CdCl_2$ e da NAC no intuito de observar-se os efeitos tóxicos causados por esse metal e os possíveis efeitos benéficos do antioxidante. Entretanto, sabe-se que a população em geral é exposta ao Cd ao longo do tempo, principalmente, através da ingestão de alimentos contaminados. Sendo assim, no **Capítulo 4** avaliou-se os efeitos da ingestão à longo prazo de $CdCl_2$ bem como de Cd proveniente de batatas contaminadas sobre o desempenho cognitivo, motor e de ansiedade e, ainda, sobre a atividade das enzimas AChE e Na^+,K^+-ATPase em diferentes estruturas cerebrais de ratos. Para tal, os ratos ingeriram, durante 5 meses, dieta a base de bolo contendo 1, 5 ou 25 mg/kg de $CdCl_2$ ou Cd presente em tubérculos de batatas crescidas na presença de 10 μM de $CdCl_2$. Os resultados demonstraram um decréscimo na latência dos ratos intoxicados quando submetidos à tarefa de esquiva inibitória sugerindo prejuízos na memória e aprendizagem desses animais. Também verificou-se o

comportamento locomotor e a sensibilidade ao choque desses animais a fim de identificar qualquer disfunção motora ou aspectos motivacionais de aprendizagem, tais como sensibilidade ao choque, que pudessem influenciar o desempenho na esquiva inibitória (SCHMATZ et al., 2009). Os resultados demonstraram que a atividade locomotora bem como a sensibilidade ao choque não foram afetadas tanto pelo $CdCl_2$ quanto pelo Cd proveniente de batatas contaminadas uma vez que não foram alteradas nos animais tratados com metal, excluindo a possibilidade que a atividade locomotora ou a sensibilidade ao choque possam ter contribuído para as latências de descida no teste de esquiva inibitória em todos os animais utilizados nos estudos dessa tese.

Além disso, avaliou-se o desempenho de ansiedade dos ratos utilizando-se o teste do labirinto em cruz elevado (BELZUNG & GRIEBEL, 2001). Os ratos que foram alimentados somente com batata crescida na ausência do metal apresentaram um comportamento de ansiedade menor. Por outro lado, os animais que receberam 25 mg/kg de $CdCl_2$ apresentaram um efeito contrário. Sendo assim, pode-se dizer que a batata *per se* apresentou atividade ansiolítica enquanto a maior dose de Cd apresentou efeito ansiogênico. Sabe-se que os compostos benzodiazepínicos são os fármacos mais frequentemente utilizados como tranquilizantes e ansiolíticos (RUDOLPH et al., 1999). Além disso, Wildmann (1988) e Kavvadias et al. (2000) reportaram a formação endógena de benzodiazepínicos em tecidos de batatas, incluindo o tubérculo. Além disso, alguns autores têm reportado que outras substâncias com atividade gabaérgica e que são potenciais ansiolíticos, como vários flavonoides, têm sido encontradas em batatas (MUCENIECE et al., 2008; GIORGETTI & NEGRI, 2011). Em relação ao Cd, pouca pesquisa tem sido realizada em relação ao comportamento de ansiedade. Leret et al. (2003) sugeriram que a maior ansiedade observada em ratos co-expostos ao Cd (10 mg/L) e ao Pb (300 mg/L) poderia estar relacionada aos neurônios serotonérgicos e dopaminérgicos uma vez que foi detectado um aumento nos níveis de serotonina e dopamina no hipocampo desses animais. Entretanto, Minetti & Reale (2005) demonstraram que fetos expostos prenatalmente a 0,6 mg/kg de Cd apresentaram menor ansiedade. Mas, é importante ressaltar que a dose de Cd utilizada por esses autores é cerca de cinco vezes menor se comparada à dose que apresentou efeito ansiogênico nessa tese.

Na presente tese, também, foi encontrado um aumento na atividade da AChE em hipocampo, hipotálamo, cerebelo e estriado de ratos expostos ao $CdCl_2$ ou ao Cd proveniente de batatas contaminadas. No córtex cerebral não houve diferença significativa entre os grupos. A ativação da AChE leva a rápida degradação de ACh e uma baixa estimulação dos receptores causando efeitos indesejáveis nas funções cognitivas (SOREQ & SEIDMAN, 2001). Assim, um aumento na atividade da AChE leva a uma redução na eficiência da neurotransmissão colinérgica devido ao decréscimo nos níveis de ACh na fenda sináptica, contribuindo para um progressivo prejuízo cognitivo e outras disfunções neurológicas observadas em animais experimentais e em humanos intoxicados por esse metal, similarmente ao reportado por Carageorgiou et al. (2004), os resultados apresentados nos capítulo 1 e 4 indicam que a exposição ao Cd em curto prazo causa uma diminuição da atividade da AChE enquanto a exposição em longo prazo causa o efeito contrário e, ambos os tipos de alterações são prejudiciais aos processos de neurotransmissão e comportamentais. Além dessas consequências, existem evidências de que níveis aumentados de ACh apresentam propriedade ansiolítica (SMYTHE et al., 1998; DEGROOT et al., 2001; DEGROOT & TREIT, 2002). Sendo assim, os presentes resultados sugerem que o sistema colinérgico pode estar envolvido no comportamento de ansiedade bem como no dano de memória observado em ratos que foram alimentados com a maior dose de $CdCl_2$ (25 mg/kg) visto que esses animais apresentaram uma atividade aumentada da AChE, o que provavelmente diminuiu os níveis de ACh em quatro estruturas cerebrais, incluindo o hipocampo, região particularmente importante nos processos de ansiedade e cognição.

Em relação à enzima Na^+,K^+-ATPase observou-se uma diminuição da sua atividade em córtex cerebral, hipotálamo e cerebelo de ratos expostos ao $CdCl_2$ ou ao Cd proveniente de batatas contaminadas. No hipocampo e no estriado não houve diferença significativa entre os grupos. Acredita-se que essa inibição pelo Cd seja devido à competição desse metal com locais de ATP e Na^+ na estrutura da enzima, à interferência do estado de fosforilação da enzima, ou a mudanças nos grupos tióis dessa molécula (RAJANNA et al., 1983; CHETTY et al., 1992; ANTONIO et al., 2002; BORGES et al., 2005). Além disso, sabe-se que a intoxicação por Cd pode aumentar a produção de radicais livres que reagem com os lipídios de membrana, causando uma desestruturação da bicamada lipídica com consequente inibição da atividade da Na^+,K^+-

ATPase (KAKO et al., 1988; MATTÉ et al., 2006). Sabe-se que essa enzima desempenha uma função crucial na comunicação dos sinais extracelulares com o meio intracelular nos tecidos neurais e, que a sua inibição prejudica os processos de aprendizado e memória (ANTONIO et al., 2002; SATO et al., 2004; ZHAN et al., 2004). Sendo assim, a inibição dessa enzima em algumas estruturas cerebrais poderia estar envolvida no prejuízo cognitivo verificado nesse capítulo.

Em linhas gerais, os resultados obtidos na presente tese demonstram que apesar de todos os mecanismos propostos pelos quais o Cd poderia afetar o comportamento continuarem a gerar controvérsia, é evidente que o exposição ao $CdCl_2$ tanto a curto quanto à longo prazo bem como ao Cd proveniente de batatas contaminadas é prejudicial para as funções cerebrais e, consequentemente para o comportamento animal, tais como memória e ansiedade. Os dados da presente tese sugerem que os danos ao comportamento exibidos pelos ratos intoxicados pelo Cd são associados com a diminuição ou o aumento na atividade da AChE e a diminuição na atividade da Na^+,K^+-ATPase em diferentes estruturas cerebrais. Também, verificou-se que a lipoperoxidação parece estar envolvida na ocorrência desses danos. Além disso, verificou-se que o Cd afeta sistematicamente o organismo dos animais estando presente tanto no plasma, quanto nos órgãos linfóides e no encéfalo alterando, assim, a atividade das enzimas NTPDase, 5'-nucleotidase, AChE e BChE de diferentes tipos celulares. É importante ressaltar a importância dos achados relacionados à capacidade do antioxidante NAC em reverter ou amenizar vários efeitos danosos causados pelo Cd sugerindo que este fármaco possa ser, após estudos adicionais, considerado um importante aliado em terapias contra a intoxicação por esse metal. É interessante salientar que animais intoxicados com baixas concentrações de Cd proveniente da dieta durante longo período exibem várias complicações mimetizando de forma mais realista a condição de exposição da população em geral.

8. CONCLUSÕES

Os resultados obtidos nestes estudos nos permitem concluir que:

1. A intoxicação por $CdCl_2$ causa aumento na concentração de Cd, nos níveis de peroxidação lipídica e na atividade da enzima AChE em diferentes estruturas cerebrais o que pode estar relacionado ao prejuízo da memória em ratos. Além disso, a NAC é capaz de diminuir os níveis de peroxidação lipídica e subsequentemente restaurar a atividade da AChE possivelmente modulando assim, a neurotransmissão colinérgica e melhorando os processos cognitivos. Sendo assim, sugere-se que a NAC possa ser um fármaco promissor em terapias alternativas contra a neurotoxicidade induzida pelo Cd;

2. A exposição ao $CdCl_2$ promove um aumento na concentração de Cd no plasma, baço e timo, causa danos hematológicos, não altera a atividade da enzima NTPDase em linfócitos, diminui a atividade da AChE em linfócitos e sangue total bem como da BChE em soro de ratos. Além disso, a NAC foi eficaz em diminuir os efeitos danosos do Cd provavelmente por diminuir os níveis de Cd nos órgãos linfóides, reverter ou amenizar os danos hematológicos e relacionados aos leucócitos mesmo sem influenciar na atividade das enzimas colinesterases;

3. O $CdCl_2$ aumenta a hidrólise de nucleotídeos de adenina em sinaptossomas de córtex cerebral e diminui em plaquetas de ratos. Provavelmente, o aumento na atividade das enzimas NTPDase e 5'-nucleotidase no SNC causado pelo Cd seja uma resposta compensatória do organismo uma vez que a rápida hidrólise de ATP e ADP favorece a produção de adenosina, uma molécula neuroprotetora. Por outro lado, em plaquetas o Cd causou uma diminuição na atividade da NTPDase e, não alterou a atividade da 5'-nucleotidase sugerindo que a possível elevação no nível de ATP e ADP conduza a um estado hipercoagulável nos ratos intoxicados por esse metal. Além disso, a NAC restaura a atividade dessas enzimas no SNC, mas não apresenta interferência sobre elas em plaquetas;

4. A dieta prolongada com $CdCl_2$ ou com Cd proveniente de batatas contaminadas ocasiona aumento no comportamento de ansiedade e prejuízo à memória de ratos o que provavelmente, seja resultado de uma concentração aumentada de Cd, atividade aumentada da AChE e diminuída da Na^+,K^+-ATPase em diferentes estruturas cerebrais devido a esse metal.

9. PERSPECTIVAS

1. Avaliar os efeitos do $CdCl_2$ e da NAC sobre a expressão e a atividade *in vitro* das enzimas já estudadas *ex vivo* nessa tese com a finalidade de obter um maior esclarecimento sobre os mecanismos pelos quais esse metal atua sobre essas proteínas.

2. Determinar o possível efeito protetor de outros antioxidantes, tal como a curcumina, em relação à intoxicação por $CdCl_2$ através de verificações bioquímicas e comportamentais em ratos expostos a esse metal e tratados com curcumina.

3. Verificar os efeitos a longo prazo do $CdCl_2$ bem como do Cd proveniente de batatas contaminadas sobre a atividade das enzimas NTPDase, 5'-nucleotidase, AChE e BChE em diferentes tecidos de ratos.

4. Verificar o papel do $CdCl_2$ como sendo um fator ambiental contribuinte para o possível agravamento de patologias tal como a epilepsia investigando-se os efeitos desse metal em convulsões induzidas pela injeção de pentilenotetrazol em ratos através da avaliação de parâmetros eletroencefalográficos, comportamentais e bioquímicos.

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