UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA TOXICOLÓGICA

AVALIAÇÃO COMPORTAMENTAL, PERFIL OXIDATIVO E ATIVIDADE DE ATPases E COLINESTERASES EM RATOS EXPOSTOS AO CÁDMIO E TRATADOS COM QUERCETINA

TESE DE DOUTORADO

Fátima Husein Abdalla

Santa Maria, RS, Brasil
2014

AVALIAÇÃO COMPORTAMENTAL, PERFIL OXIDATIVO E ATIVIDADE DE ATPases E COLINESTERASES EM RATOS EXPOSTOS AO CÁDMIO E TRATADOS COM QUERCETINA

por

Fátima Husein Abdalla

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

Orientadora: Dr.ª Cinthia Melazzo de Andrade
Co-orientadora: Dr.ª Roberta Schmatz

SANTA MARIA, RS, BRASIL

2014

Universidade Federal de Santa Maria
Centro de Ciências Naturais e Exatas
Programa de Pós-Graduação em Ciências Biológicas:
Bioquímica Toxicológica

A Comissão Examinadora, abaixo assinada, aprova a Tese de Doutorado

AVALIAÇÃO COMPORTAMENTAL, PERFIL OXIDATIVO E ATIVIDADE DE ATPases E COLINESTERASES EM RATOS EXPOSTOS AO CÁDMIO E TRATADOS COM QUERCETINA

elaborada por

Fátima Husein Abdalla

como requisito parcial para a obtenção do grau de

Doutora em Ciências Biológicas: Bioquímica Toxicológica

COMISSÃO EXAMINADORA:

_____

Prof.ª Dr.ª Cinthia Melazzo de Andrade
(Presidente/Orientador)

_____

Prof.ª Dr.ª Roselia Maria Spanevello

_____

Prof.ª Dr.ª Daniela Bitencourt Rosa Leal

_____

Prof.ª Dr.ª Patrícia Wolkmer

_____

Prof. Dr. Félix Antunes Soares

Santa Maria, 29 de setembro de 2014

É melhor tentar e falhar,
que preocupar-se e ver a vida passar.
É melhor tentar, ainda que em vão,
que sentar-se fazendo nada até o final.
Eu prefiro na chuva caminhar,
que em dias tristes em casa me esconder.
Prefiro ser feliz, embora louca,
que em conformidade viver.

(Martin Luther King)

DEDICATÓRIA

A minha família

Pelo amor, carinho, apoio,
compreensão, companheirismo e
dedicação durante toda minha vida.

AGRADECIMENTOS

Gostaria de agradecer primeiramente a Deus pelas bênçãos alcançadas ao longo destes anos de estudo, e, por apesar dos obstáculos, iluminar meu caminho e fazer com que desse tudo certo.

À minha família, em especial aos meus pais, Fernando e Samira, por me darem a vida, e me ensinar a trilhá-la com dignidade, pela educação que me concederam; por estarem sempre presentes em todos os momentos de minha vida, participando de minhas decisões, me apoiando, me compreendendo e confiando em meu potencial. Agradeço aos melhores pais, aos quais tenho um orgulho imenso e uma admiração infinita. Agradeço aos meus irmãos, cunhado e cunhadas e aos meus sobrinhos fofos por todos os momentos de alegrias que me proporcionaram, transformando momentos simples em inesquecíveis; por todo o amor e carinho que sempre me concederam, enfim, por tudo.

Ao meu marido, Thiago, por me incentivar a lutar pelos meus sonhos sempre demostrando total apoio e sendo muito compreensivo comigo, por estar sempre ao meu lado me apoiando nas horas mais difíceis, pelo seu amor que me da força para continuar sempre em busca dos meus objetivos. Te amo muito!

À professora Cinthia Melazzo de Andrade, minha orientadora, pela oportunidade de ingressar em seu grupo de pesquisa, por todos os ensinamentos, atenção e dedicação; a minha eterna gratidão, pois sem esta oportunidade e confiança depositada em mim hoje eu não estaria aqui.

Às minhas co-rientadoras, Roberta Schmatz e a Jamile Fabbrin Gonçalves pela dedicação, pelo apoio, atenção e por todos os ensinamentos que foram fundamentais para a realização deste trabalho.

Às professoras Maria Rosa Chitolina Schetinger e Vera Maria Morch pela colaboração e pela oportunidade de trabalhar com o grupo de pesquisa do enzitox, por todos os ensinamentos, atenção e dedicação.

Aos membros da banca examinadora desta tese, pela disponibilidade em avaliar este trabalho e pelas contribuições que foram fundamentais para o aprimoramento do mesmo.

Aos todos os meus amigos e colegas do laboratório Enzitox e do Bio-Ox pela colaboração para elaboração deste trabalho, pelos momentos compartilhados e pela amizade durante o período de convívio.

Aos professores e funcionários do Programa de Pós-Graduação em Bioquímica Toxicológica, pelos ensinamentos, amizade e dedicação.

À CAPES, pela bolsa concedida e pela oportunidade de realizar uma parte do trabalho no exterior.

E a todas as pessoas que, de uma forma ou de outra, contribuíram para a realização deste trabalho, gostaria de expressar minha profunda gratidão.

RESUMO

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

AVALIAÇÃO COMPORTAMENTAL, PERFIL OXIDATIVO E ATIVIDADE DE ATPases E COLINESTERASES EM RATOS EXPOSTOS AO CÁDMIO E TRATADOS COM QUERCETINA

AUTORA: FÁTIMA HUSEIN ABDALLA
ORIENTADORA: CINTHIA MELAZZO DE ANDRADE
CO-ORIENTADORA: ROBERTA SCHMATZ

Data e Local da Defesa: Santa Maria, 29 de setembro de 2014.

O cádmio (Cd) é considerado um dos metais pesados de maior toxicidade devido a sua capacidade de afetar diferentes tecidos, incluindo o encéfalo, bem como o sistema imunológico. Os mecanismos moleculares de toxicidade do Cd ainda não estão bem estabelecidos, contudo, sabe-se que uma das consequências da exposição ao Cd é a geração de estresse oxidativo. Por outro lado, a quercetina, um flavonoide presente em vários alimentos, exerce diversas funções terapêuticas no organismo, como atividade antioxidante, anti-inflamatória e ação neuroprotetora. Sendo assim, o objetivo do presente estudo foi investigar os efeitos da quercetina sobre os testes comportamentais, a atividade das enzimas acetilcolinesterase (AChE), a Na^+,K^+-ATPase e a δ-desidratase aminolevulinato (δ-ALA-D), bem como os parâmetros de estresse oxidativo no sistema nervoso central de ratos machos wistar adultos expostos ao $CdCl_2$. Também foi avaliada, no sistema periférico destes animais, a atividade das enzimas AChE, NTPDases e adenosina desaminase (ADA) de linfócitos periféricos, butirilcolinesterase (BuChE) do soro e a mieloperoxidase (MPO) do plasma. Os ratos foram expostos ao $CdCl_2$ (2,5 mg/kg) e quercetina (5, 25 ou 50 mg/kg) por gavagem (1ml/kg) durante 45 dias. Para isso, os animais foram distribuídos em oito grupos (n=10-14): salina/controle, salina/Querc 5mg/kg, salina/Querc 25 mg/kg, salina/Querc 50 mg/kg, Cd/etanol, Cd/ Querc 5mg/kg, Cd/Querc 25mg/kg e Cd/Querc 50 mg/kg. Os grupos tratados com Cd e quercetina receberam a solução antioxidante após 30 minutos da administração da solução de Cd. No final do período de 45 dias de tratamento os animais foram submetidos aos treinos e aos testes comportamentais. Posteriormente, foram anestesiados, através da inalação de halotano, e foi realizada a coleta de sangue e separação de soro, plasma e linfócitos periféricos. Em seguida os animais foram submetidos à eutanásia, com parte do encéfalo sendo retirada para a análise da atividade da enzima δ-ALA-D, enquanto que outra parte foi dissecada em córtex cerebral, estriado, cerebelo, hipocampo e hipotálamo, para posteriores ensaios enzimáticos. Os resultados obtidos mostraram que o Cd é capaz de atravessar a barreira hematoencefálica, pois, embora a quantidade de Cd acumulada nas diferentes estruturas encefálicas estudadas tenha sido baixa, ainda assim, foi significativamente maior que o controle. O tratamento concomitante da quercetina nos animais expostos ao Cd foi ineficiente em diminuir estes níveis de Cd. A exposição ao Cd causou prejuízos na aprendizagem e memória, além de causar um aumento no comportamento do tipo ansiogênico. Por outro lado, o tratamento com a quercetina preveniu os efeitos indesejáveis causados pela exposição ao metal na ansiedade

e memória. Em relação às atividades enzimáticas no encéfalo, verificou-se que a exposição ao Cd reduziu a atividade da enzima AChE no córtex cerebral e no hipocampo, enquanto que uma ativação da enzima foi observada no hipotálamo. Além disso, observou-se uma diminuição na atividade da enzima Na^+, K^+-ATPase no córtex cerebral, hipocampo e hipotálamo, bem como uma diminuição na atividade da δ-ALA-D no encéfalo total de animais expostos ao Cd. Interessantemente, a co-administração com a quercetina em animais expostos ao Cd impediu as alterações na atividade das enzimas AChE e Na^+, K^+-ATPase em diferentes estruturas encefálicas, embora não tenha restaurado a a atividade da enzima δ-ALA-D. Verificou-se, também, um aumento na produção de ROS, na lipoperoxidação, na oxidação de proteínas, nos níveis de DNA dupla fita e alterações no sistema antioxidante, como a diminuição na atividade da enzima glutationa redutase (GR), nos níveis de tióis totais (T-SH) e glutationa reduzida (GSH), e um aumento na atividade da enzima glutationa S-transferase (GST) no córtex cerebral, hipocampo e hipotálamo dos animais expostos ao Cd. A co-administração da quercetina nos ratos expostos ao Cd foi capaz de impedir totalmente ou parcialmente as alterações causadas pela exposição ao metal nos parâmetros do estresse oxidativo. Sugere-se que a quercetina é capaz de diminuir o dano oxidativo causado pela exposição ao metal e, subsequentemente, restaurar a atividade da AChE e Na^+, K^+-ATPase, modulando, assim, a neurotransmissão colinérgica e melhorando os processos cognitivos. Em relação ao sistema periférico, verificou-se um aumento na atividade das enzimas NTPDase, ADA, AChE, BuChE e MPO nos ratos expostos ao Cd. A partir desse resultado pode-se inferir que o aumento na atividade da NTPDase seja um efeito compensatório devido ao aumento dos níveis de ATP na circulação. Sugere-se que níveis diminuídos de ACh estão disponíveis na circulação devido ao aumento na atividade das colinesterases periféricas. Quando os ratos foram tratados com quercetina o flavonoide foi capaz de modular a atividade dessas enzimas provavelmente devido à propriedade anti-inflamatória do composto. Deste modo, propõe-se que a quercetina previne ou ameniza a toxicidade causada pela exposição ao metal devido a sua atividade antioxidante e anti-inflamatória. Logo, acredita-se que este flavonoide possa ser um fármaco promissor em terapias alternativas contra a toxicidade induzida pelo metal no sistema nervoso central e periférico.

Palavras chave: NTPDase, 5´-nucleotidase, ADA, AChE, Na^+, K^+-ATPase, processo inflamatório, neurotoxicidade.

ABSTRACT

Doctoral Thesis
Post-Graduate Program in Biological Science: Toxicological Biochemistry
Federal University of Santa Maria

BEHAVIORAL ASSESSMENT, OXIDATIVE PROFILE AND ATPases CHOLINESTERASE ACTIVITIES IN CADMIUM EXPOSED RATS AND TREATED WITH QUERCETIN

AUTOR: FÁTIMA HUSEIN ABDALLA
ADVISOR: CINTHIA MELAZZO DE ANDRADE
CO-ADVISOR: ROBERTA SCHMATZ

Place and date of defense: Santa Maria, September 29th, 2014.

Cadmium (Cd) is considered one of the most toxic heavy metals for its ability to affect different tissues, including the brain and the immune system. The molecular mechanisms of toxicity of Cd are not well established, however, it is known that one of the consequences of Cd exposure is the generation of oxidative stress. Conversely, the quercetin, one flavonoid present in various foods performs various therapeutic functions in the body, such as antioxidant activity, anti-inflammatory and neuroprotective action. Thus, the aim of this study was to investigate the effects of quercetin on the behavioral tests, the activity of the enzymes acetylcholinesterase (AChE), Na^+, K^+-ATPase and the δ-dehydratase aminolevulinic acid (δ-ALA-D), as well as parameters of oxidative stress in the central nervous system of adult male wistar rats exposed to $CdCl_2$. The activities of enzymes AChE, NTPDase and adenosine deaminase (ADA) of peripheral lymphocytes, butyrylcholinesterase (BuChE) in the serum and myeloperoxidase (MPO) in plasma were also measured in the peripheral system of these animals. The rats were exposed to $CdCl_2$ (2.5 mg / kg) and quercetin (5, 25 or 50 mg / kg) by gavage (1 ml/kg) for 45 days. Hence, the animals were divided into eight groups (n = 10-14): saline/control, saline/Querc 5 mg/kg, saline/Querc 25 mg/kg, saline/Querc 50 mg/kg, Cd/ethanol, Cd/Querc 5 mg/kg, Cd/Querc 25 mg/kg and Cd/Querc 50 mg/kg. The groups treated with Cd and quercetin, received the antioxidant quercetin solution after 30 minutes of the administration of Cd solution. At the end of 45 days of the treatment the animals were submitted to training and behavioral tests. After, they were anesthetized by halothane inhalation, and blood collection was performed to set serum, plasma and peripheral lymphocytes apart. Then the animals were euthanized, with part of the brain being removed for analysis of the enzyme δ-ALA-D activity, while the other part of brain was dissected into, cerebral cortex, striatum, cerebellum, hippocampus and hypothalamus, for future enzymatic assays. The results showed that Cd is able to cross the blood brain barrier, therefore, although the amount of Cd accumulated in the different brain structures studied was low, it was significantly higher than in control. Simultaneous treatment of quercetin in Cd exposed animals was ineffective to decrease these levels of Cd. The Cd exposure caused impairment on learning and memory, besides causing an increase in the anxiogenic behavior type. Nevertheless, the treatment with quercetin prevented the undesirable effects caused by exposure to the metal in the anxiety and memory. In relation to enzymatic activities in the brain, it was observed that Cd exposure reduced AChE activity in cerebral cortex and hippocampus, while as activation of the enzyme was observed in hypothalamus. Furthermore, a decrease in the Na^+, K^+-ATPase enzyme activity in cerebral cortex,

hippocampus and hypothalamus was observed, as well as a decrease in the δ-ALA-D activity in total brain of Cd exposed animals. Interestingly, the quercetin co-administration in the Cd exposed animals prevented the changes in the activity of the enzymes AChE and Na^+, K^+-ATPase in different brain structures, though has not restored the δ-ALA-D enzyme activity. It was also observed an increase in ROS production, in lipid peroxidation, in protein oxidation, the levels of double stranded DNA and changes in the antioxidant system, such as, reduction in the glutathione reductase (GR) activity, levels of total thiols (T-SH) and reduced glutathione (GSH), and an increase in the glutathione S-transferase (GST) enzyme activity in cerebral cortex, hypothalamus and hippocampus of Cd exposed animals. Co-administration of quercetin in Cd exposed rats was able to prevent totally or partially the changes caused by metal exposure in oxidative stress parameters. It is suggested that quercetin is able to reduce the oxidative damage caused by exposure to these metal and subsequently restore the AChE and Na^+, K^+-ATPase activities, modulating cholinergic neurotransmission and improving cognitive processes. In relation to the peripheral system, there was an increase in the NTPDase, ADA, AChE, BuChE and MPO activities in Cd exposed rats. Based on these results it is possible to infer that the increase in NTPDase activity is a compensatory effect due to the increase in ATP levels in circulation. It is suggested that decreased levels of ACh are available in the circulation due to increase in the peripheral cholinesterase activity. When rats were treated with the quercetin, flavonoid was able to modulate the activities of these enzymes probably due to the anti-inflammatory property of the compound. Accordingly, it is suggested that quercetin prevents or eases the toxicity caused by exposure to metal due to its antioxidant and anti-inflammatory activities. Therefore, it is believed that the flavonoid may be a promising drug in alternative therapies against toxicity induced by the metal in the central nervous system and peripheral system.

Keywords: NTPDase, 5'-nucleotidase, ADA, AChE, Na^+, K^+-ATPase, inflammation process, neurotoxicity.

LISTA DE ILUSTRAÇÕES

INTRODUÇÃO

ARTIGO 1

ARTIGO 2

LISTA DE TABELAS

INTRODUÇÃO

ARTIGO 1

ARTIGO 2

LISTA DE ABREVIATURAS E SIGLAS

ACh – Acetilcolina

AChE – Acetilcolinesterase

AChRm – Receptores de acetilcolina muscarínicos

AChRn – Receptores de acetilcolina nicotínicos

ADA – Adenosina Desaminase

ADO – Adenosina

ADP – Nucleotídeo de Adenina Difosfato

AMP – Nucleotídeo de Adenina Monofosfato

ATP – Nucleotídeo de Adenina Trifosfato

BHE – Barreira Hematoencefálica

BuChE – Butirilcolinesterase

Cd - Cádmio

ChAT – Colina acetiltransferase

E-NPPs - Ecto-nucleosídeo pirofosfatase/fosfodiesterase

E-NTPDases - Ecto-nucleosídeo trifosfato difosfoidrolase

EROS – Espécies Reativas de Oxigênio

GR - Glutationa Redutase

GSH - Glutationa Reduzida

GST - Glutationa S-Transferase

INO – Inosina

IL-1α - Interleucina 1α

MPO – Mieloperoxidase

MTs – Metalotioneínas

SNC – Sistema Nervoso Central

SNP – Sistema Nervoso Periférico

TAChV– Transportadores de acetilcolina vesícular

TCH – Transportadores de colina

TNF-α - Fator de necrose tumoral

T-SH - Tióis Totais

δ -ALA-D - δ-desidratase aminolevulinato

LISTA DE ANEXOS

SUMÁRIO

1. INTRODUÇÃO

Os metais pesados são substâncias presentes no nosso dia a dia, devido, principalmente, ao processo de industrialização. Esses compostos possuem a característica de serem altamente reativos e bioacumuláveis (TAVARES, 1992). Entre os metais pesados encontrados no meio ambiente e utilizados industrialmente, o cádmio (Cd) é o que apresenta maior interesse clínico, uma vez que a intoxicação por este metal é de difícil tratamento (JONES e CHERIAN, 1990).

O elemento químico Cd foi descoberto por Friedrich Strohmeyer em 1817, e apresenta cor branca acinzentada, alta ductilidade e maleabilidade, pertencente ao grupo 12 da tabela periódica sendo, classificado como um metal de transição externa. 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 e LAUWERYS, 1984). É um elemento não essencial, encontrado na natureza como um composto mineral associado, geralmente, com outros elementos como, ao zinco, chumbo ou cobre, e em baixas concentrações (0,15 - 0,20 mg/kg^{-1}) (LINGREL, 1992, OSPAR, Commission, 2002).

A distribuição normal deste metal pode ser alterada, naturalmente, 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, com cerca de 820 toneladas/ano (OSPAR Commission, 2002). Além disso, atividades antropogênicas, como a queima de combustíveis fósseis, efluentes industriais, aplicação de fertilizantes e mineração, também alteram a distribuição do metal no meio ambiente (JARUP, 2003; BENAVIDES et al., 2005). Salienta-se que estudos relatam que o Cd é amplamente utilizado na fabricação de baterias recarregáveis, de pilhas, de esmaltes, de tinturas, de alguns tipos de soldas, em televisores e, ainda, em fertilizantes e cigarros (JARUP et al., 1998; OSPAR Commission, 2002; NORDBERG, 2009).

Durante o século XX, a produção, o consumo e a emissão de Cd no ambiente atmosférico, aquático e terrestre têm aumentado drasticamente, devido, principalmente, aos processos de industrialização, tais como os processos de galvanização e aplicações militares e aeroespaciais (OSPAR Commission, 2002;

NORDBERG, 2009). Por consequência, a contaminação do meio ambiente ocorre devido ao descarte inadequado de produtos contendo Cd, os quais são despejados, principalmente junto com o lixo doméstico ou em rios, por efluentes industriais contaminados. Desta forma, o Cd pode ser absorvido pelos tecidos animais e vegetais, contaminando os ecossistemas terrestre e aquático, entrando, desta maneira, na cadeia alimentar (JARUP et al., 1998).

A maior fonte antropogênica de liberação de Cd na atmosfera é a queima de combustíveis fósseis e a incineração de lixos municipais. Já as concentrações do Cd em solos não poluídos são variáveis, dependendo das fontes minerais e do material orgânico. Na crosta terrestre, o Cd é encontrado em baixas concentrações, podendo alcançar níveis de 1 mg/kg, enquanto que em solos de origem vulcânica estes valores podem ser na faixa de 4,5 mg/kg (OSPAR Commission, 2002). Já em países com solos altamente poluídos por Cd, como a China e a França, as concentrações de Cd chegam a cerca de 100 mg/kg (ALLOWAY e STEINNES, 1999; KABATAPENDIAS e PENDIAS, 2001). Em relação ao nível de Cd nas águas, há referências que relatam concentrações de Cd, em mar aberto, entre 0,01 a 0,1 µg/L, e em águas profundas, valores maiores que nas águas superficiais, devido à captação do metal pelos organismos aquáticos. Outras referências relatam valores próximos ou maiores que 1 mg/L de Cd em águas oriundas de locais próximos a depósitos de minerais (ATSDR, 1997). Ressalta-se que o limite permitido de concentração de Cd na água potável é em torno de 5 µg/L (JARUP et al., 1998). Em relação à concentração de Cd no ar, sabe-se que a população está exposta, geralmente, a valores menores que $5x10^{-6}$ mg/m^3. Porém, concentrações de até $5x10^{-4}$ mg/m3 foram detectadas no ar em áreas próximas de locais com atividades emissoras de Cd (AZEVEDO e CHASIN, 2003).

Vários fatores podem influenciar a toxicidade do Cd no organismo. Quando se trata da exposição humana ao metal, deve-se considerar como rota o estilo de vida de cada pessoa, uma vez que esses fatores afetam o nível de Cd absorvido, bem como a toxicidade causada pelo mesmo, que pode variar conforme a via de contaminação, dose, forma química, duração da exposição e idade do exposto ao Cd (CASALINO et al., 1997). Há três formas principais de exposição ao Cd: a exposição cutânea, a gastrointestinal e a pulmonar, sendo que as principais fontes

de intoxicação humana pelo metal são ocupacionais, alimentares e pelo tabagismo (GODT et al., 2006; BERNARD, 2008).

Ressalta-se que a dieta é a mais importante fonte de exposição ao Cd na população não fumante (WHO, et al., 1992). Algumas pesquisas 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 (JARUP et al., 1998). Assim, considerando-se uma taxa de absorção gastrointestinal de 5%, acredita-se que cerca de 0,4 a 2,0 µg de Cd sejam retidos diariamente pelas pessoas a partir da alimentação (WHO, 1992).

A absorção gastrointestinal do Cd pode ser influenciada por fatores nutricionais. Uma baixa ingestão de cálcio, ferro, zinco e cobre pode, por exemplo, aumentar a absorção gastrointestinal do metal (NORDBERG et al., 1985; FOX, 1988). Apesar da baixa absorção gastrintestinal do Cd, a exposição oral assume importantes proporções devido à bioacumulação do metal na cadeia alimentar. Devido à meia vida biológica longa, em torno de 30 anos, e a baixa taxa de excreção do Cd no organismo, o metal torna-se prejudicial ao meio ambiente e para os seres humanos (MORSELT, 1991; WHO, 1992; SANTOS et al., 2006; BORGES et al., 2008), pois pode acumular-se em vários órgãos, como o fígado, rins, pulmões, ovários, ossos, testículos, encéfalo e órgãos linfoides, como baço e timo (SANTOS et al., 2004; SANTOS et al., 2005a; SANTOS et al., 2005b; SANTOS et al., 2006; LUCHESE et al., 2007; BORGES et al., 2008; GONÇALVES et al., 2012a).

No sangue, a meia-vida biológica do Cd é estimada em 2 a 3 meses. Desta maneira, a concentração do metal na circulação sanguínea é um indicador útil de exposição ao metal durante os últimos meses. Geralmente é encontrado em baixas concentrações abaixo de 1 µg/L em individuos não fumantes e alguns microgramas por litro em fumantes. Em individuos expostos ao Cd em longo prazo, o nível sanguineo de 10 µg de Cd/L levará a um acúmulo do metal principalmente nos rins (WHO, 1980). Portanto, uma exposição crônica ao Cd produz dano renal, osteotoxicidade, neurotoxicidade, infertilidade, enquanto que uma intoxicação aguda por Cd produz, primariamente, dano hepático e testicular (RIKANS e YAMANO, 2000).

O Cd, quando absorvido pelo trato gastrointestinal, é transportado pelo sangue ligado à albumina até o fígado, onde parte do metal é excretado na bile ligada à glutationa (NORDBERG et al., 1977). Entretanto, sob condição de exposição mais prolongada, a excreção na bile diminui e o metal induz a produção de proteínas de baixo peso molecular, as metalotioneinas (MTs), as quais se ligam formando o complexo Cd-MT que é, lentamente, liberado na circulação sistêmica (TOHYAMA e SHAIKH, 1981; JARUP et al., 1998). No plasma, este complexo é filtrado pelos glomérulos renais e liberado na urina. Seguidamente, o complexo Cd-MT é reabsorvido pelos túbulos renais por endocitose absortiva mediada pelo transportador ZIP8 na superfície apical das células tubulares renais (BERNARD et al., 1984). As lisozimas e as células tubulares degradam rapidamente este complexo, e liberam os íons Cd livre no citoplasma. O metal liberado se liga a MTs renais preexistentes ou àquelas recentemente sintetizadas pelas células tubulares (CHERIAN, 1978). Contudo, quando a quantidade de Cd presente no córtex renal excede a capacidade de ligação às MTs, os íons Cd liberados podem causar danos nos tecidos (NOMIYAM e NOMIYAMA, 1986), provavelmente pela geração de espécies reativas de oxigênio (EROs), uma vez que o Cd não participa diretamente de reações de oxidação-redução (Figura 1) (HASSOUN e STOHS, 1996; CASALINO et al., 1997).

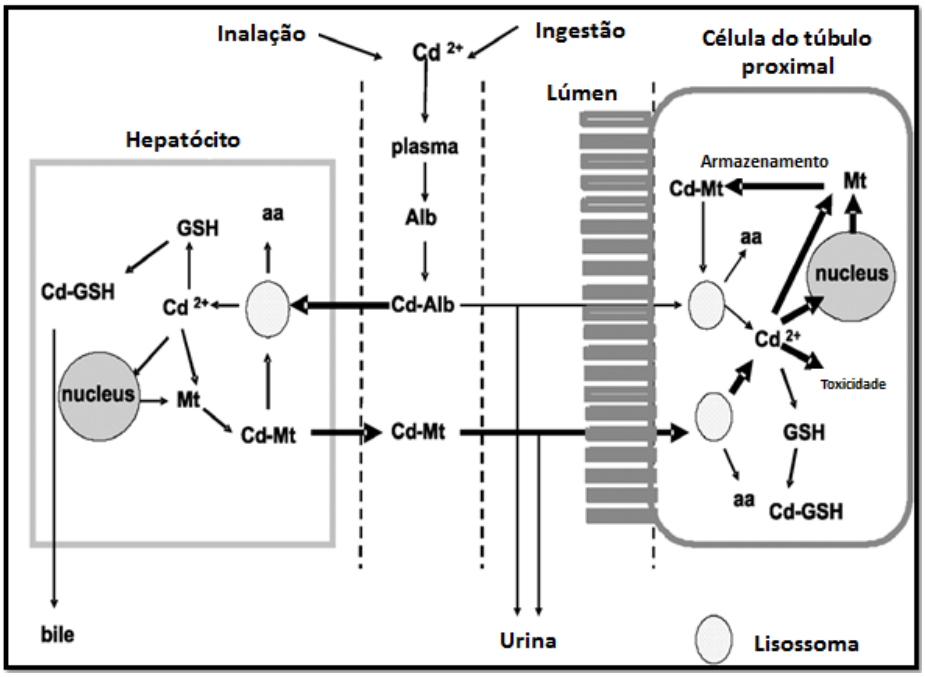


Figura 1: Esquema Ilustrando o mecanismo responsável pela acumulação seletiva de Cd nas células tubulares proximais. Alb: albumina, Mt: metalotioneína, GSH: glutationa; aa: aminoácido. Adaptado de BERNARD (2008).

Os mecanismos moleculares de toxicidade do Cd ainda não estão bem definidos. Contudo, sabe-se que uma das consequências da exposição ao Cd é a geração de estresse oxidativo por meio de um desequilíbrio entre o sistema antioxidante e as EROs, já que estudos demonstraram um aumento dos níveis de lipoperoxidação, de carbonilação proteica, uma inibição ou redução da atividade de enzimas antioxidantes como catalase, a superóxido dismutase, a glutationa redutase, a glutationa peroxidase e enzimas de reparo do DNA induzido pelo metal (Figura 2) (CASALINO et al., 1997; SANTOS et al., 2004; SANTOS et al., 2005a; SANTOS et al., 2005b; SANTOS et al., 2006; HALLIWELL e GUTTERIDGE, 2007; LUCHESE et al., 2007; BORGES et al., 2008). Com a instalação do estresse oxidativo vários processos fisiológicos são alterados levando à uma série de danos, como mutações, deleções, apoptose, neurotoxicidade, neurodegeneração, dano às membranas, entre outras (LÓPES et al., 2006; MÉNDEZ-ARMENTA e RÍOS, 2007). 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 encéfalo) sofreram alterações enzimáticas e aumento da peroxidação lipídica devido à toxicidade do Cd.

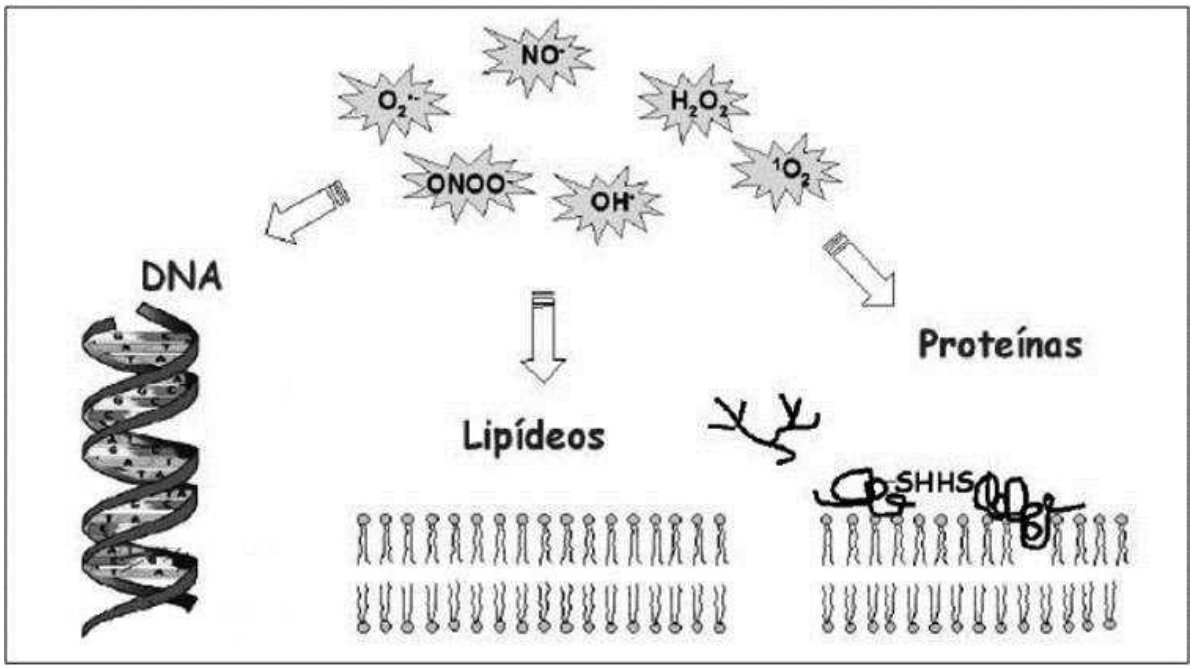


Figura 2. Dano oxidativo em macromoléculas biológicas (TORRES, 2003).

Sabe-se que o Sistema Nervoso Central (SNC) é protegido de muitos efeitos tóxicos por uma barreira anatômica denominada barreira - hematoencefálica (BHE) (MÉNDEZ-ARMENTA e RÍOS, 2007). Devido à permeabilidade seletiva da BHE, baixas quantidades de Cd podem chegar ao encéfalo. Entretanto, concentrações mais elevadas podem ser alcançadas se um veículo for empregado, como, por exemplo, o etanol. Devido à capacidade deste veículo se difundir através das membranas biológicas, ele permite uma maior penetração do metal através BHE (MÉNDEZ-ARMENTA et al., 2001; MÉNDEZ-ARMENTA e RÍOS, 2007). Além disso, o encéfalo, em especial, é mais susceptível à lipoperoxidação devido a sua alta utilização de oxigênio, abundante suprimento de ácidos graxos poli-insaturados, defesa antioxidante deficiente, bem como, alto conteúdo de metais de transição, como o cobre e o ferro (CALABRESE et al., 2000).

Outro possível mecanismo de entrada de íons de Cd nos neurônios é através dos canais de cálcio (Figura 3) (KUMAR et al., 1996). Consequentemente, supõe-se que, uma vez dentro das células, o Cd induz a uma diminuição na atividade de antioxidantes, produzindo um aumento de radicais livres. Esta produção de radicais livres é capaz de causar uma saturação nos lipídeos de diversas áreas das regiões do encéfalo resultando na lipoperoxidação, que causa alteração na fluidez da membrana e nas concentrações intracelulares de cálcio. Acredita-se que este aumento intracelular de Ca^{2+} cause o rompimento das membranas (celular e mitocondrial) e, assim, seja a principal causa de morte celular (ORRENIUS e NICOTERA, 1994; CASALINO et al., 1997).

Por outro lado, estudos têm demonstrado que a exposição ao Cd causa alterações na resposta imune humoral e mediada por células, bem como um aumento dos níveis de citocinas pró-inflamatórias, como o fator de necrose tumoral (TNF-α), interleucina 1α (IL-1α), e interleucina-6 (IL-6) (LAFUENTE et al., 2003). Além disso, sabe-se que o Cd causa alterações na atividade de importantes enzimas, como a acetilcolinesterase (AChE), δ - aminolevulínico desidratase (δ - ALA-D) e ATPases (LUCHESE et al., 2007; GONÇALVES et al., 2012b, GONÇALVES et al., 2013; ABDALLA et al., 2013), que podem levar a uma disfunção celular e ao agravamento da neurotransmissão sináptica (HOLLOWAY e THOR, 1988b; PROVIAS et al., 1994; MENDEZ ARMENTA et al., 2001), bem como produzir déficits

de atenção, aprendizagem e memória além de aumentar o comportamento agressivo e ansiogênico (HOLLOWAY e THOR, 1988b,a; TECARIOL et al., 2011).

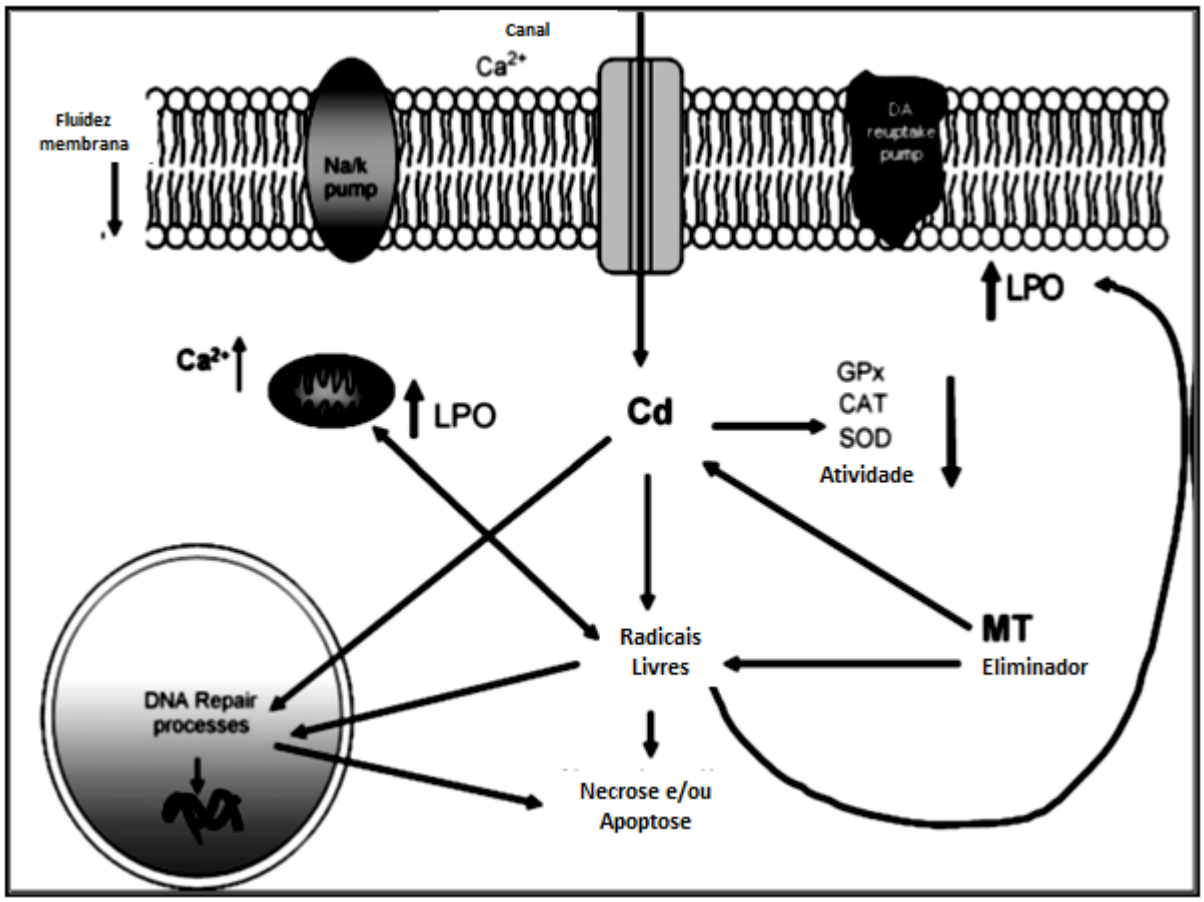


Figura 3: Efeito do Cd nos neurônios. Adaptado MENDEZ-ARMENTA e RÍOS (2007).

Atualmente, tem-se direcionado muita atenção para o efeito da exposição ao Cd na atividade da AChE modulando o sistema imune e o SNC (ZARROS et al., 2013; GONÇALVES et al., 2012a,b). Estudos *in vitro* e *ex vivo* relataram os efeitos do Cd na atividade da AChE em diferentes modelos experimentais, mas estes resultados são controversos e podem ser explicados de acordo com a dose, veículo, via e a duração de administração de Cd (ZARROS et al., 2013; GONÇALVES et al., 2010).

Ressalta-se, aqui, que recentes estudos realizados pelo nosso grupo de pesquisa relatam a toxicidade do Cd no encéfalo e também no sistema imunológico (GONÇALVES et al., 2012a,b; ABDALLA et al., 2013; GONÇALVES et al., 2013). Estas proposições foram confirmadas e reafirmadas em estudos recentes desenvolvidos em nosso laboratório. Gonçalves et al., (2010), observou que a

administração crônica de cloreto de Cd (2 mg/Kg de $CdCl_2$) leva à uma diminuição na atividade da AChE no hipocampo, no cerebelo e no hipotálamo de ratos. Além disso, Gonçalves et al., (2012) observou que a exposição ao cloreto de Cd (2 mg/Kg de $CdCl_2$) causa uma diminuição na atividade da AChE no sangue total, bem como em linfócitos, além de uma diminuição da atividade da butirilcolnesterase (BuChE) no soro. Por outro lado, Abdalla et al., (2013) observou que a administração crônica de 2,5 mg de $CdCl_2$ aumentou a atividade da AChE em sinaptossomas do córtex cerebral de ratos.

Sabe-se que o sistema colinérgico é uma das mais importantes vias modulatórias do sistema nervoso periférico (SNP) e do SNC (DECARRIES et al., 1997; DESCARRIES et al., 1997; SOREQ et al., 2001; PRADO et al., 2002). Há aproximadamente cem anos atrás, a acetilcolina (ACh) foi proposta como o agente químico responsável pela transmissão nervosa na sinapse, a área de conexão entre um neurônio e sua célula alvo. Desde a comprovação de que a ACh exerce um papel fundamental no funcionamento do sistema nervoso, a transmissão colinérgica se tornou um dos campos de maior importância para a neurociência (ANGLADE e LARAH-GODINOT, 2010). Vários trabalhos demonstraram que, no SNC, esta molécula atua agindo nos processos de aprendizagem e de memória, assim como no controle locomotor e no fluxo sanguíneo cerebral (KIEHN, 2006; DEIANA et al., 2011; HUT e VAN DER ZEE, 2011; KLINKERBERG et al., 2011). Além disso, estudos também tem salientado a importância da ACh circulante como um modulador do sistema imune, uma vez que a medida das atividades das enzimas AChE e BuChE têm sido descritas como marcadores fidedignos de inflamação sistêmica de baixo grau (DAS, 2007).

A histoquímica da sinapse colinérgica nasceu em 1949, quando Koelle e Friedenwald localizaram a atividade da AChE *in situ* a partir de uma ACh artificial. A partir daí, os outros componentes do sistema também foram caracterizados (ANGLADE e LARAH-GODINOT, 2010). Neste contexto, é relevante destacar os principais componentes que constituem o sistema colinérgico: a ACh, a colina-acetiltransferase (ChAT; EC 2.3.1.6), o transportador de colina (TCH), o transportador de acetilcolina vesicular (TAChV), os receptores de acetilcolina muscarínicos (AChRm) e nicotínicos (AChRn), e AChE (E.C. 3.1.1.7) (KAWASHIMA e FUJII 2000; SARTER e PARIKH, 2005).

A ChAT é a enzima responsável pela síntese da ACh a partir de acetil-coenzima A, um produto do metabolismo celular, e da colina, um importante produto do metabolismo dos lipídios da dieta (SOREQ e SEIDMAN, 2001; PRADO et al., 2002). Uma vez sintetizada, a ACh é transportada pelo TAChV, que armazena a ACh dentro de vesículas, até a sua liberação por exocitose. Ao ser liberada, a ACh se difunde na fenda sináptica e ativa os receptores específicos de ACh (AChRm ou AchRn) que são sensibilizados, causando a despolarização e a propagação do potencial de ação na célula pós-sináptica (KAWASHIMA e FUJII 2000; SARTER e PARIKH, 2005). Os receptores colinérgicos, AChRm e AChRn, transmitem os sinais por mecanismos diferentes (RANG et al., 2004). Até o momento, cinco subtipos de AChRm foram identificados (M1-M5). Estes receptores 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 e LUITEN, 1999), enquanto que os receptores nicotínicos são compostos por cinco subunidades conhecidas por α1, α2, β, γ e δ e atuam como canais iônicos regulados por ligante e localizam-se, predominantemente, nas sinapses ganglionares (ARIAS, 1998). Por fim, a ACh que permanece na fenda é hidrolisada em acetato e colina pela enzima AChE, presente na fenda sináptica, evitando, assim, uma “super-estimulação” pós-sináptica, modulando, portanto, a sinalização colinérgica (KAWASHIMA e FUJII 2000; SARTER e PARIKH, 2005). Parte da colina resultante da hidrólise é recaptada pelo TCH para o terminal pré-sináptico, onde poderá ser reutilizada na síntese de novas moléculas de ACh (Figura 4) (MESULAM et al., 2002).

Há diferentes tipos de colinesterases, que diferem por sua localização nos tecidos, afinidade pelo substrato e funções fisiológicas. As principais dessas colinesterases são a AChE e a butirilcolinesterase (BuChE) (MESULAM et al., 2002; COKUGRAS, 2003), as quais daremos ênfase, dentre todos os componentes do sistema colinérgico, pois foram alvos deste estudo.

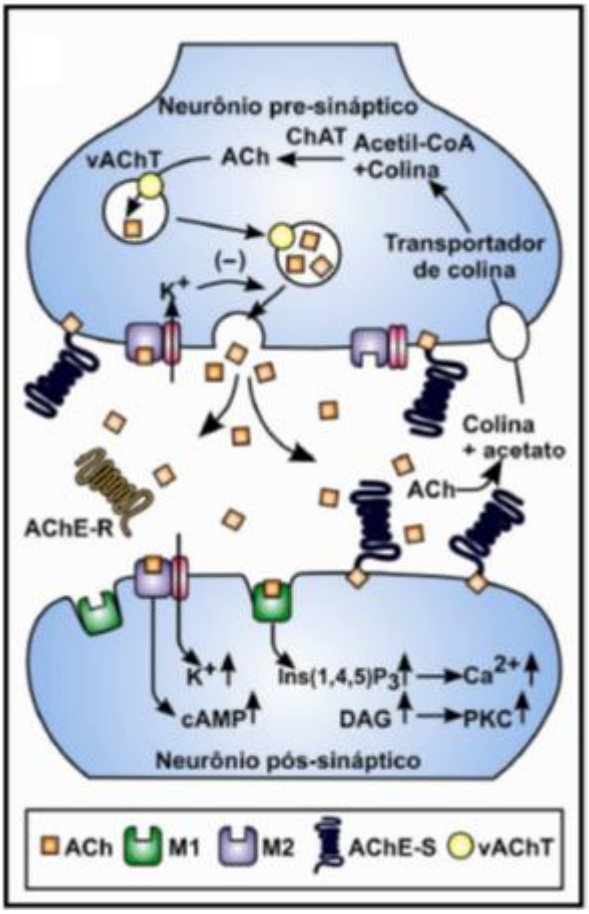


Figura 4: Esquema de uma sinapse colinérgica: Acetilcolina (ACh), acetilcolinesterase sináptica (AChE-S), receptor muscarínico do tipo 1 (M1), receptor muscarínico do tipo 2 (M2), transportador de ACh vesicular (TAChV). Adaptado de Soreq & Seidman, (2001).

A AChE, ou colinesterase verdadeira, é predominantemente encontrada no encéfalo (em concentrações 10 vezes mais abundante que a BuChE), junção neuromuscular e eritrócitos (COKUGRAS, 2003). Pode ser classificada como uma enzima serina hidrolase regulatória, que é extremamente importante e que hidrolisa, preferencialmente, ésteres com grupamento acetil, como a ACh. Deste modo, a enzima AChE modula a concentração de ACh tanto na sinapse colinérgica quanto na junção neuromuscular, finalizando, assim, a transmissão do impulso (SOREQ e SEIDMAN, 2001). Esta enzima apresenta uma atividade muito versátil, podendo desempenhar os mais variados papéis no organismo, uma vez que o sistema colinérgico abrange totalmente o mesmo (TAYEBATI et al., 2002).

A AChE está presente em diversas formas moleculares no organismo, como a globular e a assimétrica, dependendo da sua conformação espacial (Figura 5). A forma globular apresenta-se como uma montagem homomérica de subunidades catalíticas que aparecem como monômeros, dímeros ou tetrâmeros, originando, respectivamente, as seguintes formas globulares (G): G1, G2 e G4, sendo, cada uma delas, predominante em alguns tecidos. Outra classe de AChE é a forma assimétrica, que apresenta-se como uma montagem heteromérica das subunidades estrutural e catalítica, onde a ligação através de pontes dissulfeto de uma molécula tríplice helicoidal de colágeno a um, dois, ou três tetrâmeros catalíticos resultam nas formas estruturais assimétricas A4, A8 ou A12, respectivamente (MASSOULIÉ et al., 1993).

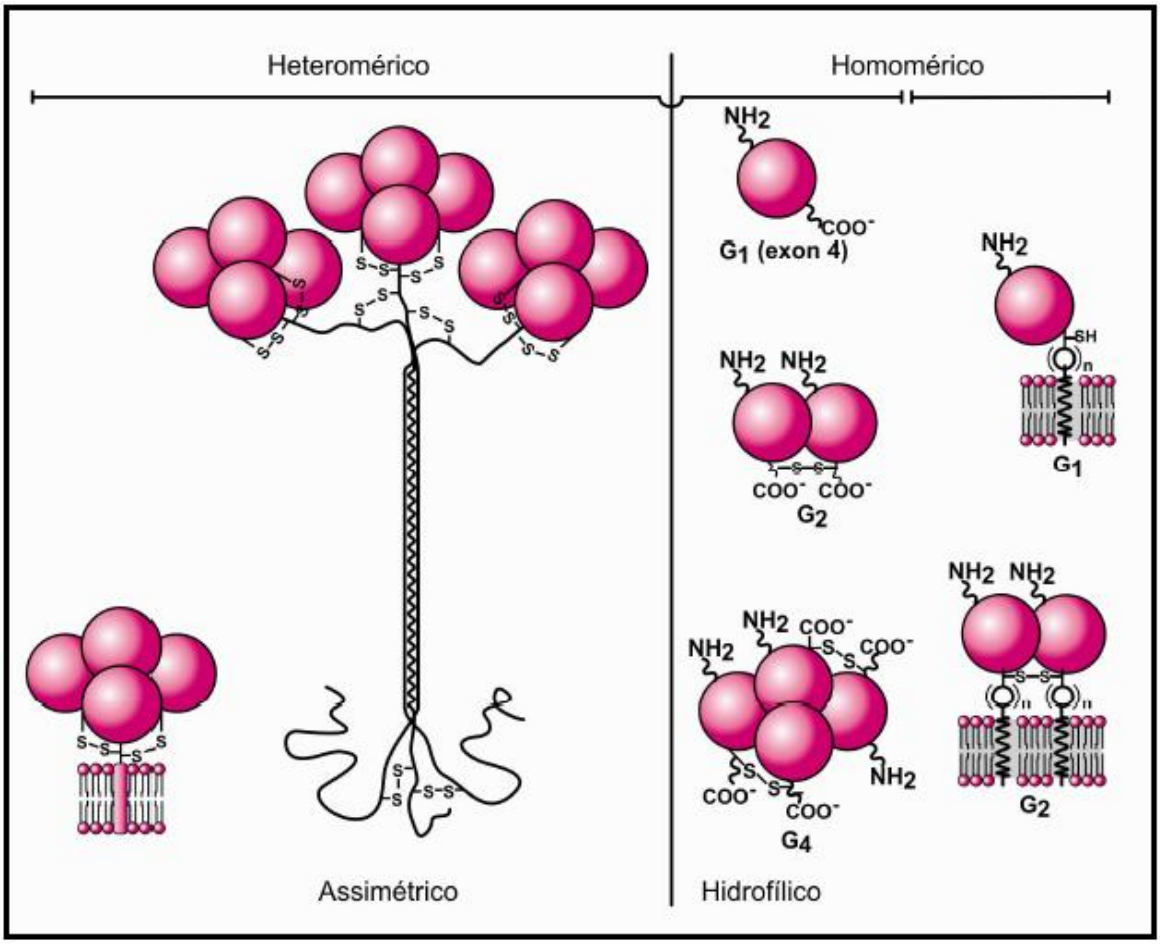
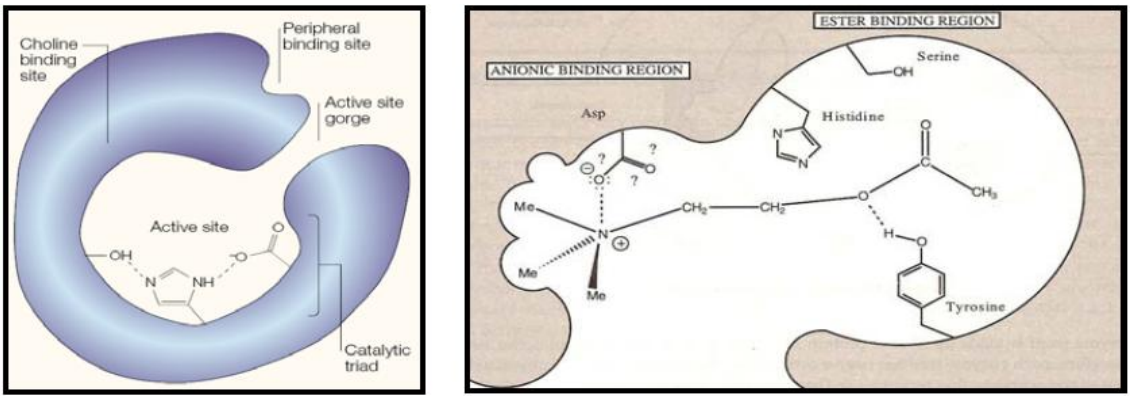


Figura 5: Isoformas da acetilcolinesterase. Estrutura Assimétrica (A12) e molecular (G1, G2, G4) da AChE. (http://www.chemistry.emory.edu/ach_inactivation.htm).

As formas globulares podem ser solúveis ou ancoradas à membrana por sequências de aminoácidos hidrofóbicos, enquanto que as formas assimétricas

estão incluídas na matriz extracelular por uma cauda colagênica (ColQ) (ALDUNATE et al., 2004). Contudo, todas essas formas apresentam uma alta taxa de hidrólise enzimática, de aproximadamente 5.000 moléculas de ACh/s (JÓSÊ et al., 2009). As formas globulares encontram-se, predominantemente, no SNC, enquanto que as formas assimétricas são encontradas, principalmente, no SNP e no músculo (RAKONCZAY et al., 2005). Além disso, a maior parte da AChE encontrada no tecido nervoso é do tipo G4, ligada à membrana (MASSOULIÉ et al., 1993).

A estrutura tridimensional da AChE demonstra que seu sítio ativo é encontrado no interior de um estreitamento semelhante a uma garganta (*gorge*), a uma profundidade de 20 Aº, alinhado com resíduos hidrofóbicos, os quais parecem ser importantes na orientação do substrato ao sítio ativo (Figura 6) (JOHNSON e MOORE, 1990). Este sítio ativo consiste de dois subsítios de ligação: um subsítio carregado negativamente ou aniônico, ao qual a cadeia de nitrogênio quaternário [-$N+(CH_3)_3$] da ACh carregada positivamente se liga, e um sítio esterásico contendo uma tríade catalítica formada por serina 203, histidina 447 e glutamato 334, o qual aloja o grupamento éster e carbonila da ACh (TAYLOR e BROWN, 1999).



(A) (B)

Figura 6: Sítio catalítico da AChE. A) Ilustração do sítio esterásico contendo a tríade catalítica, externamente o sítio periférico (PAS) Adaptado de Soreq & Seidman (2001). B) Interação do substrato com o sítio esterásico da AChE. Adaptado de Patrick (2001).

Na borda ou superfície 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 composto

bi-quaternários (BOURNE et al., 2005). Tem sido proposto que este sítio periférico possa estar envolvido na ação de determinados inibidores da enzima ou na inibição por excesso de substrato (NUNES-TAVARES et al., 2002; SILMAN e SUSSMAN, 2005). A ACh é hidrolisada a partir de sua ligação ao resíduo de serina no sítio ativo da enzima, formando o intermediário acetil-AChE, liberando colina. Em sequência, há a hidrólise deste intermediário, liberando acetato, e permitindo o "*turnover*" da enzima (SOREQ e SEIDMAN, 2001).

Diferentemente da AChE, a BuChE é uma enzima sérica com ampla distribuição no organismo (KUTTY, 1980; CHATONNET e LOCKDRIDE, 1989; ORESVIC e KUNEC-VAJIC, 1992), encontrada, principalmente, no soro, rins, fígado, intestino, coração e pulmão, e que possui uma distribuição neuronal muito mais restrita em relação a AChE (MESULAM et al., 2002; COKUGRAS, 2003). Além disso, sabe-se que a BuChE encontrada no encéfalo não deriva do soro, mas é produzida no próprio tecido encefálico (PRODY et al., 1987; McTIERNAN et al., 1987). A BuChE, ou pseudocolinesterase, hidrolisa, além da ACh, outros tipos de ésteres de colina, como a butirilcolina, os relaxantes musculares succinilcolina, mivacúrio, ésteres alifáticos que não contêm colina, assim como o ácido acetilsalicílico e muitas drogas, como a cocaína e a procaína (BOECK et al., 2002).

Além das colinesterases, a enzima mieloperoxidase (MPO) também contribui para a regulação do processo inflamatório (HAMPTON et al., 1998; REGASINI et al., 2008). A MPO é uma hemeproteína liberada a partir de leucócitos no local da lesão tecidual, e reflete, desta maneira, a ativação de ambos os neutrófilos e linfócitos (HANSSON et al., 2006). Esta enzima é responsável pela geração de ácido hipocloroso, responsável por oxidar compostos endógenos, como microrganismos, alguns medicamentos e toxinas. Basicamente, a MPO converte o peróxido de hidrogênio em ácido hipocloroso e água. Além disso, o ácido hipocloroso gera produtos secundários como o oxigênio singlete e o radical hidroxila, agentes oxidantes que possuem amplas ações biológicas em eventos como apoptose e condução do processo inflamatório (Figura 7) (REGASINE et al., 2008).

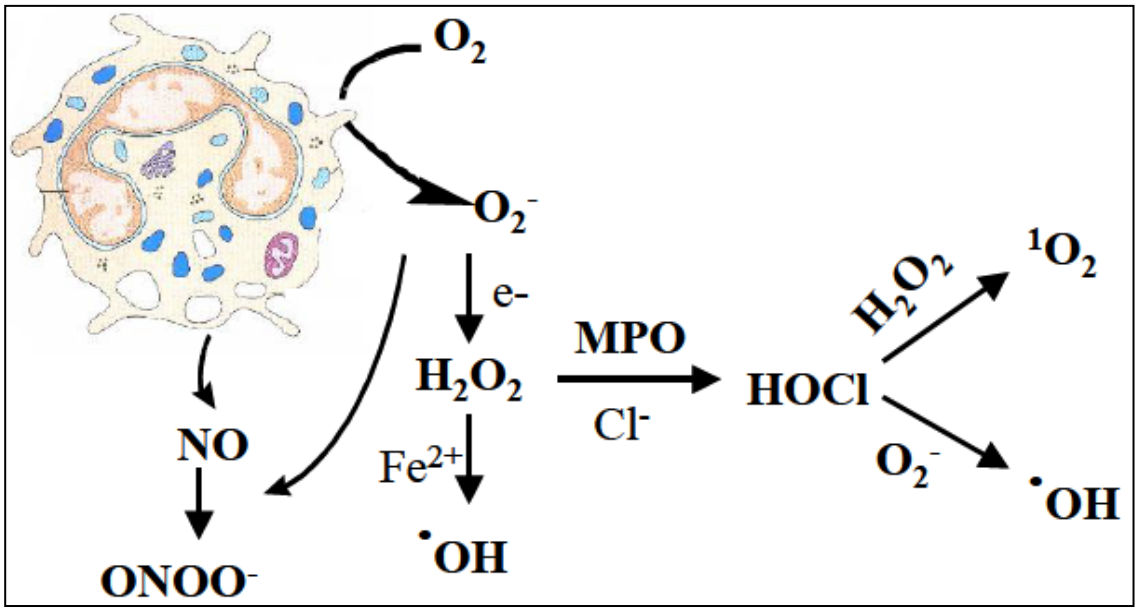


Figura 7: Ação da mieloperoxidase liberada a partir do leucócito.

Outro sistema importante para a regulação da neurotransmissão e da resposta imune é o sistema purinérgico. A sinalização purinérgica é mediada, principalmente, pelos nucleotídeos e nucleosídeo de adenina extracelulares, pelos receptores através dos quais estes nucleotídeos exercem seus efeitos, e pelas ectoenzimas (YEGUTKIN, 2008).

Os nucleotídeos extracelulares de adenina trifosfato (ATP) e nucleotídeo monofosfato (AMP), a nucleotídeo difosfato (ADP) e o nucleosídeo adenosina (ADO) são considerados importantes moléculas sinalizadoras, mediando seus efeitos através de receptores purinérgicos localizados na superfície celular (ILLES e RIBEIRO, 2004) (Figura 8). Em condições fisiológicas, os nucleotídeos e nucleosídeos estão presentes no meio extracelular em baixas concentrações, normalmente em quantidades nanomolares, podendo chegar até as quantidades micromolares em determinadas situações (DI VIRGILIO, 2001). Entretanto, as concentrações de ATP, ADP, AMP e ADO são influenciadas por vários fatores, tais como: secreção e/ou lise celulares, efeito da diluição no espaço extracelular e pela ação catalítica das ecto-nucleotidases (RATHBONE et al., 1999; MALMSJO et al., 2000).

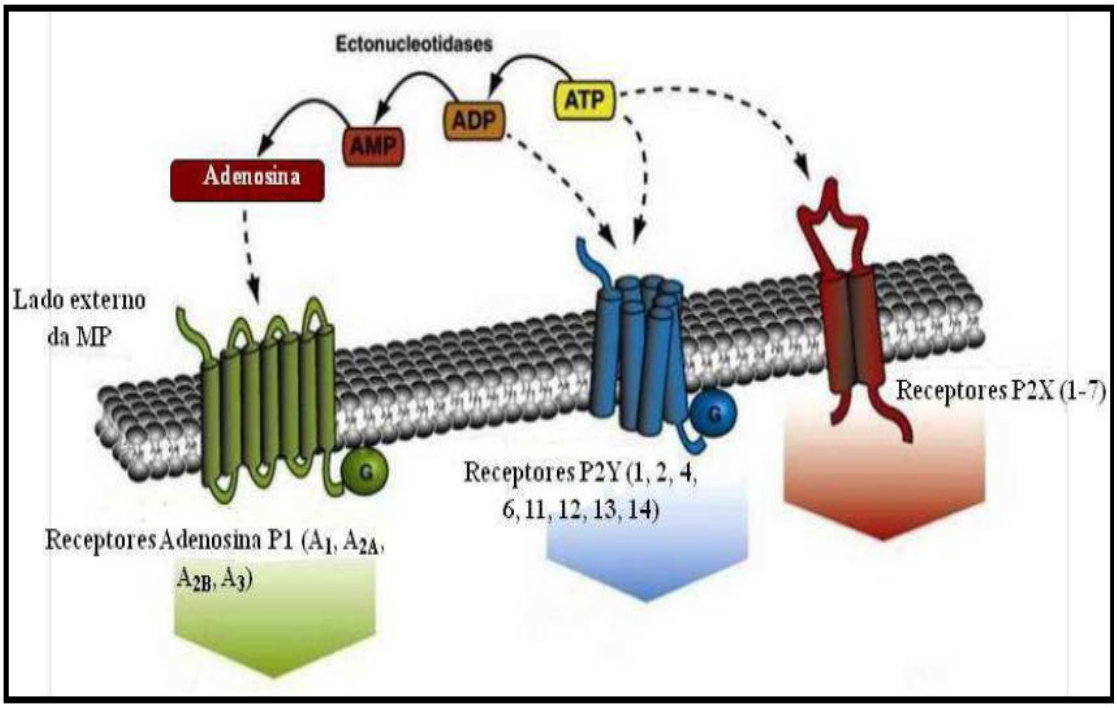


Figura 8: Receptores envolvidos na resposta fisiológica dos nucleotídeos e nucleosídeos extracelulares de adenina (http://ars.sciencedirect.com/content/image/1-s2.0-S0005273612000065-gr1.jpg)

Os níveis extracelulares de nucleotídeos e nucleosídeos de adenina, e a consequente sinalização purinérgica por eles induzida através dos receptores, são regulados através de uma variedade de enzimas localizadas na superfície celular ou solúveis no meio intersticial, denominadas ecto-nucleotidases (ZIMMERMANN et al., 2007). Este conjunto de enzimas inclui a família das E-NTPDases (ecto-nucleosídeo trifosfato difosfoidrolase), a família das E-NPPs (ecto-nucleosídeo pirofosfatase/fosfodiesterase), as fosfatases alcalinas, a ecto-5'-nucleotidase e a adenosina desaminase (ADA) (ROBSON et al., 2006; YEGUTKIN, 2008).

Estas enzimas atuam em conjunto, formando uma cadeia enzimática que tem início com a ação da E-NTPDase e da E-NPP, que catalisam a hidrólise de ATP e do ADP formando AMP (ZIMMERMANN et al., 2007). A seguir, a enzima 5'-nucleotidase hidrolisa a molécula de AMP formando a adenosina, que, posteriormente, sofre desaminação pela ação da ADA, formando a inosina (INO) (Figura 9) (YEGUTKIN, 2008).

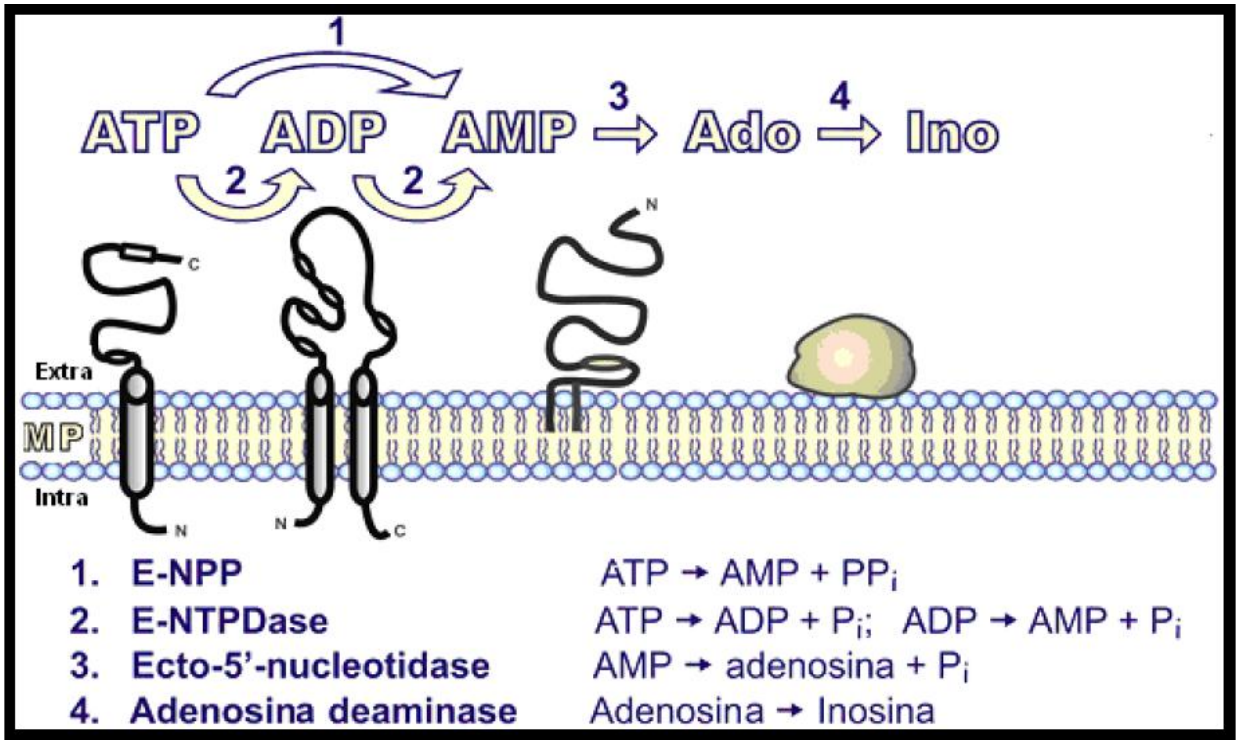


Figura 9: Enzimas envolvidas na degradação extracelular de nucleotídeos e nucleosídeos de adenina. Adaptado de Yegutkin (2008).

Sabe-se que E-NTPDase é o termo genérico para designar uma família de enzimas presentes na membrana plasmática de diversos tecidos, que catalisam a hidrólise de nucleotídeos difosfatos e trifosfatos até suas formas monofosfato (ZIMMERMANN et al., 2007). Atualmente, oito membros da família das E-NTPDases já foram identificadas (Figura 10), as quais são denominadas E-NTPDases 1-8 e diferem entre si quanto à especificidade ao substrato (NDP ou NTP) e a cátions divalentes (ROBSON et al., 2006).

A NTPDase 1 degrada o ATP e ADP em iguais proporções (1:1), diferindo da NTPDase 2, que hidrolisa preferencialmente o ATP (BALZ et al., 2003). As NTPDases 3 e 8, por sua vez, apresentam uma preferência maior pelo substrato ATP em relação ao ADP (ROBSON et al., 2006). Além disso, ao contrário da NTPDase 1 e 2, as NTPDases 3 e 8 são, preferencialmente, ativadas pelo Ca^{2+} em relação ao Mg^{2+} (BIGONNESSE et al., 2004; LAVOIE et al., 2004; VORHFF et al., 2004). Estas diferenças entre os subtipos nas propriedades catalíticas podem ser devido à diferenças na sequência de aminoácidos, como, também, nas estruturas secundária, terceária e quaternária das enzimas (GRINTHAL e GUIDOTTI, 2004).

Além das diferenças quanto à preferência a substratos e a cátions divalentes, estas enzimas apresentam diferenças em relação à distribuição tecidual e localização celular. Um grupo de E-NTPDases (NTPDase 1, 2, 3, 8) estão localizadas na superfície das células, com um sítio catalítico extracelular. Outro grupo é composto pelas enzimas NTPDase 4, 5, 6 e 7 e caracterizam-se por apresentar localização intracelular (ZIMMERMAN, 2001; ROBSON et al., 2006).

Tem-se relatado que a E-NTPDase é expressa em sinaptossomas isolados, bem como em cultura de neurônios primários de córtex cerebral, astrócitos e células do sistema imune (BATTASTINI et al., 1991; WANG et al., 2007). Estudos imuno-histoquímicos têm demonstrado que esta enzima é amplamente distribuída no encéfalo de ratos, encontrando-se presente em neurônios de córtex cerebral, hipocampo, cerebelo, células gliais e células endoteliais (WANG e GUIDOTTI, 1998).

A NTPDase 1 (ATP difosfoidrolase, Apirase, Ecto/CD39) foi a primeira enzima da família E-NTPDase a ser descrita, e já foi identificada em uma ampla variedade de organismos, tais como: protozoários, plantas, invertebrados, peixes, aves, ratos e humanos (ANICH et al., 1990; VASCONCELOS et. al., 1993; FRASSETO et al., 1995; MATOS et al., 2001; SCHETINGER et al., 2001; RICO et al., 2003; LEAL et al., 2005). Está ancorada na superfície celular através de duas regiões transmembranas próximas ao grupamento amino e carboxi terminal, com o seu sítio catalítico voltado para o meio extracelular (Figura 10). Esta enzima hidrolisa tanto ATP como ADP, formando AMP na presença de íons Ca^{2+} e Mg^{2+} (ZIMMERMAN, 2001).

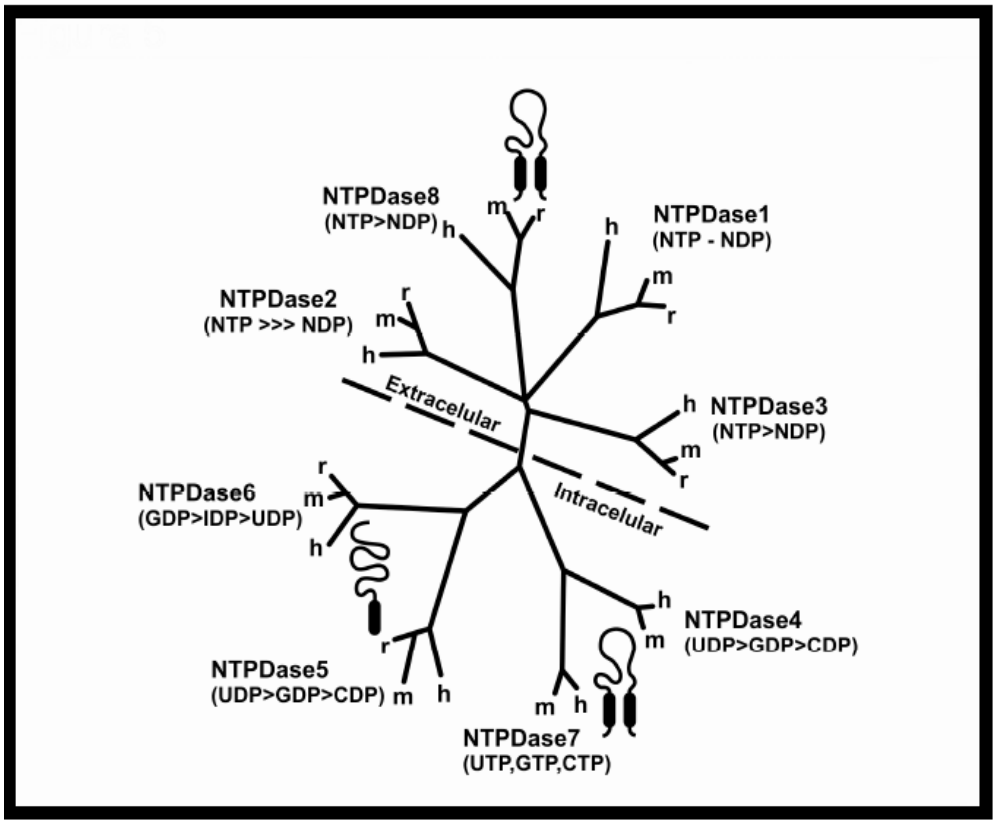


Figura 10: Membros da família da E-NTPDase (Robson et al*.,* 2006).

Já a ecto-5’-nucleotidase é uma glicoproteína ancorada à membrana plasmática via uma molécula de glicofosfatidil inositol (GPI), com seu sítio catalítico voltado para o meio extracelular (Figura 11). Por outro lado, formas solúveis e clivadas desta enzima também já foram descritas (ZIMMERMANN, 2001; HUNSUCKER et al., 2005). Ela é amplamente encontrada em uma variedade de tecidos, como rins, fígado, encéfalo, pulmão, endotélio vascular, plaquetas e células do sistema imune (COLGAN et al., 2006).

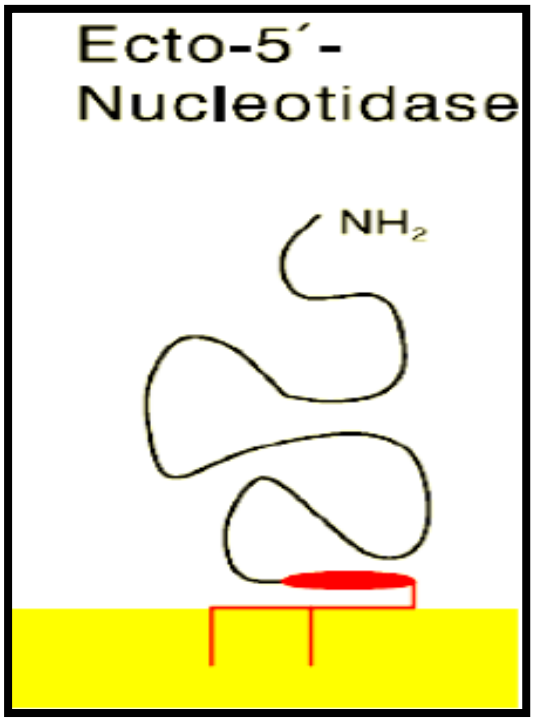


Figura 11: Estrutura da ecto 5'-nucleotidase ancorada a membrana plasmática via uma molécula de GPI. Adaptado de Zimmermann (2001).

A reação catalisada pela ecto 5'-nucleotidase é a desfosforilação de vários nucleotídeos 5'-monofosfatados, como CMP, IMP, UMP, GMP e AMP a seus respectivos nucleosídeos (ZIMMERMANN, 1996). No entanto, foi demonstrado que a 5´-nucleotidase hidrolisa mais eficientemente o AMP, sendo, por isto, considerada a principal enzima responsável pela formação de adenosina (ZIMMERMANN, 1996; ZIMMERMAN, 1998; ZIMMERMANN, 2001).

A enzima ADA também faz parte do conjunto de enzimas responsáveis pela degradação sequencial dos nucleotídeos e nucleosídeos de adenina (YEGUTKIN, 2008). É responsável por realizar a desaminação da ADO com consequente produção de inosina (INO), seu metabolito inativo, regulando, assim, as concentrações extracelulares deste nucleosídeo (FREDHOLM et al., 2005). A ADO pode ser sintetizada tanto no meio intracelular quanto extracelular, cujas ações são exercidas através de um grupo de receptores metabotrópicos classificados como A_1, A_{2A}, A_{2B}, A_3, sendo que a maioria do conhecimento sobre a distribuição e

funcionalidade dos purinoreceptores P1 corresponde a estudos referentes aos receptores A_1 e A_{2A} (FREDHOLM et al., 2001; BURNSTOCK, 2006).

Portanto, as ecto-enzimas, junto com os nucleotídeos e nucleosídeos de adenina, 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 e GUIDOTTI, 1998; CUNHA e RIBEIRO, 2000; DUNWIDDIE e MASINO, 2001), uma vez que o ATP hidrolisado por estas enzimas é, essencialmente, um neurotransmissor excitatório 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+} (ILLE e RIBEIRO, 2004). Já a ADO formada pela hidrólise do AMP, reação catalisada pela enzima 5'-nucleotidase, é capaz de regular a liberação de vários neurotransmissores, agindo tanto pré-sinapticamente quanto pós-sinapticamente (CUNHA, 2001; DNWIDDIE e MASIMNO, 2001; RIBEIRO et al., 2003). Além disso, a ação da ADO 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 e 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 no sistema imunológico. As enzimas NTPDase e 5'-nucleotidase são encontradas, também, 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).

Nos últimos anos, o papel da NTPDase, 5'-nucleotidase e ADA tem sido avaliado em várias condições patológicas e toxicológicas, como no diabetes (LUNKES et al., 2003; SCHMATZ ET AL., 2009a), 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., 2010 a,b) e na intoxicação por alumínio (KAIZER et al., 2007).

Além disso, alguns trabalhos foram realizados, também, *in vitro,* 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), demonstrando a importância destas no processo de neurotransmissão (ABDALLA et al., 2003; GONÇALVES et al., 2013). Abdalla et al., (2013) observou um aumento na atividade das enzimas NTPDase, 5'-nucleotidase e ADA no sinaptossoma do córtex cerebral de animais expostos cronicamente ao Cd (2,5 mg/kg durante 45 dias), sugerindo que o aumento na atividade destas enzimas poderia levar à conversão final de INO e, consequentemente, com a diminuição de moléculas neuroprotetoras, como o ATP e ADO, o qual poderia contribuir para os efeitos neurotóxicos do metal no SNC. Estes resultados estão de acordo com os observados por Gonçalves et al., (2013), que encontrou um aumento na atividade das enzimas NTPDase e 5'-nucleotidase em sinaptossomas do córtex cerebral de ratos expostos ao Cd (2 mg/kg) durante 30 dias.

Levando em consideração os dados acima mencionados, torna-se relevante o estudo da atividade das ecto enzimas do sistema purinérgico em diferentes tipos celulares de animais expostos ao Cd, bem como faz-se necessário associar a atividade dessas enzimas com outras respostas bioquímicas e comportamentais relacionadas à intoxicação por esse metal.

Neste contexto, destacamos a enzima Na^+, K^+-ATPase (E.C. 3.6.1.3), que também possui um papel importante na neurotransmissão sináptica por atuar na manutenção do gradiente eletrolítico de íons Na^+ e K^+, que contribuem para a homeostase intracelular de eletrólitos, além da manutenção do volume e do pH celular (LINGER, 1992; LINGER et al., 1994a,b). Sabe-se que uma diminuição na atividade ou expressão dessa enzima pode prejudicar, diretamente, a sinalização de

neurotransmissores, com consequências deletérias sobre a memória, o comportamento e a ansiedade (DOS REIS et al., 2002; MOSELEY et al., 2007). Além disso, estudos *in vitro* demonstram que inibidores da enzima, como a ouabaína, aumenta o influxo de Ca^{2+}, induz a liberação do glutamato por transporte reverso de Na^{2+} e causa toxicidade em neurônios do hipocampo (FJISAWA et al., 1965; LI e STYS, 2001; LEES et al., 1990).

Vários estudos demonstraram que certos íons metálicos bivalentes são potentes inibidores da enzima Na^+, K^+-ATPase no encéfalo (HEXUM, 1974; PRAKASH et al., 1973; RAJANNA et al., 1983; CHETTY et al., 1992; ANTONIO et al., 2002). Há relato de que a exposição ao Cd causou uma diminuição na atividade da enzima no córtex cerebral, no hipotálamo e ceberelo de animais expostos a tubérculos de batata com Cd, sugerindo que esta inibição da Na^+, K^+-ATPase poderia ser prejudicial para os neurônios, levando ao edema e degeneração celular (GONÇALVES et al., 2012).

Além da Na^+, K^+-ATPase, diversas enzimas podem ter suas atividades reduzidas pelo aumento na concentração de metais tóxicos no organismo. Esses metais podem ligar-se à grupos sulfidrílicos (-SH) e/ou substituir metais essenciais de algumas enzimas, alterando suas atividades catalíticas (CASALINO et al., 2000). Desta maneira, outra enzima importante a ser avaliada é a enzima δ-ALA-D, amplamente utilizada como marcador de intoxicação por vários metais, como o mercúrio (ROCHA et al., 2000), chumbo (RODRIGUES et al., 1996) e Cd (LUCHESE et al., 2007). A δ-ALA-D é uma metaloenzima que requer íons zinco para sua atividade catalítica máxima (JAFFE et al., 2000). Esta enzima catalisa a condensação assimétrica de duas moléculas do ácido δ-aminolevulínico (δ-ALA), formando o porfobilinogênio (PBG), em um dos passos iniciais da biossíntese do heme (GIBSON et al., 1955). A inibição da δ-ALA-D pode prejudicar a rota biossintética do heme (SASSA et al., 1989; GOERING, 1993) e resultar no acúmulo do substrato ALA, o qual pode estar relacionado com a superprodução de EROs (PEREIRA et al., 1992; BECHARA et al., 1993).

Estudos vêm sendo realizados na tentativa de reverter ou minimizar os efeitos causados pelo Cd, principalmente aos danos relacionados ao SNC e ao sistema imunológico. Destaca-se que o interesse pela prevenção e cura de doenças através da alimentação vem aumentando a cada dia, e cada vez com mais embasamento

científico. Neste contexto, um crescente número de estudos epidemiológicos tem associado o consumo de compostos flavonoides a uma variedade de efeitos benéficos para a saúde. Além da atuação antioxidante, já foi descrito que estes compostos possuem, ainda, diversas propriedades farmacológicas, tais como: propriedade anticarcinogênica, vasodilatadora, anti-inflamatória, imunoestimulante, antialérgica, vasoprotetora, neuroprotetora, entre outras (COOK e SAMMAN 1996; RICE-EVANS et al., 1995; MIDDLETON et al., 2000; CHO et al., 2003; PRIOR, 2003; KANG et al., 2005).

Os flavonoides são uma classe de compostos que diferem entre si pela sua estrutura química e características particulares (NIJVELDT, 2001). Apesar de apresentarem diferenças estruturais, todos incluídos nesta classe, apresentam uma organização genérica composta por 15 átomos de carbono reunidos em um núcleo fundamental tricíclico, ou seja, consistem de um esqueleto de difenil propano com dois anéis benzênicos (A e B) ligados a um anel pirano (C) (Figura 12) (AHLENSTIEL, 2003). Estima-se que existam em torno de 8.000 variedades de flavonoides, sendo que a estrutura de cada um, a partir de seu núcleo fundamental, é o que permite classificá-los em: antocianidina, flavanóis, flavanona, flavonas, flavonóis e isoflavonas (Tabela 1) (RICE-EVANS *et al.,* 1995; YAO *et al.,* 2004).

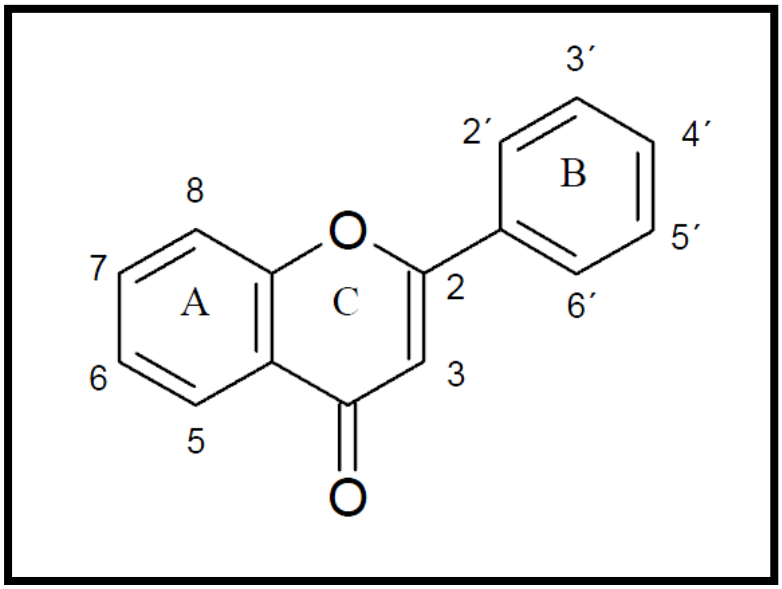


Figura 12: Núcleo genérico de um flavonóide. Os anéis A e B são aromáticos e o anel C é um heterociclo (COOK & SAMMMAN, 1996).

Os flavonoides compõem uma ampla classe de substâncias de origem natural e não podem ser sintetizados pelo metabolismo humano, fazendo com que sejam obtidos através da alimentação (MIDDLETON et al., 2000; CHO et al., 2003; PRIOR, 2003; KANG et al., 2005). Estes compostos são encontrados em frutas, legumes, vegetais, açúcares, grãos, vinho tinto, chás de ervas, café e chocolate (SCALBERT e WILLIAMSON, 2000; AHERNE e O'BRIEN, 2002). De acordo com Pierpoint (1986) o teor de flavonoides em alimentos consumidos diariamente é: 44 mg em cereais, 79 mg em batatas, 45 mg em grãos e nozes e 162 mg em vegetais e ervas. Além disso, sabe-se que maior parte dos flavonóides consumidos, aproximadamente 420 mg/dia, provém do cacau, da cola, do café, do chá preto, da cerveja e do vinho, com um adicional de 290 mg/dia provenientes de frutas e sucos.

Tabela 1. Sub-Classes dos flavonoides, características e fontes. Adaptada de (Pedriali, 2005).

Sub-Classes	Cor	Flavonóides representativos	Fontes
Antocianidina	Azul, vermelho, violeta	Cianidina	Frutas e flores
Flavanol	Incolor	Catequinas, epicatequinas	Maçãs, chá, cerveja
	Amarelo	Procianidina	Sucos de frutas, vinho
Flavanona	Incolor, amarelo	Hesperidina, Naringenina	Frutas cítricas
Flavona	Amarelo claro	Apigenina, luteolina	Cereais, frutas,flores,vegetais
Flavonol	Amarelo claro	Quercetina, Miricetina e rutina	Cebolas, maçãs,tomates,vinho tinto, trigo sarraceno, chá
Isoflavona	Incolor	Genisteína, daizeína	Legumes (derivados de soja)

Os flavonoides são considerados substâncias lipossolúveis e hidrossolúveis, sendo encontrados em forma livre de açúcares, mais apolares, e em formas glicosiladas, mais polares (BRAVO, 1998). A maioria deles se apresentam na forma glicosilada, sendo mais comumente encontrados os carboidratos D-glicose, L-ramnose, glicoramnose, galactose e arabinose ligados principalmente na posição 3 (RICE-EVANS et al., 1995). Sabe-se que, dificilmente, a forma intacta, a glicosilada, é absorvida no intestino delgado, devido ao favorecimento da sua hidrofilicidade

(CRESPY, 1999). O metabolismo dos flavonoides é amplamente conhecido em animais, porém não se tem muitas informações do seu mecanismo em humanos. Hoje se tem um interesse grande em esclarecer esse mecanismo, assim como o comportamento destes compostos no organismo, visto que apresentam inúmeras propriedades farmacológicas (MIDDLETON et al., 2000; MUROTA, 2003).

Estes compostos são moléculas especialmente grandes e passam por um processo de quebra, auxiliados por enterobactérias, liberando a aglicona, uma molécula que pode ser absorvida com maior facilidade pelas células epiteliais do intestino grosso devido a sua lipofilicidade (HOLLMAN e KATAN, 1999; MIDDLETON et al., 2000; HAVSTEEN, 2002; MUROTA, 2003; BHATIA e JAIN, 2004). Após passarem pelo trato digestivo, estas moléculas migram pela corrente sanguínea até o fígado, onde são submetidos à metilação, glucoronidação e/ ou sulfatação e, por fim, encontrarem-se livres no sangue. Uma vez livres, podem alterar o estado redox de órgãos, melhorar a atividade antioxidante enzimática, modificar a expressão de algumas proteínas, alterar o crescimento celular, promover reparo ao material genético danificado, desencadear apoptose de células aberrantes entre outros efeitos (YAO et al*.,* 2004).

Alguns estudos têm apontado a capacidade dos flavonoides em atravessar a BHE através da quantificação dos mesmos no SNC (MÉNDEZ-ARMENTA et al., 2001; MÉNDEZ-ARMENTA e RÍOS, 2007). A BHE possui certa seletividade quanto à passagem de moléculas polares e, também, de moléculas grandes como polímeros. Todavia, apesar desta seletividade, já foi registrada a presença de catequinas, antocianidinas, naringeninas, hesperetina, quercetina, rutina, dentre outros flavonoides e derivados, no encéfalo de roedores que receberam oralmente estes compostos (YOUDIM et al*.,* 2003).

O efeito neuroprotetor dos flavonoides foi evidenciado em vários estudos que demonstraram que estes compostos protegem as células neuronais da morte celular em modelos animais de doenças neurodegenerativas, como a doença de Parkinson, a doença de Alzheimer, a isquemia cerebral unilateral ou global e, ainda, em modelos de estresse oxidativo por peróxido de hidrogênio (ARUOMA et al., 2003; KANG et al., 2005; MANDEL et al., 2005). Além disso, os efeitos benéficos dos flavonoides no SNC, atuando como neuroprotetores, podem ser atribuídos, principalmente, pela atividade antioxidante do composto, através da redução das

EROs, bem como evitando a neurotoxicidade de substâncias, e agindo pela modulação de atividades de enzimas, como as ectonucleotidases e a AChE (ABDALLA et al., 2013; SCHMATZ et al., 2009a; SCHMATZ et al., 2009b; GONÇALVES et al., 2010). Além dos efeitos destes compostos no SNC, têm sido amplamente reportado os efeitos benéficos dos flavonoides ao sistema imunológico, devido à propriedade antioxidante, anti-inflamatória e antitumorais, como apotenciação de apoptose, inibição da proliferação de células e inibição da metástase, que já esta bem descrita na literatura (CHINEMBIRI et al., 2014).

Dentre todos os flavonoides, destaca-se um em especial, a quercetina, que é considerada um dos compostos (3,3',4',5,7-pentaidroxiflavona) mais abundantes, sendo encontrados em frutas, legumes e vegetais (Figura 13). Também é um dos componentes mais conhecidos na *Ginkgo biloba* e na erva de São João (*Hypericum perforatum*) (PRIOR, 2003; WILLIANS et al., 2004). Acredita-se que a cebola (284-486 mg/kg), a maçã (21-72 mg/kg) e o brócolis (30mg/Kg) são as fontes majoritárias de quercetina (HERTOG et al., 1993; MUROTA, 2003).

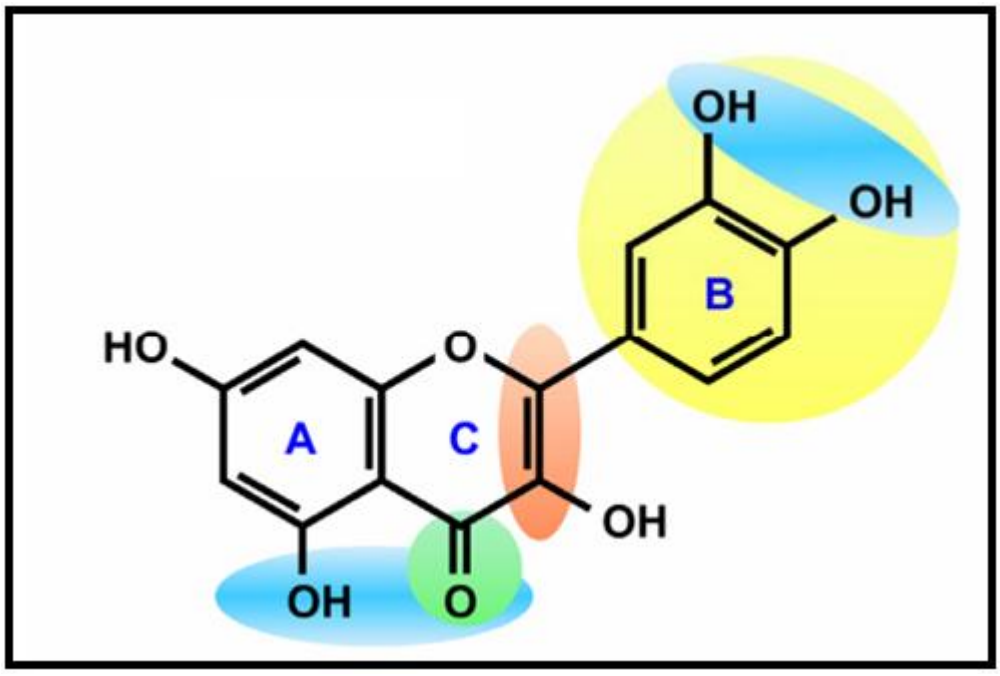


Figura 13. Estrutura do flavonoide Quercetina. Destaque para as estruturas envolvidas na sua atividade farmacológica e antioxidante. O grupamento mais importante é o catecol (amarelo) outros grupamentos importantes: presença de insaturação no anel C (vermelho), presença de função 4-oxo no anel C (verde). O grupo catecol e as outras funções (azul) possuem a habilidade em quelar metais de transição como o cobre e o ferro. (Adaptado de Spencer *et al*., 2003).

A quercetina é um composto não carcinogênico e possui baixa toxicidade (NATIONAL TOXICOLOGY PROGRAM, 1992, CHOI et al., 2003). Em experimentos animais, a dose oral segura da quercetina é de 1000 mg/dia e, por via intravenosa, 756 mg/dia (HARWOOD et al., 2007). Já em humanos, 4 g/dia de quercetina não apresentam efeitos colaterais. Além disso, a estimativa calculada de ingesta por indivíduo é de aproximadamente 25 mg/dia (NATIONAL TOXICOLOGY PROGRAM, 1992; COOK e SAMMAN, 1996; CHOI et al., 2003). Este flavonoide é um composto facilmente absorvido por pequenas células intestinais, onde sofre hidrólise para, posteriormente, entrar na corrente sanguínea e seus metabólitos não utilizáveis serem excretados pela urina. Uma vez atingido a corrente sanguínea, a quercetina será metabolizada no fígado e distribuída através da circulação sanguínea para todos os tecidos, onde, por sua vez, realizará uma gama de efeitos, em sua grande maioria, benéficos (MUROTA e TERAO, 2003; MANACH et al., 2005). No que diz respeito à biodisponibilidade, sabe-se que apenas 60 % da quercetina ingerida é absorvida, e a sua eliminação é lenta (GUGLER et al., 1975).

Pesquisas têm descrito várias aplicações terapêuticas para este flavonoide, incluindo a atividade antioxidante, antiinflamatória e neuroprotetora (CHO et al., 2003, CHOI et al., 2003, ABDALLA et al., 2013). Acredita-se que em muitas situações, estas ações, provavelmente, envolvem sua propriedade antioxidante (PRIOR, 2003), a qual pode ser explicada por sua participação em inibir enzimas como a ciclooxigenase, lipoxigenase e xantina oxidase, que estão envolvidas na citotoxicidade oxidativa (KAHRAMAN, 2003).

Estudos *in vitro* demonstram o efeito antitumoral da quercetina em cultura de células de melanoma, relatando que a quercetina, em baixas doses, afeta a viabilidade celular e, em altas doses, induz a apoptose celular (HOLLMAN et al., 1997). Zhang e colaboradores (2005) sugerem que a quercetina induz a apoptose de células de melanoma (B16-BL6) através do aumento da atividade da caspase-3. Além disso, já foi relatado que a quercetina inibe o crescimento e a metastase do melanoma *in vivo* (CAO et al., 2013).

Em relação ao efeito protetor da quercetina no SNC vários estudos relatam o efeito neuroprotetor do composto. PU et al., (2007) observaram uma ação protetora da quercetina em testes de memória espacial e a redução da morte neuronal em ratos. Existem, também, registros da ação protetora da quercetina na discinesia

orofacial induzida por tratamento crônico com neurolépticos (haloperidol) (NAIDU et al., 2003), no modelo de isquemia (SARKAR e DAS 2006), em insultos por privação de glicose e oxigênio (HA et al., 2003) e na injúria de células corticais causada por EROs (HA et al., 2003). Destaca-se que, recentemente, o nosso grupo de pesquisa avaliou o efeito da quercetina na atividade de enzimas do sistema purinérgico e colinérgico em sinaptossomas do córtex cerebral de ratos expostos ao Cd e confirmou-se que, de fato, a quercetina exerce um efeito neuroprotetor em relação à toxicidade do Cd nas enzimas do sistema colinérgico e purinérgico (ABDALLA et al., 2013).

Com base no acima mencionado e considerando-se os efeitos tóxicos da exposição ao Cd ao SNC e SNP, o objetivo do presente trabalho foi investigar os parâmetros comportamentais e bioquímicos, como a atividade das enzimas AChE, Na^+, K^+-ATPase, δ - ALA - D, NTPDases , ADA, BuChE e MPO, bem como o perfil oxidativo em ratos submetidos à intoxicação por Cd. Em adição, torna-se relevante avaliar o efeito protetor da quercetina, composto com ação antioxidante, anti-inflamatória e neuroprotetora, em ratos submetidos a este tipo de exposição.

2. OBJETIVOS

2.1 Objetivo geral

O objetivo do presente estudo foi investigar alterações nos testes comportamentais e de memória bem como os parâmetros bioquímicos em ratos expostos ao Cd e tratados com quercetina.

2.2 Objetivos específicos

- Investigar o comportamento do tipo ansiogênico e a memória em ratos expostos ao $CdCl_2$ e/ou tratados com quercetina;

- Determinar a presença de Cd em diferentes estruturas encefálicas de ratos expostos ao $CdCl_2$ e/ou tratados com quercetina a fim de verificar a possibilidade do metal em acumular-se no encéfalo além das propriedades quelantes da quercetina;

- Avaliar a atividade das enzimas AChE e Na^+, K^+-ATPase em diferentes estruturas encefálicas de ratos expostos ao $CdCl_2$ e/ou tratados com quercetina a fim de relacionar os resultados dos parâmetros comportamentais com as possíveis alterações enzimáticas;

- Avaliar a atividade da enzima δ-ALA–D no encéfalo de ratos expostos ao $CdCl_2$ e/ou tratados com quercetina com o propósito de confirmar a toxicidade induzida pelo metal;

- Determinar os parâmetros de estresse oxidativo como: os níveis intracelulares de EROs, níveis de peroxidação lipídica, oxidação de proteínas, a presença de DNA dupla fita bem como os níveis de vitamina C, tióis totais, glutationa

reduzida, a atividade das enzimas glutationa redutase e glutationa transferase em diferentes estruturas encefálicas de ratos expostos ao $CdCl_2$ e/ou tratados com quercetina com o propósito de relacionar estes resultados com as possíveis alterações enzimáticas e comportamentais, além de esclarecer os possíveis mecanismos de toxicidade induzida pela exposição ao Cd;

- Avaliar a atividade das enzimas NTPDase, ADA e AChE em linfócitos periféricos de ratos expostos ao $CdCl_2$ e/ou tratados com quercetina a fim de verificar possíveis alterações no sistema purinérgico e colinérgico destes animais;

- Analisar a atividade da enzima BuChE no soro e da MPO no plasma de ratos expostos ao $CdCl_2$ e/ou tratados com quercetina para confirmar a presença do processo inflamatório e relacionar com a atividade anti-inflamatória da quercetina;

- Verificar o efeito da quercetina *in vitro*, nas atividades da NTPDase, da ADA, da AChE em linfócitos periféricos, atividade da BuChE no soro e da MPO no plasma a fim de comparar com os resultados *in vivo*.

APRESENTAÇÃO

Os resultados desta tese estão apresentados sob a forma de dois artigos, os quais encontram-se no item **"ARTIGOS**". As seções Materiais e Métodos, Resultados, Discussão e Referências encontram-se nos próprios artigos e representam a íntegra deste estudo.

Os itens **DISCUSSÃO** e **CONCLUSÕES,** encontrados no final desta tese, apresentam interpretações e comentários gerais a respeito dos resultados apresentados nos artigos. As **REFERÊNCIAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO** e **DISCUSSÃO** desta tese.

3. ARTIGOS

3.1 Artigo 1

Quercetina protege o comprometimento da memória e comportamento do tipo ansiogênico em ratos expostos ao cádmio: Possível envolvimento da atividade da acetilcolinesterase e Na^+, K^+-ATPase

Quercetin protects the impairment of memory and anxiogenic-like behavior in rats exposed to cadmium: Possible involvement of the acetylcholinesterase and Na^+,K^+-ATPase activities

Fátima H. Abdalla, Roberta Schmatz, Andréia M. Cardoso, Fabiano B. Carvalho, Jucimara Baldissarelli, Juliane Sorraila de Oliveira, MichelleM. Rosa, Matheus Augusto Gonçalves Nunes,Maribel A. Rubin, Ivana B.M. da Cruz, Fernanda Barbisan, Valderi L. Dressler, Luciane B. Pereira, Maria Rosa C. Schetinger, VeraM.Morsch, Jamile F. Gonçalves, Cinthia M. Mazzanti

Publicado na revista "Physiology & Behavior"

Physiology & Behavior 135 (2014) 152–167

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Quercetin protects the impairment of memory and anxiogenic-like behavior in rats exposed to cadmium: Possible involvement of the acetylcholinesterase and Na^+,K^+-ATPase activities

Fátima H. Abdalla [a,b,*], Roberta Schmatz [a,**], Andréia M. Cardoso [a], Fabiano B. Carvalho [a,b], Jucimara Baldissarelli [a], Juliane Sorraila de Oliveira [a], Michelle M. Rosa [a], Matheus Augusto Gonçalves Nunes [a], Maribel A. Rubin [a], Ivana B.M. da Cruz [a], Fernanda Barbisan [a], Valderi L. Dressler [a], Luciane B. Pereira [a], Maria Rosa C. Schetinger [a], Vera M. Morsch [a], Jamile F. Gonçalves [a], Cinthia M. Mazzanti [b,***]

[a] Programa de Pós Graduação em Ciências Biológicas: Bioquímica Toxicológica, Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil
[b] Programa de Pós Graduação em Ciências Biológicas: Bioquímica Toxicológica, Setor de Bioquímica e Estresse Oxidativo do Laboratório de Terapia Celular, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil

HIGHLIGHTS

- Cd impairs memory has anxiogenic effect and alters AChE and Na^+, K^+-ATPase activities.

ARTICLE INFO

Article history:
Received 22 March 2013
Received in revised form 10 June 2014
Accepted 11 June 2014
Available online 18 June 2014

Keywords:
AChE
Anxiety
Cd
Oxidative stress
Memory
Na^+
K^+-ATPase
Quercetin

ABSTRACT

The present study investigated the effects of quercetin in the impairment of memory and anxiogenic-like behavior induced by cadmium (Cd) exposure. We also investigated possible alterations in acetylcholinesterase (AChE), Na^+,K^+-ATPase and δ-aminolevulinate dehydratase (δ-ALA-D) activities as well as in oxidative stress parameters in the CNS. Rats were exposed to Cd (2.5 mg/kg) and quercetin (5, 25 or 50 mg/kg) by gavage for 45 days. Animals were divided into eight groups (n = 10–14): saline/control, saline/Querc 5 mg/kg, saline/Querc 25 mg/kg, saline/Querc 50 mg/kg, Cd/ethanol, Cd/Querc 5 mg/kg, Cd/Querc 25 mg/kg and Cd/Querc 50 mg/kg. Results demonstrated that Cd impaired memory has an anxiogenic effect. Quercetin prevented these harmful effects induced by Cd. AChE activity decreased in the cerebral cortex and hippocampus and increased in the hypothalamus of Cd-exposed rats. The Na^+,K^+-ATPase activity decreased in the cerebral cortex, hippocampus and hypothalamus of Cd-exposed rats. Quercetin prevented these effects in AChE and Na^+,K^+-ATPase activities. Reactive oxygen species production, thiobarbituric acid reactive substance levels, protein carbonyl content and double-stranded DNA fractions increased in the cerebral cortex, hippocampus and hypothalamus of Cd-exposed rats. Quercetin totally or partially prevents these effects caused by Cd. Total thiols (T-SHs), reduced glutathione (GSH), and reductase glutathione (GR) activities decreased and glutathione S-transferase (GST) activity increased in Cd exposed rats. Co-treatment with quercetin prevented reduction in T-SH, GSH, and GR activities and the rise of GST activity. The present findings show that quercetin prevents alterations in oxidative stress parameters as well as AChE and Na^+,K^+-ATPase activities, consequently preventing memory impairment and anxiogenic-like behavior displayed by Cd exposure. These results may contribute to a better understanding of the neuroprotective role of quercetin, emphasizing the influence of this flavonoid in the diet for human health, possibly preventing brain injury associated with Cd intoxication.

© 2014 Elsevier Inc. All rights reserved.

* Correspondence to: F.H. Abdalla, Programa de Pós Graduação em Ciências Biológicas: Bioquímica Toxicológica, Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil. Tel./fax: +55 55 3220 8814.
** Correspondence to: R. Schmatz, Programa de Pós Graduação em Ciências Biológicas: Bioquímica Toxicológica, Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria/,RS 97105--900, Brazil. Tel./fax: +55 55 3220 8814.
*** Corresponding author. Tel./fax: +55 55 3220 8814.
E-mail addresses: faha.biomed@hotmail.com (F.H. Abdalla), betaschmatz@hotmail.com (R. Schmatz), cmelazzomazzanti@gmail.com (C.M. Mazzanti).

http://dx.doi.org/10.1016/j.physbeh.2014.06.008
0031-9384/© 2014 Elsevier Inc. All rights reserved.

1. Introduction

Cadmium (Cd) is a heavy metal with a long biological half-life (15–30 years) in humans which presents low rate of excretion [1]. The natural and anthropogenic sources of Cd may lead to the contamination of soils and to increased uptake of this metal by crops and vegetables grown for human consumption [2]. Based on these evidences, diet is the most important source of the Cd exposure in the general population (non-occupational and non-smoking), since Cd is not degraded in the environment and can enter into the food chain [1]. In this way, the prolonged exposure to Cd has been linked to toxic effects triggered by the accumulation of this metal in a variety of structures of the central nervous system (CNS) [3].

In fact, Cd is able to cross the blood–brain barrier (BBB) and accumulate in the brain [3] leading to cerebral edema, cellular dysfunction and also the worsening of the synaptic neurotransmission and the antioxidant levels [4–6]. Some studies have demonstrated that the exposure to Cd can produce impairment of attention, learning and memory as well an increase in aggressive and anxiogenic-like behaviors [7–11]. In addition, Cd can cause changes in important enzymes of the CNS including acetylcholinesterase (AChE), Na^+,K^+-ATPase and δ-aminolevulinate dehydratase (δ-ALA-D) [10,12–14].

Acetylcholine (ACh) is a neurotransmitter with an important role in many functions of both the peripheral and central nervous systems [15] acting in the learning and memory processes as well as locomotor control and cerebral blood flow [16–20]. ACh levels in synaptic cleft are regulated by AChE activity. It has been shown that the AChE activity is implicated in cell proliferation [21] and neurite outgrowth [22]. Interestingly, AChE responds to various insults including oxidative stress, an important event that has been related to the pathogenesis and progression of a variety of CNS disorders [23]. Thus, this enzyme is a target for the emerging therapeutic strategies to treat cognitive disorders like Alzheimer's disease (AD) [24].

Furthermore, the enzyme Na^+,K^+-ATPase also plays an important role in synaptic neurotransmission. This enzyme plays a key role in the maintenance of electrolytic gradient of Na^+ and K^+ ions, contributing to the intracellular electrolyte homeostasis as well as to the maintenance of volume and cellular pH [25–27]. It has been reported that a decrease in this enzyme activity or expression directly impairs neurotransmitter signaling with deleterious consequences on memory and anxiety behavior [28,29]. Moreover, ouabain, a Na^+,K^+-ATPase inhibitor, increases Ca^{2+} influx in brain slices [30] and causes hippocampal cell death in rats [31].

Another important target enzyme in our study is the δ-ALA-D. This metalloenzyme requires zinc ions for its activity, and it catalyzes the asymmetric condensation of two molecules of δ-aminolevulinic acid (δ-ALA) to porphobilinogen in the initial steps of heme biosynthesis [32]. δ-ALA-D is a sulfhydryl enzyme and numerous metals such as mercury [33], lead [34], Cd [13] and other compounds can oxidize sulfhydryl groups, modifying its activity [35]. Therefore, δ-ALA-D is inhibited by substances that compete with zinc and/or oxidize the – SH groups [36], and it is linked to situations associated with oxidative stress [13].

The molecular mechanisms of Cd toxicity are not yet well defined; however, the accumulation of Cd can damage various tissues including the brain [14,37]. Studies have shown increased levels of lipid peroxidation, protein carbonylation, inhibition or reduction of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx) and DNA damage induced by Cd [38,39]. Therefore, we can assume that the toxicity effects of this metal probably occur due generation of reactive oxygen species (ROS), since Cd does not directly participate in oxidation–reduction [40].

On the other hand, antioxidant compounds have an important role in the neuroprotection against the establishment of oxidative stress. In this context, the quercetin is a natural antioxidant present in a variety of vegetables and fruits which are regularly consumed in our diet. Quercetin belongs to the flavonoid family and has phenolic groups in their structure [41]. This compound has been studied due to its wide therapeutic benefits that involve its antioxidant effects, chelating properties of some elements as metals [42] and neuroprotective capacity [41].

In this context, considering the toxic effects and neurological damage caused by Cd exposure and that quercetin has important antioxidant and neuroprotective actions, the aim of this study was to evaluate the possible protective effects of this flavonoid in memory and anxiogenic-like behavior, AChE, Na^+,K^+-ATPase and δ-ALA-D activities in Cd exposed rats. In addition we also checked oxidative stress parameters, such as protein and lipid oxidation, intracellular ROS production and double-stranded DNA (dsDNA) level, as well as, antioxidant enzymes activities and endogenous non-enzymatic antioxidant content.

2. Material and methods

2.1. Chemicals

Acetylthiocholine iodide, cadmium chloride monohydrate ($CdCl_2$), 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), Tris-(hydroxymethyl)-aminomethane, ouabain octahydrate, Coomassie Brilliant Blue G and quercetin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used in the experiments were of analytical grade and of the highest purity.

2.2. Animals

Adult male Wistar rats (70–90 days; 220–300 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 (protocol under number: 031/2011).

2.3. Experimental procedure

Cd is derived primarily from the ingestion of food and water contaminated with Cd. $CdCl_2$ is the principal form of Cd associated with oral exposure, since it is highly soluble in water [43]. Thus, in the present study rats received by oral administration (gavage): Cd as $CdCl_2$ (Cd 2.5 mg/kg) dissolved in saline and/or quercetin (5, 25 and 50 mg/kg) dissolved in ethanol 25%. The choice of Cd doses was made based on previous works of our research group, in which Cd-exposed rats showed a small but significant amount of Cd in their brain structures enough to cause brain injury and hematological injuries [10,14,44,45]. The choice of the doses 5, 25 or 50 mg/kg of quercetin was made based on previous studies that showed beneficial effects of this compound in the CNS. Moreover, our research group showed that these doses of quercetin prevented the toxic effect caused by Cd exposure on important enzymes of the CNS [37,41,46,47]. Both solutions were administered 5 days a week for 45 days. Animals were randomly divided into eight groups of 10–14 animals each: saline/control, saline/Querc 5 mg/kg, saline/Querc 25 mg/kg, saline/Querc 50 mg/kg, Cd/ethanol 25%, Cd/Querc 5 mg/kg, Cd/Querc 25 mg/kg, and Cd/Querc 50 mg/kg.

Quercetin was administered 30 min after Cd. The solutions were freshly prepared. Cd was diluted in saline and the quercetin in ethanol 25% and both were administered (1 ml/kg) between 9 and 11 a.m.

In the last day of treatment (after 45 days), animals were subjected to training and behavioral parameter estimation. After, the animals were submitted to euthanasia being previously anesthetized with halothane. The total brain was collected to δ-ALA-D assay, and the cerebral

cortex, hippocampus, striatum, hypothalamus and cerebellum were collected for the subsequent enzymatic assays.

It is important to note that controls for all ex vivo tests were performed to correct for vehicle (ethanol) interference. However, no significant differences between the results obtained to the vehicle and to the control were observed in the parameter analyzed in this study (data not shown).

2.4. Behavioral procedure

2.4.1. Inhibitory avoidance task

At the final day of treatment (45 days), animals were subjected to training in a step-down inhibitory avoidance apparatus as previously described. Next, 24 h after training the animals were subjected to a test in a step-down inhibitory avoidance task. 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 high. The rat was placed gently on the platform facing the rear left corner, and when the rat stepped down with all four paws on the grid, a 3-s 0.4-mA shock was applied to the grid. 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 300 s was established.

2.4.2. Open field

Immediately after the inhibitory avoidance test session, animals were transferred to an open-field measuring 56 × 40 × 30 cm, with the floor divided into 12 squares measuring 12 × 12 cm each. The open field session lasted for 5 min and during this time, an observer, who was not aware of the treatments, recorded the number of crossing responses and rearing responses manually. 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 motor disabilities, which might influence inhibitory avoidance performance at testing.

2.4.3. Foot shock sensitivity test

Reactivity to shock was evaluated in the same apparatus used for inhibitory avoidance, except that the platform was removed and was used to determine the flinch and jump thresholds in experimentally naive animals [48,49]. 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 0.5 mA in 0.1 mA increments. The adjustments in shock intensity were made in accordance with each animal response. The intensity was raised by one unit when no response occurred and lowered by one unit when a response was made. 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, and jump) were made, and the mean of each score was calculated for each animal.

2.4.4. Elevated plus maze task

Anxiolytic-like behavior was evaluated using the elevated plus maze task as previously described [50,51]. The apparatus consisted of a wooden structure raised to 50 cm from the floor. This apparatus was composed of 4 arms of the same size, with two closed-arms (walls of 40 cm) and two open-arms. Initially, the animals were placed on the central platform of the maze in front of an open arm. The animal had 5 min to explore the apparatus. The time spent and the number of entries in open and closed arms were recorded. The apparatus was thoroughly cleaned with 30% ethanol between each session.

2.5. Brain tissue preparation

After behavioral tests, animals were anesthetized with halothane and submitted to euthanasia. The cranium was opened and the structures were gently removed and separated into the cerebral cortex, hippocampus, striatum, hypothalamus and cerebellum. To verify the Cd concentration in the brain structures, six or seven animals per group were randomly chosen. For the others all the brain structures were homogenized in a glass potter in a solution of 10 mM Tris–HCl, with 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 AChE and δ-ALA-D activities, oxidative stress parameters, antioxidant enzymes activities and endogenous non-enzymatic antioxidant content.

2.6. Cadmium concentration in the brain tissue

For Cd determination, digestion of the cerebral cortex, hippocampus, striatum, hypothalamus and cerebellum (up to 0.2 g) was performed by a conventional heating block (Velp Scientifica, model DK, Italy) with open glass vessels. In this case, samples were weighed inside the vessels and 6 ml of concentrated nitric acid (Merck, Darmstadt, Germany) was added. Digestion procedure was carried out for 2 h up to 120 °C. In a second step, 1 ml of 30% (w/w) H_2O_2 was added and the digests were heated up to 80 °C for 1 h. After cooling, digests were diluted with purified water (Milli-Q system, Millipore Corp., Bedford, USA) to 25 ml in polypropylene vessels for further analysis.

A spectrometer Spectro Ciros CCD (Spectro Analytical Instruments, Kleve, Germany) was used for the determination of Cd at 226.502 nm by inductively coupled plasma optical emission spectrometry (ICP-OES). This instrument was equipped with an axial view configuration and a cross flow nebulizer coupled to a Scott type double pass nebulization spray chamber. Argon (99.996%, White Martins-Praxair, São Paulo, SP, Brazil) was used for plasma generation, for nebulization and as auxiliary gas. For the accuracy evaluation, a certified reference material (CRM) of dogfish liver (DOLT-3) from the National Research Council Canada was used.

Sample	Cd measured ($\mu g\ g^{-1}$)	Cd certified ($\mu g\ g^{-1}$)
DOLT-3	19.6 ± 0.8	19.4 ± 0.6

2.7. AChE enzymatic assay

The AChE enzymatic assay was determined as previously described [52] with a modification of the spectrophotometric method as described previously [53]. The reaction mixture (2 ml final volume) contained 100 mM K^+-phosphate buffer, with 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 of incubation at 25 °C. The enzyme was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh). The protein content was adjusted for each structure: cerebral cortex (0.8 mg/ml), hippocampus (0.8 mg/ml), striatum (0.4 mg/ml), hypothalamus (0.6 mg/ml) and cerebellum (0.6 mg/ml). All samples were run in triplicate and 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 [54] with minor modifications [55]. Briefly, the assay medium consisted of 30 mM Tris–HCl buffer (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$ and 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 [56], using KH_2PO_4 as reference standard. Specific Na^+,K^+-ATPase activity was calculated by subtracting the ouabain-insensitive activity from the overall activity (in the absence of ouabain) and expressed in nmol of Pi/mg of protein/min.

2.9. δ-Aminolevulinic acid dehydratase activity (δ-ALA-D)

Brain δ-ALA-D activity was assayed according to the method of Sassa [57] by measuring the rat of porphobilinogen (PBG) formation. δ-ALA-D activity was expressed as nmol porphobilinogen (PBG)/mg of protein/h.

2.10. Measurement of intracellular reactive oxygen species (ROS) production

2′-7′-Dichlorofluorescein (DCF) levels were determined as an index of the reactive species production by the cellular components [58]. Aliquots (50 μl) of brain supernatants were added to a medium containing Tris–HCl buffer (10 mM; pH 7.4) and 2′-7′-dichlorofluorescein diacetate DCFH-DA (1 mM). After DCFH-DA addition, the medium was incubated in the dark for 1 h until fluorescence measurement procedure (excitation at 488 nm and emission at 525 nm, and both slit widths used were at 1.5 nm). DCF levels were determined using a standard curve of DCF, and results were corrected by the protein content.

2.11. Thiobarbituric acid reactive substance (TBARS) measurement

TBARS levels were determined according to Ohkawa et al. [59] by measuring of 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 sulfate (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 of protein.

2.12. Protein carbonyl levels

The carbonylation of proteins was determined by a modified Levine method [60]. Firstly, from 1 ml of brain supernatants, proteins were precipitated using 0.5 ml of 10% trichloroacetic acid (TCA) and centrifuged at 1800 *g* for 5 min, discarding the supernatant. One half milliliter of 10 mmol l^{-1} 2,4-dinitrophenylhydrazine (DNPH) in 2 mol l^{-1} HCl was added to this protein precipitate and incubated at room temperature for 30 min. During incubation, the samples were mixed vigorously every 15 min. After incubation, 0.5 ml of 10% TCA was added to the protein precipitate and centrifuged at 1800 *g* for 5 min. After discarding the supernatant, the precipitate was washed twice with 1 ml of ethanol/ethylacetate (1:1), centrifuging out the supernatant in order to remove the free DNPH. The precipitate was dissolved in 1.5 ml of protein dissolving solution (2 g sodium dodecyl sulfate and 50 mg EDTA in 100 ml 80 mmol l^{-1} phosphate buffer, with pH 8.0) and incubated at 37 °C for 10 min. The color intensity of the supernatant was measured using a spectrophotometer at 370 nm against 2 mol l^{-1} HCl. Carbonyl content was calculated by using the molar extinction coefficient (21 x 10^3 1/mol cm), and results were expressed as nmol/mg of protein.

2.13. Double-stranded DNA (dsDNA) assay

To evaluate the cytotoxic effect of Cd in different brain structures, the presence of double-stranded DNA (dsDNA) in supernatant was determined, using a Quant-IT TM PicoGreen® dsDNA kit (Invitrogen-Life Technologies) according to manufacturer's instructions. Briefly, the assay is based on quantifications of cell death. Because of that, when the cell dies the membrane is disrupted and dsDNA fractions are released into the extracellular medium [61]. The DNA PicoGreen® dye presents high affinity with the dsDNA and is able to quantify the dsDNA released. In relation to the dsDNA, it was measured by using 50 μl of the sample and 50 μl of the DNA PicoGreen® dissolved in TE buffer, 1× (1:1; v/v), following the incubation for 5 min in a dark room. The fluorescence was measured at an excitation of 485 nm and an emission of 520 nm recorded at room temperature.

2.14. Measurement of vitamin C

Vitamin C levels were determined by the method of Jacques-Silva et al. [62]. Proteins of the brain were precipitated in a cold 10% trichloroacetic acid (TCA) solution at a proportion of 1:1 (v/v) and submitted to centrifugation again. This supernatant was then used for analysis.

A 300 μl aliquot of the sample in a final volume of 575 μl of solution was incubated for 3 h at 37 °C then 500 μl of H_2SO_4 at 65% (v/v) was added to the medium. The reaction product was determined using a color reagent containing 4.5 mg/ml dinitrophenyl hydrazine (DNPH) and $CuSO_4$ (0.075 mg/ml). Vitamin C levels are expressed as mg ascorbic acid/g tissue.

2.15. Determination of total thiols

Total thiol groups (T-SHs) were assayed spectrophotometrically by the method of Boyne and Ellman [63] with some modifications. An aliquot of 200 μl for brain supernatants (S1) in a final volume of 900 μl of solution was used for the reaction. The reaction product was measured at 412 nm after the addition of 10 mM 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB) (0.05 ml). A standard curve using cysteine was added to calculate the content of thiol groups in samples, and was expressed as nmol T-SH/g tissue.

2.16. Measurement of reduced glutathione (GSH)

GSH was measured spectrophotometrically with Ellman's reagent [64]. An aliquot of 200 μl for supernatants in a final volume of 900 μl of solution was used for the reaction. The reaction product was measured at 412 nm after the addition of 10 mM 5-5-dithio-bis(2-nitrobenzoic acid) (DTNB) (0.05 ml). A standard curve using cysteine was added to calculate the content of thiol groups in samples, and was expressed as nmol GSH/g tissue.

2.17. Assay of reductase glutathione (GR)

For the measurement of GR activity, we used a method previously described by Carlberg and Mannervik [65], with some modifications. The method is based on the utilization the enzyme oxidized glutathione (GSSG), to convert GSSG to GSH in the presence of the cofactor NADPH. Briefly, brain supernatant (0.050 ml) was added to medium containing 0.2 M phosphate buffer (0.2 M K_2HPO_4 and 2 mM EDTA, pH 7.0) and NADPH (2 mM). The reaction was initiated by adding substrate GSSG (20 mM). The measurement of GR levels was measured by absorbance at 340 nm during 2 min of incubation. GR activity was determined using the molar extinction coefficient 6220 M^{-1} cm^{-1} and expressed as μmol/min/mg of protein.

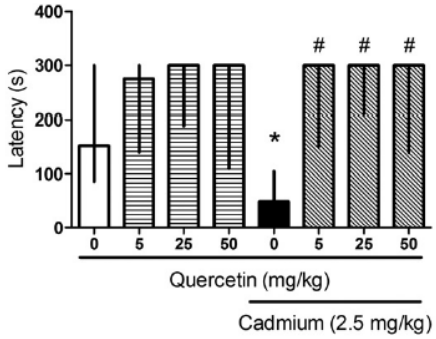


Fig. 1. Effect of Cd-exposed (2.5 mg/kg) rats and treated with quercetin in different doses (5, 25 or 50 mg/kg) on step-down latencies. After one treatment-free day, animals were tested in a step-down latency test. Data are median ± interquartile range of training and test. *$p < 0.05$ compared with the saline/control, and #$p < 0.05$ compared with the Cd/ethanol group at testing by the Dunn's non-parametric multiple comparison task ($n = 10–14$).

2.18. Assay of glutathione S-transferase (GST)

The GST enzymatic assay was determined as previously described by Habig et al. [66] with a modification of the spectrophotometric method. GST activity was quantified in tissue homogenates in a reaction mixture containing 1-chloro-2,4-dinitrobenzene (1 mM CDNB) and GSH (1 mM) as substrates in 0.1 M K^+-phosphate buffer, with pH 7.5, at 37 °C. The reaction was initiated by adding GSH substrate. Enzyme activity was calculated by the change in the absorbance value from the slope of the initial linear portion of the absorbance time curve at 340 nm for 2 min of incubation. Enzyme activity was determined using the molar extinction coefficient 9.6 mM^{-1} cm^{-1} and expressed as μmol/CDNB/min/mg of protein.

2.19. Protein determination

Protein content was determined colorimetrically according to the Bradford [67] method using bovine serum albumin (1 mg/ml) as standard solution.

2.20. Statistical analyses

Statistical analyses of training and test step-down latencies were carried out by Kruskal–Wallis followed by post-hoc analyses (non-parametric Dunn's test). Crossing, rearing and foot shock sensitivity test responses were analyzed by one-way ANOVA. $p < 0.05$ was used as the criterion for statistical significance. All other parameters evaluated were analyzed by one or two-way ANOVA, followed by the Tukey test where $p < 0.05$ was considered to represent a significant difference in all experiments. The retention latencies are expressed as the median and interquartile ranges and all other data are expressed as mean ± S.E.M.

3. Results

3.1. Behavioral tests

3.1.1. Quercetin prevents memory impairment induced by Cd exposure

Fig. 1 shows the effect of the treatment with quercetin per se and Cd-exposed rats on step-down latencies. Statistical analyses (non-parametric two-way ANOVA) showed a significant Cd (2.5 mg/kg) vs. quercetin (5, 25 or 50 mg/kg) or vehicle interaction (control), revealing that the treatment with quercetin was able to reverse the impairment of memory induced by Cd [$H(7)$: 31.79; $p < 0.001$].

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 quercetin affected shock threshold or locomotor ability of the animals. Statistical analysis of open-field data (one-way ANOVA) revealed that Cd did not alter the number of crossing ($p > 0.05$) or rearing ($p > 0.05$) responses in a subsequent open-field test session, suggesting that neither Cd nor quercetin caused gross motor disabilities at testing (Table 1). Moreover, Cd did not alter foot shock sensitivity as demonstrated by the similar flinch (unpaired t test, $p > 0.05$) and jump (unpaired t test, $p > 0.05$) thresholds exhibited by the animals. These data suggest that neither Cd intoxication nor quercetin treatment administered before training of inhibitory avoidance caused motor disabilities or altered foot shock sensitivity (Table 1).

3.1.2. Effect of Cd exposure and quercetin treatment on anxiolytic-like behavior

Fig. 2 shows the effect of Cd exposure and quercetin treatment on the anxiolytic-like behavior in the elevated plus-maze task. Statistical analysis of testing (one-way ANOVA) showed that rats exposed to Cd spent more time in closed arms and entered the closed arms more times compared with those of the other groups indicating a possible behavior induced by Cd (Fig. 2A and B). Moreover, quercetin 5, 25 or 50 mg/kg was able to prevent this increase of time in closed arms and the number of entries in closed arms induced by Cd (Fig. 2A and B). Fig. 2C shows that Cd decreased the time in center, and quercetin 5, 25 or 50 also was able to prevent this anxiogenic effect induced by Cd. Fig. 2D shows that Cd and quercetin did not alter the number of entries in arms in the elevated plus-maze task, showing that the treatments did not impair locomotor activity in this test.

Table 1
Effect of oral administration of quercetin (Querc 5, 25 or 50 mg/kg), cadmium (Cd) and cadmium (Cd) plus quercetin (Querc) on the latency to training in the inhibitory avoidance apparatus, on the behavior of rats (number of crossing and rearing responses) in the open-field immediately after the inhibitory avoidance testing session and on foot shock sensitivity (flinch, jump and vocalization).

Group	Latency of training (s)	Crossing	Rearing	Flinch (m·A)	Jump (m·A)	Vocalization (m·A)
Control	9.80 ± 2.63	23.50 ± 3.90	18.10 ± 3.40	0.42 ± 0.02	0.28 ± 0.01	0.42 ± 0.02
Querc 5	7.30 ± 3.25	21.50 ± 2.92	17.40 ± 2.59	0.40 ± 0.02	0.27 ± 0.02	0.40 ± 0.03
Querc 25	4.90 ± 0.93	23.60 ± 3.00	19.80 ± 2.48	0.41 ± 0.01	0.25 ± 0.01	0.35 ± 0.02
Querc 50	8.20 ± 2.54	24.30 ± 2.98	19.85 ± 2.84	0.37 ± 0.01	0.27 ± 0.01	0.32 ± 0.01
Cd	4.92 ± 0.85	27.85 ± 2.31	23.54 ± 2.19	0.39 ± 0.02	0.27 ± 0.01	0.37 ± 0.02
Cd + Q5	5.70 ± 0.67	28.21 ± 2.51	24.71 ± 2.49	0.37 ± 0.00	0.28 ± 0.01	0.41 ± 0.01
Cd + Q25	7.25 ± 2.22	26.25 ± 2.26	21.92 ± 2.11	0.37 ± 0.01	0.27 ± 0.01	0.39 ± 0.02
Cd + Q50	5.15 ± 0.84	26.69 ± 2.70	23.23 ± 2.07	0.40 ± 0.01	0.30 ± 0.01	0.40 ± 0.02
Statistical analysis	$F_{(7,90)} = 1.25$ $p > 0.05$	$F_{(7,90)} = 0.92$ $p > 0.05$	$F_{(7,90)} = 1.16$ $p > 0.05$	$F_{(7,90)} = 1.06$ $p > 0.05$	$F_{(7,90)} = 0.85$ $p > 0.05$	$F_{(7,90)} = 1.99$ $p > 0.05$

Data are means ± S.E.M. for 10–14 animals in each group.

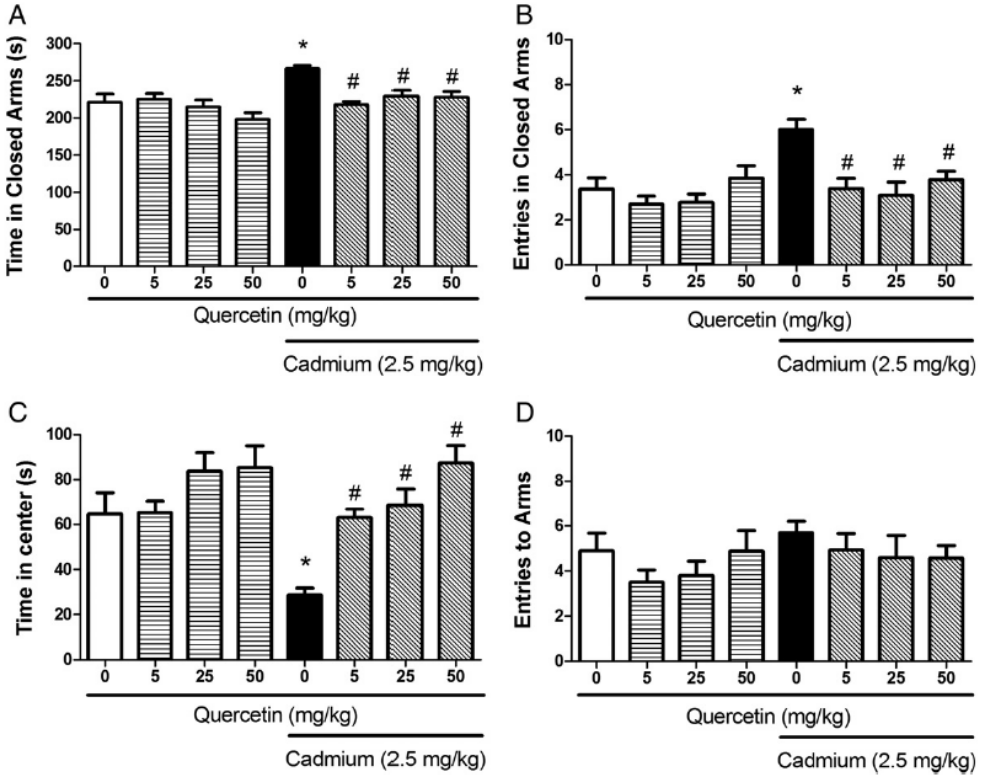


Fig. 2. Effect of Cd-exposed rats and treated with quercetin in different doses (mg/kg) on time in closed arms (A), number of entries in closed arms, time spent in center (C) and number of total entries in arms (D) measured in the elevated plus maze over the 5 min test. *$p < 0.05$ compared with the saline/control, and #$p < 0.05$ compared with the Cd/ethanol group. Bars represent means ± S.E.M. One-way ANOVA–Student, followed by Newman–Keuls (SNK) ($p < 0.05$) ($n = 10–14$).

3.2. Atomic absorption

All the brain structures analyzed showed a similar Cd concentration in the same group of rats. Control rats (saline) and rats treated with quercetin alone presented Cd concentrations lower than 0.20 $\mu g/g^{-1}$ in the different brain structures. Rats exposed to Cd alone or Cd plus quercetin showed Cd concentrations greater than 0.20 $\mu g/g^{-1}$ in the different brain structures. Moreover, a significant effect of Cd ($p < 0.05$) and quercetin ($p < 0.05$) on Cd concentrations in the different brain structures was observed (data not shown). Results demonstrated that Cd exposure alone or in combination with quercetin caused an increase in Cd brain concentration. Therefore, the quercetin administration was ineffective in restoring Cd levels to normal values in the cerebral cortex, hippocampus, striatum, hypothalamus, and cerebellum evaluated.

3.3. Quercetin prevented the alterations induced by Cd in AChE activity in the cerebral cortex, hippocampus and hypothalamus

Fig. 3 shows the effect of Cd and/or quercetin on the activity of AChE in different brain structures. The exposure to Cd decreased (53%) the AChE activity in the cerebral cortex when compared with control, while the treatment with quercetin 5, 25 or 50 mg/kg prevented this decrease ($p < 0.05$) (Fig. 3A). AChE activity was decreased (51%) in the hippocampus of rats exposed to Cd, while the treatment with quercetin 5, 25 or 50 mg/kg prevented this reduction ($p < 0.05$) (Fig. 3B). On the other hand, the AChE activity in the hypothalamus increased (58%) in Cd-exposed rats; however, the treatment with quercetin 5, 25 or 50 mg/kg was able to prevent this elevation ($p < 0.05$) (Fig. 3C). There were no significant alterations in the AChE activity in the striatum and cerebellum (Fig. 3D and E).

3.4. Quercetin prevented the decrease in Na^+,K^+-ATPase activity induced by Cd in the cerebral cortex, hippocampus and hypothalamus

Results obtained for Na^+,K^+-ATPase activity in different brain structures are presented in Fig. 4. The exposure to Cd decreased Na^+,K^+-ATPase activity in the cerebral cortex (36%) and the treatment with quercetin 5, 25 or 50 mg/kg ($p < 0.05$) prevented this reduction (Fig. 4A). On the other hand, the exposure to Cd decreased the Na^+, K^+-ATPase activity in the cerebral cortex (36%), hippocampus (28%) and hypothalamus (27%). The treatment with quercetin 5, 25 or 50 mg/kg prevented this effect on the cerebral cortex but only the treatment with quercetin 25 or 50 mg/kg prevented this effect in the hippocampus and hypothalamus ($p < 0.05$) (Fig. 4A, B and C). No significant differences ($p < 0.05$) were observed in the Na^+,K^+-ATPase activity in the striatum and cerebellum (Fig. 4D and E).

3.5. δ-Aminolevulinic acid dehydratase activity

Fig. 5 shows the effect of Cd and/or quercetin on the activity of δ-ALA-D in the brain. The exposure to Cd decreased the δ-ALA-D activity (53%) in the brain when compared with the saline/control group. The quercetin administration was ineffective in restoring δ-ALA-D activity to normal values.

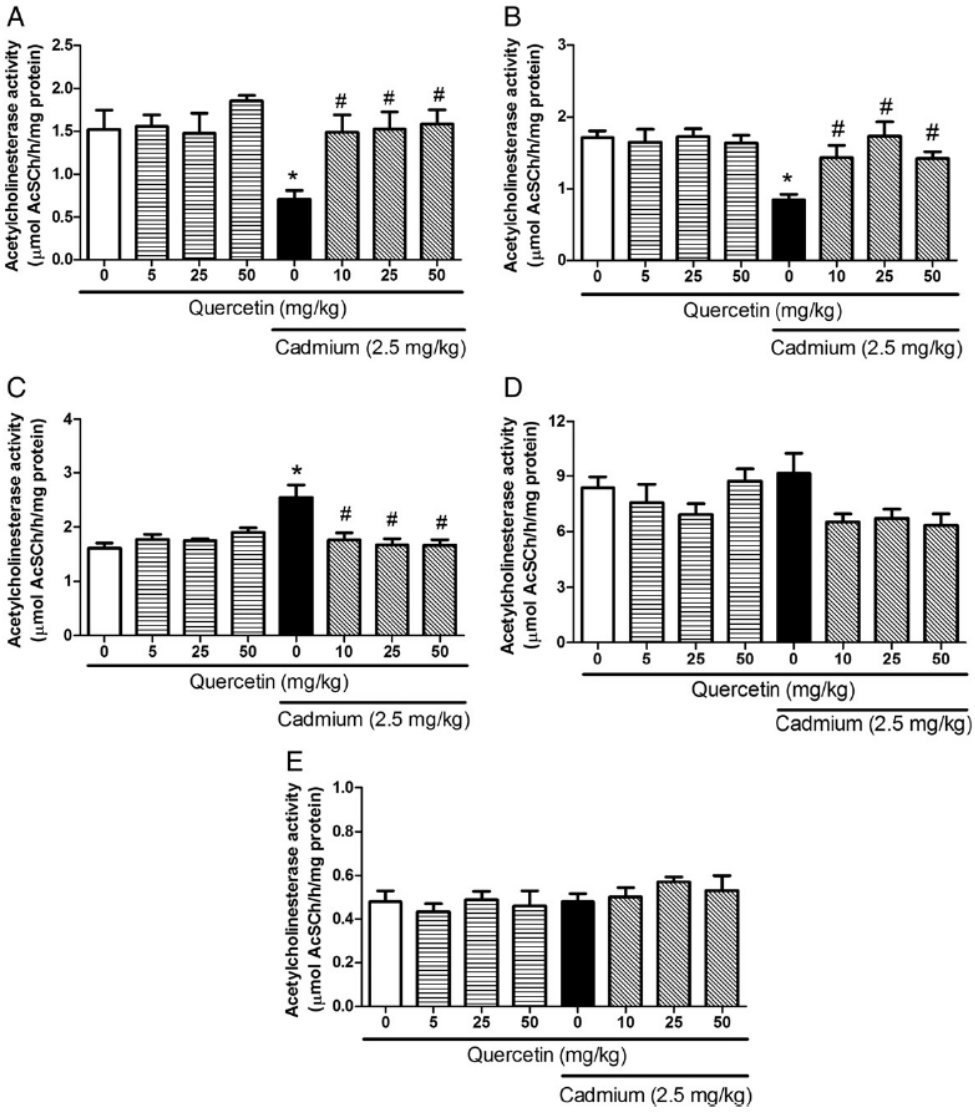


Fig. 3. Acetylcholinesterase (AChE) activity in cerebral cortex (A), hippocampus (B), hypothalamus (C), striatum (D) and cerebellum (E) of Cd-exposed rats and treated with quercetin. *p < 0.05 compared with the saline/control, and #p < 0.05 compared with the Cd/ethanol group. Bars represent means ± S.E.M. (Two-way ANOVA followed by Tukey test; n = 10–14.)

3.6. Effect of Cd exposure and quercetin treatment on ROS production in the cerebral cortex, hippocampus, hypothalamus, striatum and cerebellum

Table 2 shows the effect of Cd exposure and quercetin treatment on ROS production. As can be seen, in the cerebral cortex, hippocampus and hypothalamus, ROS production was significantly increased in the Cd/ethanol group when compared to the saline/control group (84.2%, 88.1%, 92.8% respectively) ($p < 0.05$). However, the treatment with quercetin 5, 25 or 50 mg/kg was able to prevent the increase on ROS production in the cerebral cortex, hippocampus and hypothalamus ($p < 0.05$) when compared to the Cd/ethanol group ($p < 0.05$). No significant differences ($p < 0.05$) were observed in the ROS production in the striatum and cerebellum. When quercetin was given per se, no significant difference in the cerebral cortex, hippocampus, hypothalamus, striatum and cerebellum were observed in the saline/Querc 5, saline/Querc 25 or saline/Querc 50 groups when compared to the saline/control group.

3.7. Quercetin prevented the alterations in lipid peroxidation levels induced by Cd exposure in the cerebral cortex, hippocampus, hypothalamus, striatum and cerebellum

TBARS levels in the different brain structures are given in Table 3. As can be observed, the exposure to Cd increased TBARS levels in the cerebral cortex (82%) hippocampus (64%) and hypothalamus (132%), while the treatment with quercetin 5, 25 or 50 mg/kg prevented this increase ($p < 0.05$). No significant differences in the TBARS levels were observed in the striatum and cerebellum. Moreover, when quercetin was given

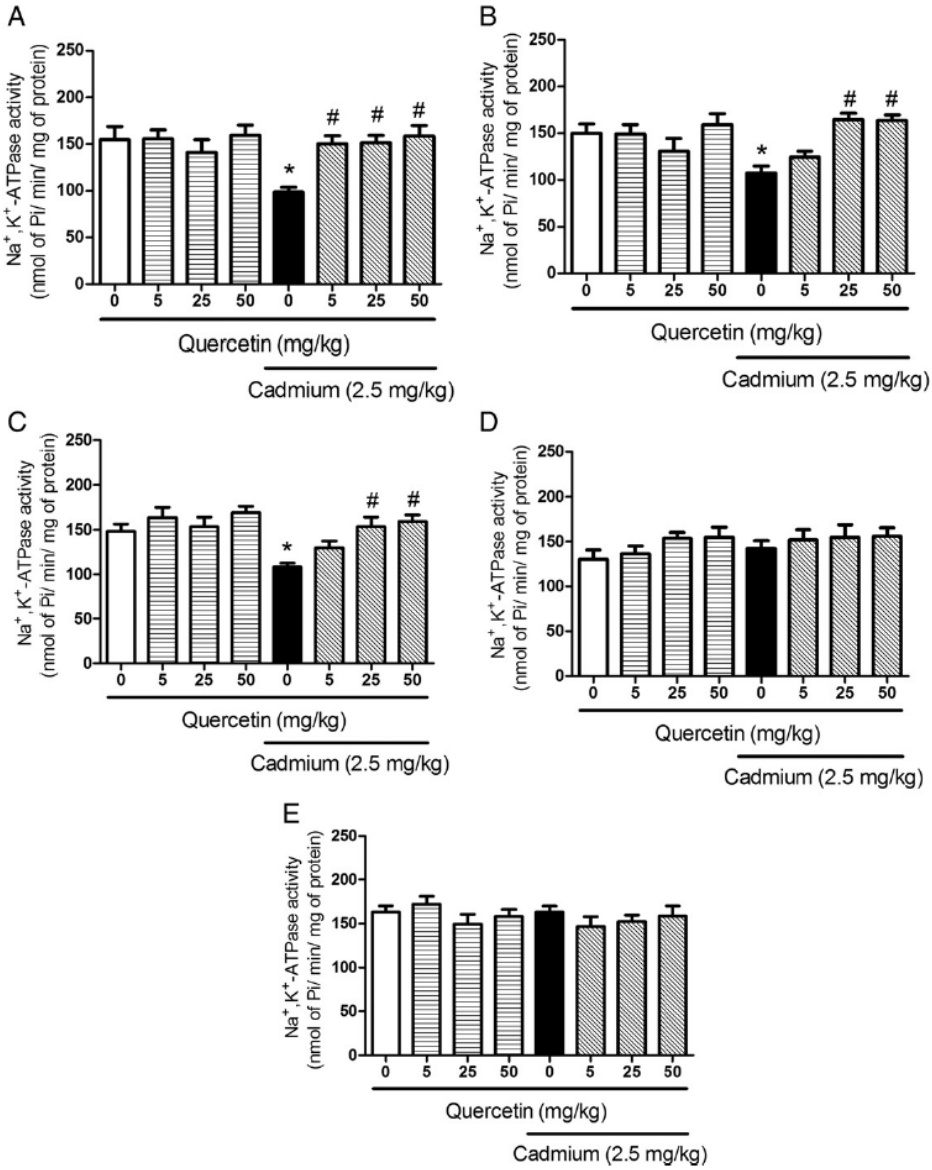


Fig. 4. Na^+,K^+-ATPase activity in homogenate of cerebral cortex (A), hippocampus (B), hypothalamus (C), striatum (D) and cerebellum (E) of cadmium (Cd)-exposed rats and treated with quercetin. *$p < 0.05$ compared with the saline/control, and #$p < 0.05$ compared with the Cd/ethanol group. Bars represent means ± S.E.M. One-way ANOVA–Student–Newman–Keuls (SNK) ($p < 0.05$) ($n = 10–14$).

per se, no significant differences in the cerebral cortex, hippocampus, hypothalamus, striatum and cerebellum were observed in the saline/Querc 5, saline/Querc 25 or saline/Querc 50 groups when compared to the saline/control group.

3.8. Quercetin prevented the alterations induced by Cd in protein carbonyl levels in the cerebral cortex, hippocampus and hypothalamus

The protein carbonyl levels in the cerebral cortex, hippocampus and hypothalamus were significantly higher in the Cd/ethanol group when compared to the saline/control group (226.3%, 178%, 140% respectively) ($p < 0.05$) (Table 4). The co-treatment with quercetin was able to prevent the rise of protein carbonyl levels in the Cd/Querc 5, Cd/Querc 25 and Cd/Querc 50 groups, when compared to the Cd/ethanol group ($p < 0.05$). No significant differences were observed in the protein carbonyl levels in the striatum and cerebellum. Moreover, when quercetin was given per se, no significant differences were observed in the saline/Querc 5, saline/Querc 25 or saline/Querc 50 groups when compared to the saline/control group.

3.9. Cytotoxic effects of Cd in the cerebral cortex, hippocampus and hypothalamus of cadmium Cd-exposed rats were prevented by quercetin treatment

The cerebral cortex, hippocampus and hypothalamus dsDNA fractions (Table 5) presented a significant increase in the Cd/ethanol

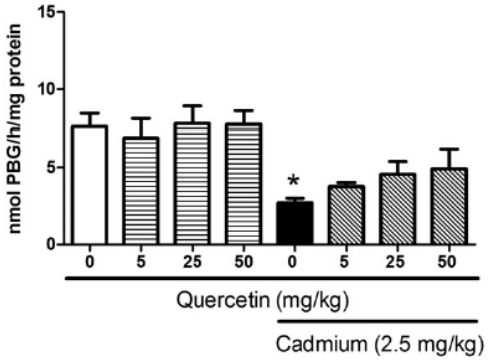


Fig. 5. δ-Aminolevulinic acid dehydratase activity in the total brain of cadmium (Cd)-exposed rats and treated with quercetin. *$p < 0.05$ compared with the saline/control, and #$p < 0.05$ compared with the Cd/ethanol group. Bars represent means ± S.E.M. (Two-way ANOVA followed by Tukey test; $n = 7–10$.)

group when compared to the saline/control group (495%, 114%, 42% respectively) ($p < 0.05$). The co-treatment with Cd and quercetin prevented this increase in dsDNA fractions in the cerebral cortex, hippocampus and hypothalamus in the Cd/Querc 5, Cd/Querc 25 and Cd/Querc 50 groups when compared to the Cd/ethanol group. No significant differences ($p < 0.05$) were observed in the dsDNA levels in the striatum and cerebellum. Moreover, when quercetin was given per se, no significant differences were observed in the saline/Querc 5, saline/Querc 25 or saline/Querc 50 groups when compared to the saline/control group.

3.10. Effect of Cd exposure and quercetin treatment on vitamin C levels in the cerebral cortex, hippocampus, hypothalamus, striatum and cerebellum

The results obtained for vitamin C levels presented no significant difference among the different groups treated with Cd alone or Cd co-treated with quercetin. Moreover, when quercetin was given per se, no significant differences were observed in the saline/Querc 5, saline/Querc 25 or saline/Querc 50 groups when compared to the saline/control group (data not shown).

3.11. Effects of quercetin on the glutathione redox system

The effects of Cd exposure and quercetin treatment on T-SH, GSH, GR and GST in the cerebral cortex, hippocampus, hypothalamus, striatum and cerebellum are shown in the Tables.

Results obtained for T-SH, GSH and GR in different brain structures are presented in Tables 6, 7 and 8 respectively. The exposure to Cd decreased T-SH, GSH and GR activities significantly in the cerebral cortex (51.3%, 67.6% and 80.6% respectively), hippocampus (56%, 42.2% and 71% respectively) and hypothalamus (55%, 59% and 72% respectively) when compared with the saline/control group. On the other hand, co-treatment with quercetin 5, 25 or 50 mg/kg was able to prevent these reductions ($p < 0.05$). On the other hand, GST activity was significantly higher in the Cd/ethanol group in the cerebral cortex (163%), hippocampus (70%) and hypothalamus (61%) when compared with the saline/control group ($p < 0.05$) (Table 9). Interestingly, the co-treatment with quercetin prevented significantly the rise of GST activity in the Cd/Querc 5, Cd/Querc 25 and Cd/Querc 50 groups in both structures ($p < 0.05$).

When quercetin was given per se, no significant differences were observed in the saline/Querc 5, saline/Querc 25 and saline/Querc 50 groups when compared to the saline/control group. Moreover, no significant differences were observed in the T-SH, GSH, GR and GST activities in the striatum and cerebellum.

4. Discussions and conclusion

The Cd exposure to the general population occurs mainly through the ingestion of contaminated food [68]. This element is one of the most environmentally abundant and distributed toxic metals that affect many organs of the body including the CNS because of its elevated half-life in the organisms [1]. It is important to point out that the CNS has fewer antioxidant defenses besides being rich in easily oxidizable lipid and containing high levels of transition metals involved in the formation of free radicals [69]. Thus, the CNS may be seriously affected by Cd indirectly producing ROS and gradually damaging the BBB. This way, it could facilitate the accumulation of Cd in the brain and contribute to the development of cerebral edema and cellular dysfunction [70].

The aim of the present study was to investigate the possible neurotoxic mechanism caused by Cd exposure in memory and anxiogenic-like behavior as well as the therapeutic potential effect of quercetin against cerebral disorders triggered by Cd intoxication. In this context, it is important to note that Cd exposed rats showed a small but significant amount of Cd in the brain structures (data not shown), which was enough to cause brain injury as observed by alterations in oxidative stress parameters and cerebral enzymes evaluated. These alterations are probably linked with cerebral disorders triggered by Cd intoxication.

The inhibitory avoidance task is a classical model of behavior, which uses an aversive stimulus to evaluate learning and memory in rats and mice [71]. A significant decrease in the step-down latency to Cd-exposed rats was observed in our study suggesting learning and memory impairment in these animals. In addition, our results corroborate with data recently published by our research group in which Cd salt (1, 5 or 25 mg/kg) and Cd accumulated in potato tubers and promoted memory impairment in rats [14]. Really, some studies have reported that Cd induces neurotoxicity in animals with a wide spectrum of clinical alterations such as changes in the normal brain neurochemistry as well as neurological and behavioral disturbances [12,14,38].

Moreover, we observed that Cd exposed rats co-treated with quercetin presented similar results in the step-down latency in the inhibitory avoidance test when compared to the saline/control group. These findings indicate that the treatment with this flavonoid was able to prevent learning and memory impairment induced by Cd exposure. In addition,

Table 2
Intracellular reactive oxygen species (ROS) production by 2′-7′-dichlorofluorescein (DCF) levels in the cerebral cortex, hippocampus, hypothalamus, striatum and cerebellum of cadmium Cd-exposed rats and treated with quercetin.

ROS	Cortex	Hippocampus	Hypothalamus	Striatum	Cerebellum
Saline/control	13.04 ± 0.94	5.57 ± 0.34	12.93 ± 1.55	5.55 ± 1.05	2.23 ± 0.15
Saline/Querc 5	10.87 ± 0.79	6.08 ± 0.10	10.81 ± 0.72	4.47 ± 0.69	2.22 ± 0.64
Saline/Querc 25	12.15 ± 0.88	6.38 ± 0.75	11.03 ± 0.60	4.68 ± 0.92	2.55 ± 0.35
Saline/Querc 50	10.98 ± 0.78	4.90 ± 0.97	10.98 ± 0.78	4.91 ± 0.76	2.02 ± 0.18
Cd/ethanol	24.02 ± 1.30*	10.48 ± 1.39*	24.57 ± 1.63*	3.79 ± 1.19	2.02 ± 0.21
Cd/Querc 5	10.99 ± 0.85#	3.55 ± 0.75#	13.02 ± 1.71#	3.56 ± 0.62	1.57 ± 0.23
Cd/Querc 25	11.89 ± 0.77#	3.06 ± 0.72#	11.60 ± 0.31#	4.79 ± 0.84	2.32 ± 0.22
Cd/Querc 50	12.86 ± 1.96#	3.04 ± 1.12#	13.33 ± 1.07#	4.05 ± 0.49	1.57 ± 0.07

Data are expressed as means ± S.D. *$p < 0.05$ compared with the saline/control, and #$p < 0.05$ compared with the Cd/ethanol group. (Two-way ANOVA followed by Tukey test.)

Table 3
Thiobarbituric acid reactive substances (TBARSs) in the cerebral cortex, hippocampus, hypothalamus, striatum and cerebellum of cadmium Cd-exposed rats and treated with quercetin.

TBARS level	Cortex	Hippocampus	Hypothalamus	Striatum	Cerebellum
Saline/control	15.31 ± 1.77	16.06 ± 1.30	12.16 ± 1.96	17.30 ± 1.57	15.31 ± 1.77
Saline/Querc 5	15.21 ± 2.38	17.79 ± 1.86	12.12 ± 2.04	18.85 ± 2.08	15.21 ± 2.38
Saline/Querc 25	16.08 ± 1.28	15.46 ± 2.27	14.11 ± 1.04	18.16 ± 0.54	16.08 ± 1.28
Saline/Querc 50	16.04 ± 1.45	18.43 ± 2.52	12.64 ± 1.72	17.88 ± 0.84	16.04 ± 1.45
Cd/ethanol	17.39 ± 2.29*	26.39 ± 3.05*	28.20 ± 1.23*	21.03 ± 2.87	17.39 ± 2.29
Cd/Querc 5	17.40 ± 2.15#	16.16 ± 1.04#	18.48 ± 0.276#	18.92 ± 1.85	17.40 ± 2.15
Cd/Querc 25	17.32 ± 1.37#	16.18 ± 2.37#	17.73 ± 1.37#	19.31 ± 2.00	17.32 ± 1.37
Cd/Querc 50	16.69 ± 2.37#	7.359 ± 1.93#	8.797 ± 0.59#	18.74 ± 2.42	16.69 ± 2.37

Data are expressed as means ± S.D. *$p < 0.05$ compared with the saline/control, and #$p < 0.05$ compared with the Cd/ethanol group. (Two-way ANOVA followed by Tukey test.)

our results corroborate with data recently published by our research group in which treatment with quercetin and another antioxidant, N-acetylcysteine, partially prevented neurotoxicity effects caused by Cd exposure [37], suggesting a role of antioxidant compounds in the protection of the memory deficits [10].

Immediately after the session of inhibitory avoidance, animals were subjected to an open-field test in order to identify any motor disorders that could influence the performance in this task [72]. Our results demonstrated that Cd did not alter the number of crossing or rearing responses in the open-field test session, suggesting that neither Cd nor quercetin caused gross motor disabilities at testing. We also observed that Cd did not alter foot shock sensitivity as demonstrated by similar flinch and jump thresholds exhibited by the animals. Therefore, these data exclude the possibility that locomotor activity or shock sensitivity may have contributed to the alterations in step-down latencies observed in the inhibitory avoidance test in Cd-exposed rats.

Another central effect that we highlighted was the anxiolytic property of quercetin against Cd anxiogenic properties. Our results have shown that Cd exposure caused an increase in anxiogenic-like behavior, as observed in the elevated plus-maze task. In fact, some studies have shown that Cd exposure increases anxiety. Goncalves et al. [14] showed that 2 mg/kg Cd in the diet caused higher anxiety in rats. Moreover, Antonio et al. [73] showed that 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 behavior. Although the mechanism by which these metals are able to alter behavior in the elevated plus-maze is still not fully established, it is suggested that there is a link with hippocampal serotoninergic and dopaminergic neurons as well as the involvement of AChE and Na^+,K^+-ATPase activities in the anxiety alterations [14,73].

On the other hand, in this study we showed that the treatment with quercetin was able to prevent the anxiogenic effect induced by Cd exposure. These results are in accordance with several studies that have showed that quercetin produces a variety of anxiolytic-like behavioral effects [74–76]. Among the mechanisms by which the quercetin produces these effects is the modulation of neurotransmitter systems associated with anxiety and depression-like GABAergic and serotonergic systems [50]. Therefore, considering our results, the present study is in accordance with the anxiogenic effect caused by Cd exposure as well as the anxiolytic-like effect reported for quercetin. To our knowledge, this is the first work that reports the beneficial actions of quercetin against Cd-mediated anxiolytic-like behavior and memory impairment in rats.

Another important aspect to be discussed here is the effect of Cd on the cholinergic system, which plays a crucial role in regulating learning, memory, and cortical organization of movement [77]. AChE enzyme, which hydrolyzes the neurotransmitter ACh in many tissues, is responsible for correcting the cholinergic function [21]. In the present study, Cd exposure decreased the AChE activity in the cerebral cortex and hippocampus. On the other hand, the activation of AChE activity in the hypothalamus was observed in rats exposed to this metal. In the striatum and cerebellum there is no significant difference between groups. In contrast to our results, Goncalves et al. [10] showed a decrease in the AChE activity in the hippocampus, cerebellum and hypothalamus, while no alterations were observed in the cerebral cortex synaptosomes and striatum of rats exposed to Cd (2 mg/kg). However, the dose of Cd and the experimental period of exposure to this metal were less than those used in this study.

It is important to point out that the results described in literature, related to the effects of Cd on the AChE activity are controversial [12,13]. Some studies have shown that Cd can influence the AChE activity in a different way after short- and long-term administration [12,78,79]. In another study, Carageorgiou et al. [12,79] showed an increase in the AChE activity in the brain of rats exposed to 1 mg/kg Cd intraperitoneally for 14 days or intramuscularly for 4 months. Taking these studies together, it is plausible to suggest that the different results with respect to AChE activity in Cd-exposed animals in our study could be explained by the different routes and doses of Cd administration as well as differences in the biological samples assayed and different periods of exposure.

Several mechanisms have been proposed to explain the effects of Cd on AChE activity. Casalino et al. [80] suggested that Cd may alter the AChE activity either by the displacement of metallic ions from the active site or by the direct deactivation of the site from the enzyme. In addition, Appleyard [21] suggested that the inactivation of the AChE enzyme could be a result of the occupation of its active sites by heavy metals. It is important to note that an inhibition in the AChE activity caused by Cd can lead to a lesser hydrolysis of the ACh neurotransmitter in synapse and consequently an abnormal amount of this neurotransmitter can cause an overactivation of cholinergic receptors and possible toxic

Table 4
Carbonylation of proteins in cerebral cortex, hippocampus, hypothalamus, striatum and cerebellum of cadmium Cd-exposed rats and treated with quercetin.

Protein carbonyl	Cortex	Hippocampus	Hypothalamus	Striatum	Cerebellum
Saline/control	1.33 ± 0.17	1.49 ± 0.18	1.60 ± 0.19	1.46 ± 0.19	1.63 ± 0.21
Saline/Querc 5	1.30 ± 0.08	1.65 ± 0.09	1.19 ± 0.08	1.52 ± 0.14	1.44 ± 0.21
Saline/Querc 25	1.19 ± 0.85	1.17 ± 0.10	1.09 ± 0.25	1.17 ± 0.10	1.39 ± 0.05
Saline/Querc 50	1.32 ± 0.07	1.44 ± 0.14	1.27 ± 0.06	1.19 ± 0.08	1.19 ± 0.08
Cd/ethanol	4.34 ± 0.67*	4.15 ± 0.24*	3.84 ± 0.30*	1.32 ± 0.07	1.56 ± 0.18
Cd/Querc 5	1.85 ± 0.39#	2.46 ± 0.20#	1.89 ± 0.31#	1.33 ± 0.17	1.45 ± 0.16
Cd/Querc 25	1.42 ± 0.19#	2.09 ± 0.22#	1.14 ± 0.12#	1.30 ± 0.17	1.17 ± 0.10
Cd/Querc 50	1.19 ± 0.04#	1.21 ± 0.02#	1.04 ± 0.10#	1.19 ± 0.08	1.19 ± 0.85

Data are expressed as means ± S.D. *$p < 0.05$ compared with the saline/control, and #$p < 0.05$ compared with the Cd/ethanol group. (Two-way ANOVA followed by Tukey test.)

Table 5
dsDNA fractions in the cerebral cortex, hippocampus, hypothalamus, striatum and cerebellum of cadmium Cd-exposed rats and treated with quercetin.

dsDNA	Cortex	Hippocampus	Hypothalamus	Striatum	Cerebellum
Saline/control	6.69 ± 0.36	35.54 ± 0.52	4.98 ± 0.37	9.67 ± 0.14	9.64 ± 1.81
Saline/Querc 5	8.10 ± 0.33	42.69 ± 3.09	4.74 ± 0.08	9.74 ± 0.34	11.29 ± 1.15
Saline/Querc 25	7.85 ± 0.39	35.04 ± 2.62	4.47 ± 0.14	9.67 ± 0.14	9.22 ± 0.46
Saline/Querc 50	7.62 ± 0.02	40.30 ± 2.90	4.35 ± 0.22	9.74 ± 0.24	8.37 ± 1.06
Cd/ethanol	39.80 ± 1.94*	76.17 ± 2.98*	7.05 ± 0.76*	11.28 ± 0.76	8.33 ± 0.76
Cd/Querc 5	7.36 ± 0.15#	30.20 ± 3.80#	5.03 ± 0.22#	12.01 ± 0.36	9.16 ± 0.51
Cd/Querc 25	6.73 ± 0.25#	22.51 ± 2.55#	5.46 ± 0.15#	12.05 ± 0.38	9.43 ± 1.44
Cd/Querc 50	7.98 ± 0.18#	22.50 ± 1.28#	5.06 ± 0.14#	11.10 ± 0.84	7.68 ± 0.85

Data are expressed as means ± S.D. *$p < 0.05$ compared with the saline/control, and #$p < 0.05$ compared with the Cd/ethanol group. (Two-way ANOVA followed by Tukey test.)

effects [81]. On the other hand, it has been reported that the AChE activation leads to fast ACh degradation and a subsequent down stimulation of ACh receptors causing undesirable effects on cognitive functions [82,83].

In the present study the treatment with quercetin was able to abolish these undesirable effects caused by Cd exposure. Quercetin treatment prevents alterations in the AChE activity in all cerebral structures, suggesting that this compound is able to modulate the level of ACh on the CNS in Cd-exposed rats. Moreover, these results are also in accordance with those observed by our research group, which showed that cerebral AChE activity was restored by various natural compounds that have antioxidant activity such as resveratrol, N-acetylcysteine and α-tocopherol as well as quercetin, suggesting that these compounds may modulate the cholinergic neurotransmission and improve cognition [10,37,84,85]. In line with this, we can suggest that the antioxidant property of quercetin may contribute to the prevention of cholinergic dysfunction in Cd-exposed rats.

Another important enzyme evaluated in our study was Na^+,K^+-ATPase which presented a reduced activity in the cerebral cortex, hippocampus and hypothalamus in rats exposed to Cd. However, in the striatum and cerebellum there was no significant difference between the groups. These results are in agreement with those found by Goncalves et al. [14], who showed a decreased activity of this enzyme in the cerebral cortex, hypothalamus, and cerebellum of rats exposed to Cd salt or Cd from contaminated tubers. The inhibition of Na^+,K^+-ATPase activity by Cd could contribute to cerebral damage leading to an earlier stage of edema, followed by a later stage of degeneration and necrosis. Moreover, it has been reported that Na^+,K^+-ATPase inhibition can impair learning and memory in the Morris water maze and step-through passive avoidance tasks [87,88], showing an important role of this enzyme in learning and memory processes which could be associated with Cd-induced impaired cognition observed in the present investigation.

Several hypotheses have been proposed for the inhibition of Na^+, K^+-ATPase in Cd exposure. Rajanna et al. [89] proposed that Cd may compete with ATP and Na^+ sites on Na^+,K^+-ATPase inhibiting its activity in rat brain synaptosomes. On the other hand, Chetty et al. [90] reported that Cd interferes with the phosphorylation state of the enzyme which results in its inhibition. Furthermore, recent research has suggested that the Na^+,K^+-ATPase is susceptible to free radical-induced damage [31]. Thus, we suggest that the decrease in Na^+,K^+-ATPase activity found in animals exposed to Cd may be associated with the elevation of the lipid peroxidation in cerebral structures observed in this study, since similar to AChE, the Na^+,K^+-ATPase is a biological component of the membrane and therefore can respond to oxidative deterioration of this structure.

Interestingly, the treatment with quercetin was able to reverse this effect caused by Cd exposure. In fact, Stefanello et al. [91] 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. Therefore, considering that the antioxidant potential of quercetin is already well established, we suggest that the treatment with this flavonoid can protect against Cd-induced oxidative stress, as observed by the prevention of increased TBARS levels in our study. In this context, we believe that the prevention of increased TBARS levels may prevent modifications of the conformational state of Na^+,K^+-ATPase and maintain the resting membrane potential of neurons.

Another important aspect observed in this study was the decrease in δ-ALA-D activity in Cd exposed rats. Of particular importance, this inhibition of δ-ALA-D may impair the heme biosynthetic pathway [57] and lead to the accumulation of its substrate, ALA [35], which can autooxidize, leading to reactive ROS. These, in turn, are harmful to biological systems, since it can oxidize numerous biomolecules leading to tissue injury and cell death [92]. The δ-ALA-D is a metalloenzyme that requires Zn^{2+} for maximal catalytic activity and data support the hypothesis of a direct competition between bivalent metals and Zn^{2+} on δ-ALA-D from mammals and bacteria [34,40]. Thus, a probable mechanism to decrease δ-ALA-D activity in Cd exposed rats could be Zn^{2+} displacement caused by Cd increase.

Several authors have demonstrated that the use of chelating and/or antioxidants could be a good alternative in the treatment of poisoning by Cd [13,14,37]. These compounds act by increasing the excretion of these toxic elements, decreasing toxicity by preventing binding of these molecules to target cell [93]. In this context, studies in our laboratory have shown that antioxidant chelating agents such as quercetin and N-acetylcysteine are able to reverse damage caused by Cd in the CNS [10,37]. However, in the present study quercetin was not effective in reversing the inhibition of δ-ALA-D activity caused by Cd exposure.

Table 6
Effect of cadmium and treated with quercetin on total thiol groups (T-SHs) in the cerebral cortex, hippocampus, hypothalamus, striatum and cerebellum.

T-SH	Cortex	Hippocampus	Hypothalamus	Striatum	Cerebellum
Saline/control	11.85 ± 0.89	8.45 ± 0.44	8.10 ± 0.46	10.04 ± 0.65	10.51 ± 0.97
Saline/Querc 5	12.24 ± 0.93	8.66 ± 0.71	7.88 ± 0.90	10.19 ± 0.58	10.24 ± 1.48
Saline/Querc 25	13.39 ± 0.37	10.06 ± 0.11	9.52 ± 0.30	11.50 ± 0.78	11.72 ± 1.31
Saline/Querc 50	13.63 ± 0.57	10.93 ± 0.35	10.59 ± 0.92	12.01 ± 1.19	13.63 ± 0.57
Cd/ethanol	5.77 ± 0.39*	3.71 ± 0.63*	3.63 ± 0.42*	9.35 ± 0.40	10.04 ± 1.25
Cd/Querc 5	9.60 ± 0.47#	7.82 ± 0.96#	8.34 ± 0.83#	9.02 ± 0.74	10.03 ± 0.56
Cd/Querc 25	9.94 ± 0.71#	9.37 ± 1.16#	9.13 ± 0.85#	10.16 ± 0.74	10.23 ± 1.03
Cd/Querc 50	11.63 ± 1.60#	9.76 ± 1.40#	9.94 ± 0.66#	10.70 ± 0.46	9.53 ± 1.06

Data are expressed as means ± S.D. *$p < 0.05$ compared with the saline/control, #and $p < 0.05$ compared with the Cd/ethanol group. (Two-way ANOVA followed by Tukey test.)

Table 7
Effect of cadmium and treated with quercetin on reduced glutathione (GSH) in the cerebral cortex, hippocampus, hypothalamus, striatum and cerebellum.

GSH	Cortex	Hippocampus	Hypothalamus	Striatum	Cerebellum
Saline/control	1.70 ± 0.22	1.42 ± 0.07	1.45 ± 0.11	0.88 ± 0.01	1.12 ± 0.12
Saline/Querc 5	1.95 ± 0.02	1.48 ± 0.09	1.57 ± 0.07	1.06 ± 0.13	1.06 ± 0.18
Saline/Querc 25	2.06 ± 0.13	1.82 ± 0.05	1.61 ± 0.17	1.12 ± 0.11	1.14 ± 0.15
Saline/Querc 50	2.10 ± 0.06	2.26 ± 0.03	1.55 ± 0.15	1.26 ± 0.03	1.36 ± 0.05
Cd/ethanol	0.55 ± 0.06*	0.82 ± 0.04*	0.60 ± 0.06*	0.99 ± 0.09	0.97 ± 0.03
Cd/Querc 5	0.83 ± 0.05#	1.12 ± 0.07#	0.99 ± 0.05#	0.95 ± 0.03	1.03 ± 0.07
Cd/Querc 25	1.34 ± 0.14#	1.40 ± 0.20#	1.09 ± 0.10#	1.20 ± 0.14	1.12 ± 0.08
Cd/Querc 50	1.59 ± 0.16#	2.15 ± 0.10#	1.14 ± 0.12#	1.19 ± 0.16	1.23 ± 0.17

Data are expressed as means ± S.D. *$p < 0.05$ compared with the saline/control, and #$p < 0.05$ compared with the Cd/ethanol group. (Two-way ANOVA followed by Tukey test.)

These data are consistent with data found by Luchese [13] who showed that antioxidants such as ascorbic acid and NAC failed to reverse the inhibition of the activity of δ-ALA-D induced metal.

Knowing that, the potential pro-oxidant property of Cd is an important mechanism that has been proposed to explain the undesirable effects of Cd and considering the results obtained in this study related to AChE, Na^+,K^+-ATPase and δ-ALA-D activities, we investigated, further, the effects of Cd exposure on parameters of oxidative stress as well as antioxidant enzyme activities and endogenous non-enzymatic antioxidants to understand the possible mechanisms by which the Cd induced neurotoxicity and caused alterations in different brain structures. Moreover, these data can better clarify the possible mechanism by which the quercetin exerts its neuroprotective effects.

The oxidative effect of Cd is associated mainly with the depletion of the sulfhydryl (SH)-group containing compounds [94]. Thus, one of the possible effects of Cd toxicity is the indirect production of ROS [40]. In this context, it is important to note that of all the organs, the brain must be considered more sensitive to oxidative stress due to the following features: high oxygen consumption, high levels of iron crucial to lipid peroxidation of the membrane through the Fenton reaction, relatively low levels of protective antioxidants agents, presence of excitotoxic amino acids (glutamate), and ability of microglia (CNS macrophages) to produce ROS in activation and secrete inflammatory cytokines among others. Therefore, an important result to be discussed here is that, we observed an increased production of ROS in the cerebral cortex, hippocampus and hypothalamus. It is suggested that these brain structures are more vulnerable to the action of ROS compared to the cerebellum and striatum wherein change was not observed. Moreover, it is also important to note that, each brain structure presents a structural feature and functions differently, which may have contributed in different responses in relation to the cerebellum and striatum. However, although there are differences in vulnerability between the brain structures in animals exposed to Cd, there have been no studies in the literature that explain these differences.

Increased production of ROS has been observed in many crop species exposed to toxic levels of the metals Cd, Pb, Fe, Ni, Cu, As, etc., and ROS production has been considered as one of the consequences of metal toxicity [95] since the constant increase in ROS production may decrease the effectiveness of the antioxidant system installing a condition called oxidative stress. Oxidative stress affects numerous cellular components, such as proteins, DNA and lipids through oxidation reactions. These alterations in structure produce significant changes in cellular function [70].

In fact, the elevation in ROS production found in our study was accompanied by an increase in TBARS, protein carbonyl and dsDNA levels in the cerebral cortex, hippocampus and hypothalamus of Cd exposed rats. Oxidative damage to proteins can occur directly by the interaction of the protein with ROS or indirectly by the interaction of the protein with a secondary product (resulting from the interaction of the radical with lipid or sugar molecule). Protein damage can be an irreversible oxidative damage, often leading to the loss of protein function as well as cell death [96]. In the present study, Cd exposed rats showed an increase in TBARS parallel to an increase in protein carbonyl levels. Taken together these findings suggest that the decrease in δ-ALA-D activity in Cd exposed rats observed this study may be closely associated with increased production of ROS. Consequently, this increase in ROS production in Cd exposed rats induces a significant increase in oxidative stress resulting to an increase in TBARS and consequently oxidative damage to proteins, in the cerebral cortex, hippocampus and hypothalamus of Cd-exposed rats, that can alter membrane function process that can cause cell death in several tissues including the brain [4,97,98].

In this context, it is important to note that enzymes such as AChE and Na^+,K^+-ATPase are significant components of the biological membranes and, thus, can be important targets of oxidation of membrane caused by Cd exposure [99]. These results allow us to infer that, these alterations may explain the changes in the activities of these enzymes found in our study and, consequently, impairment of memory and anxiogenic-like behavior observed in Cd exposed rats (Fig. 6). Recent results published by our research group are in agreement with the results of the present study [10,14], suggesting that alterations in the lipid membrane observed after the Cd exposure could be a decisive factor in the modification of the conformational state of the AChE molecule.

Another important result to be discussed is the DNA strand breaks. In view of this, it is clear that the oxidative stress can affect DNA through oxidation reactions. Moreover, there are studies showing that Cd is able to generate apoptosis both dependently and independently of the concentration and time of exposure [100]. Cd induces DNA damage [101], interferes with DNA repair processes and enhances genotoxic damage [102]. Corroborating with these studies, our results showed an increase in dsDNA levels in the cerebral cortex, hippocampus and hypothalamus

Table 8
Effect of cadmium and treated with quercetin on reductase glutathione (GR) in the cerebral cortex, hippocampus, hypothalamus, striatum and cerebellum.

GR	Cortex	Hippocampus	Hypothalamus	Striatum	Cerebellum
Saline/control	13.51 ± 1.23	10.32 ± 0.24	0.89 ± 0.07	0.55 ± 0.09	1.20 ± 0.15
Saline/Querc 5	13.86 ± 2.07	10.31 ± 0.44	0.95 ± 0.21	0.59 ± 0.11	1.27 ± 0.24
Saline/Querc 25	15.23 ± 0.72	9.94 ± 0.84	1.05 ± 0.21	0.54 ± 0.07	1.13 ± 0.25
Saline/Querc 50	14.67 ± 2.42	10.41 ± 1.30	1.15 ± 0.14	0.71 ± 0.05	1.24 ± 0.20
Cd/ethanol	2.62 ± 0.7*	3.09 ± 0.62*	0.25 ± 0.01*	0.51 ± 0.17	1.03 ± 0.04
Cd/Querc 5	12.73 ± 0.81#	4.82 ± 2.09#	0.31 ± 0.09#	0.55 ± 0.06	1.12 ± 0.13
Cd/Querc 25	16.76 ± 2.82#	5.59 ± 1.96#	0.44 ± 0.11#	0.47 ± 0.02	1.13 ± 0.14
Cd/Querc 50	17.92 ± 1.39#	5.28 ± 1.65#	0.49 ± 0.10#	0.51 ± 0.13	1.17 ± 0.12

Data are expressed as means ± S.D. *$p < 0.05$ compared with the saline/control, and #$p < 0.05$ compared with the Cd/ethanol group. (Two-way ANOVA followed by Tukey test.)

Table 9
Effect of cadmium and treated with quercetin on glutathione S-transferase (GST) in the cerebral cortex, hippocampus, hypothalamus, striatum and cerebellum.

GST	Cortex	Hippocampus	Hypothalamus	Striatum	Cerebellum
Saline/control	1.82 ± 0.01	8.12 ± 0.44	0.92 ± 0.04	1.19 ± 0.10	1.45 ± 0.11
Saline/Querc 5	1.61 ± 0.20	8.02 ± 0.87	0.78 ± 0.03	1.07 ± 0.16	1.39 ± 0.21
Saline/Querc 25	1.75 ± 0.15	7.93 ± 1.02	0.69 ± 0.82	1.04 ± 0.03	1.10 ± 0.13
Saline/Querc 50	1.47 ± 0.26	7.33 ± 0.71	0.40 ± 0.01	1.07 ± 0.05	1.01 ± 0.21
Cd/ethanol	4.78 ± 0.38*	13.77 ± 0.99*	1.48 ± 0.15*	1.29 ± 0.19	1.54 ± 0.23
Cd/Querc 5	2.30 ± 0.52#	8.14 ± 0.96#	0.73 ± 0.07#	1.07 ± 0.14	1.06 ± 0.084
Cd/Querc 25	1.75 ± 0.09#	6.29 ± 1.42#	0.63 ± 0.06#	1.04 ± 0.08	1.04 ± 0.17
Cd/Querc 50	1.61 ± 0.16#	4.22 ± 1.25#	0.49 ± 0.05#	0.89 ± 0.14	0.97 ± 0.09

Data are expressed as means ± S.D. *$p < 0.05$ compared with the saline/control, and #$p < 0.05$ compared with the Cd/ethanol group. (Two-way ANOVA followed by Tukey test.)

of Cd exposed rats. However, in the striatum and cerebellum there was no significant difference between the groups. Taken together, these findings suggest that Cd exposure caused an increase in DNA damage indirectly due to the interaction of DNA with ROS and/or due to the diminished repair capacity of DNA in the cerebral cortex, hippocampus and hypothalamus of Cd-exposed rats.

Parallel to this increase in ROS production and increase in TBARS, protein carbonyl levels and dsDNA levels in the cerebral cortex, hippocampus and hypothalamus of Cd exposed rats, we also found alterations in the antioxidant system of cells, including an decrease in GR activity and T-SH and GSH levels, as well as an increase in GST activities. More over, it is important to note that no significant difference was observe in the striatum and cerebellum.

GSH is part of a non-enzymatic defense system. It is a central protec tive antioxidant and considered the first line of defense against oxida tive damage and free radical generation in the brain tissue. It ca directly scavenge free radicals or act as a substrate for GPx and GST i the detoxification of hydrogen peroxide [103]. Moreover, GSTs are ir volved in response to the oxidative stress including heavy metal Based on the results of the present study, we suggest that the depletio

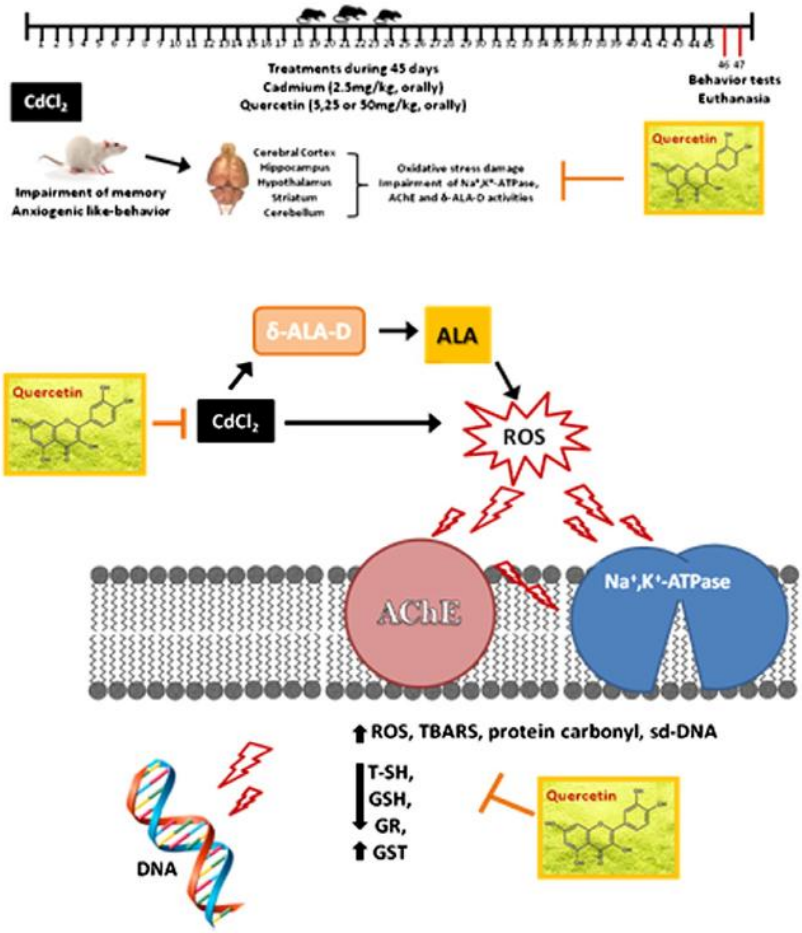


Fig. 6. Possible neuroprotective mechanism of quercetin in the brain of Cd exposed rats.

of GSH and T-SH levels and GR activity upon Cd intoxication could be due to increased utilization to overwhelm the free radicals and lipid peroxidation and protein oxidation products in the brain. Moreover, the decrease in the activities of antioxidant enzymes might be due to the binding of Cd with the –SH groups of enzymes and oxidative modifications of amino acid chains, which alter the enzyme structure and lead to the inactivation or decreased activity of enzyme. On the other hand, in the present study, we found the increase in the GST activity in the cerebral cortex, hippocampus and hypothalamus of Cd-exposed rats. It is important to note that under oxidative stress, the excessive ROS induces an increase in GST levels, and then the GSTs metabolize the toxic products of lipid peroxidation, protein oxidation, damaged DNA and other molecules [104]. Thus, we suggest that the increase in the GST activity can be a compensatory mechanism to metabolize the toxic products of lipid peroxidation, protein oxidation and damaged DNA observed in the cerebral cortex, hippocampus and hypothalamus of Cd-exposed rats.

Taking these results together, we believe that one of the mechanisms associated with differential responses to Cd in different brain structures, found in our study may be related to different structures of the vulnerability to oxidative stress. Because of that, although the cerebellum and striatum showed an increase in the levels of Cd similar to that observed in other brain structures, increased ROS production and levels of lipid peroxidation, protein oxidation and DNA damage occurred only in the hippocampus, hypothalamus and cerebral cortex. Moreover, we observed alterations of enzymatic and non-enzymatic antioxidants only in these structures.

On the other hand, the beneficial effects of flavonoids in the CNS, acting as neuroprotector, primarily by reducing ROS, as well as avoiding the neurotoxicity of other substances are well established in the literature [10,37,86]. Moreover, several studies have established that the presence of these compounds in the CNS has been found in rats supplemented with quercetin, suggesting that quercetin can cross the BBB and exert neuroprotective effects [105]. Therefore, another important point to note in this study is that the co-administration of quercetin was able to totally or partially prevent the undesirable effects caused by Cd exposure in lipid peroxidation, protein carbonyl and DNA damage as well as in the enzymatic and non-enzymatic antioxidant defenses. This effect can be reached through the antioxidant properties of quercetin or due to chelating some elements as metals involved in producing free radicals, sweeping of ROS, and, furthermore, increasing or protecting the antioxidant defenses [42]. Corroborating with these results, other studies have demonstrated that quercetin is able to decrease lipid peroxidation, improve the CAT and SOD activities [98,106], prevent the glutathione depletion as well as attenuate the neuronal death in the hippocampus, resulting in improved learning and memory in the arm maze test [86]. These observations emphasize the antioxidant potential of quercetin in the brain and allow us to suggest that the possible mechanism of the neuroprotective effects of quercetin in Cd exposed rats is the establishment of oxidative stress as well as the antioxidant system (Fig. 6). Thus, we can suggest that orally administrated quercetin can be a coadjuvant in the treatment of mental stress, depression and possible metal poisoning such as Cd.

In conclusion, to our knowledge, this is the first work that reports the possible mechanism of quercetin on effective protection against the neurotoxicity and consequent memory impairment in Cd exposed rats. Our findings suggest that quercetin can modulate cholinergic neurotransmission resting membrane potential of neurons by modulation in the enzymatic and non-enzymatic antioxidant defense system, preserving the cellular integrity. These effects, consequently improved the memory deficits and anxiogenic-like behavior observed in Cd exposure, possibly by modulation of AChE, Na^+,K^+-ATPase and δ-ALA-D activities. Thus, this study may contribute to a better understanding of the neuroprotective role of quercetin, emphasizing the influence of this polyphenol and other antioxidants in the diet for human health, possibly preventing brain injury associated with Cd poisoning.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

[1] Jarup L, Rogenfelt A, Elinder CG, Nogawa K, Kjellstrom T. Biological half-time of cadmium in the blood of workers after cessation of exposure. Scand J Work Environ Health 1983;9:327–31.
[2] Olsson IM, Bensryd I, Lundh T, Ottosson H, Skerfving S, Oskarsson A. Cadmium in blood and urine—impact of sex, age, dietary intake, iron status, and former smoking—association of renal effects. Environ Health Perspect 2002;110:1185–90.
[3] Sinha M, Manna P, Sil PC. Induction of necrosis in cadmium-induced hepatic oxidative stress and its prevention by the prophylactic properties of taurine. J Trace Elem Med Biol 2009;23:300–13. http://dx.doi.org/10.1016/j.jtemb.2009.03.010.
[4] Mendez-Armenta M, Villeda-Hernandez J, Barroso-Moguel R, Nava-Ruiz C, Jimenez-Capdeville ME, Rios C. Brain regional lipid peroxidation and metallothionein levels of developing rats exposed to cadmium and dexamethasone. Toxicol Lett 2003;144:151–7.
[5] Mendez-Armenta M, Barroso-Moguel R, Villeda-Hernandez J, Nava-Ruiz C, Rios C. Histopathological alterations in the brain regions of rats after perinatal combined treatment with cadmium and dexamethasone. Toxicology 2001;161:189–99.
[6] Provias JP, Ackerley CA, Smith C, Becker LE. Cadmium encephalopathy: a report with elemental analysis and pathological findings. Acta Neuropathol 1994;88:583–6.
[7] Holloway WR, Thor Jr DH. Social memory deficits in adult male rats exposed to cadmium in infancy. Neurotoxicol Teratol 1988;10:193–7.
[8] Holloway WR, Thor Jr DH. Cadmium exposure in infancy: effects on activity and social behaviors of juvenile rats. Neurotoxicol Teratol 1988;10:135–42.
[9] Desi I, Nagymajtenyi L, Papp A, Schulz H. Experimental model studies of pesticide exposure. Neurotoxicology 1998;19:611–6.
[10] Goncalves JF, Fiorenza AM, Spanevello RM, Mazzanti CM, Bochi GV, Antes FG, et al. N-acetylcysteine prevents memory deficits, the decrease in acetylcholinesterase activity and oxidative stress in rats exposed to cadmium. Chem Biol Interact 2010;186:53–60. http://dx.doi.org/10.1016/j.cbi.2010.04.011.
[11] Tercariol SG, Almeida AA, Godinho AF. Cadmium and exposure to stress increase aggressive behavior. Environ Toxicol Pharmacol 2011;32:40–5. http://dx.doi.org/10.1016/j.etap.2011.03.005.
[12] Carageorgiou H, Tzotzes V, Pantos C, Mourouzis C, Zarros A, Tsakiris S. In vivo and in vitro effects of cadmium on adult rat brain total antioxidant status, acetylcholinesterase, (Na^+,K^+)-ATPase and Mg^{2+}-ATPase activities: protection by L-cysteine. Basic Clin Pharmacol Toxicol 2004;94:112–8.
[13] Luchese C, Zeni G, Rocha JB, Nogueira CW, Santos FW. Cadmium inhibits delta-aminolevulinate dehydratase from rat lung in vitro: interaction with chelating and antioxidant agents. Chem Biol Interact 2007;165:127–37. http://dx.doi.org/10.1016/j.cbi.2006.11.007.
[14] Goncalves JF, Nicoloso FT, da Costa P, Farias JG, Carvalho FB, da Rosa MM, et al. Behavior and brain enzymatic changes after long-term intoxication with cadmium salt or contaminated potatoes. Food Chem Toxicol 2012;50:3709–18. http://dx.doi.org/10.1016/j.fct.2012.07.016.
[15] Scarr E. Muscarinic receptors: their roles in disorders of the central nervous system and potential as therapeutic targets. CNS Neurosci Ther 2011. http://dx.doi.org/10.1111/j.1755-5949.2011.00249.x.
[16] Kiehn O. Locomotor circuits in the mammalian spinal cord. Annu Rev Neurosci 2006;29:279–306. http://dx.doi.org/10.1146/annurev.neuro.29.051605.112910.
[17] Deiana S, Platt B, Riedel G. The cholinergic system and spatial learning. Behav Brain Res 2011;221:389–411. http://dx.doi.org/10.1016/j.bbr.2010.11.036.
[18] Hut RA, Van der Zee EA. The cholinergic system, circadian rhythmicity, and time memory. Behav Brain Res 2011;221:466–80. http://dx.doi.org/10.1016/j.bbr.2010.11.039.
[19] Klinkenberg I, Sambeth A, Blokland A. Acetylcholine and attention. Behav Brain Res 2011;221:430–42. http://dx.doi.org/10.1016/j.bbr.2010.11.033.
[20] Havekes R, Abel T, Van der Zee EA. The cholinergic system and neostriatal memory functions. Behav Brain Res 2011;221:412–23. http://dx.doi.org/10.1016/j.bbr.2010.11.047.
[21] Appleyard ME. Non-cholinergic functions of acetylcholinesterase. Biochem Soc Trans 1994;22:749–55.
[22] Chacon MA, Reyes AE, Inestrosa NC. Acetylcholinesterase induces neuronal cell loss, astrocyte hypertrophy and behavioral deficits in mammalian hippocampus. J Neurochem 2003;87:195–204.
[23] Ozkul A, Akyol A, Yenisey C, Arpaci E, Kiylioglu N, Tataroglu C. Oxidative stress in acute ischemic stroke. J Clin Neurosci 2007;14:1062–6. http://dx.doi.org/10.1016/j.jocn.2006.11.008.
[24] Shen C, Yang B, Zhou T, Duan G, Yu Y. Bioequivalence evaluation of two brands of rivastigmine of different salt forms, an acetylcholinesterase inhibitor for the treatment of Alzheimer's disease, in healthy Beagle dogs. Pharmazie 2011;66:590–3.
[25] Lingrel JB. Na,K-ATPase: isoform structure, function, and expression. J Bioenerg Biomembr 1992;24:263–70.
[26] Lingrel JB, Van Huysse J, O'Brien W, Jewell-Motz E, Askew R, Schultheis P. Structure-function studies of the Na,K-ATPase. Kidney Int Suppl 1994;44:S32–9.
[27] Lingrel JB, Van Huysse J, O'Brien W, Jewell-Motz E, Schultheis P. Na,K-ATPase: structure-function studies. Ren Physiol Biochem 1994;17:198–200.
[28] dos Reis EA, de Oliveira LS, Lamers ML, Netto CA, Wyse AT. Arginine administration inhibits hippocampal $Na^{(+)},K^{(+)}$-ATPase activity and impairs retention of an inhibitory avoidance task in rats. Brain Res 2002;951:151–7.

[29] Moseley AE, Williams MT, Schaefer TL, Bohanan CS, Neumann JC, Behbehani MM, et al. Deficiency in Na,K-ATPase alpha isoform genes alters spatial learning, motor activity, and anxiety in mice. J Neurosci 2007;27:616–26.
[30] Fujisawa H, Kajikawa K, Ohi Y, Hashimoto Y, Yoshida H. Movement of radioactive calcium in brain slices and influences on it of protoveratrine, ouabain, potassium chloride and cocaine. Jpn J Pharmacol 1965;15:327–34.
[31] Lees GJ, Lehmann A, Sandberg M, Hamberger A. The neurotoxicity of ouabain, a sodium–potassium ATPase inhibitor, in the rat hippocampus. Neurosci Lett 1990;120:159–62.
[32] Rocha JBT, Saraiva RA, Garcia SC, Gravina FS, Nogueira CW. Aminolevulinate dehydratase (δ-ALA-D) as marker protein of intoxication with metals and other pro-oxidant situations. Toxicol Res 2012;1:85.
[33] Rocha JB, Rocha LK, Emanuelli T, Pereira ME. Effect of mercuric chloride and lead acetate treatment during the second stage of rapid post-natal brain growth on the behavioral response to chlorpromazine and on delta-ALA-D activity in weaning rats. Toxicol Lett 2001;125:143–50.
[34] Rodrigues AL, Rocha JB, Pereira ME, Souza DO. Delta-aminolevulinic acid dehydratase activity in weanling and adult rats exposed to lead acetate. Bull Environ Contam Toxicol 1996;57:47–53.
[35] Emanuelli T, Rocha JB, Pereira ME, Porciuncula LO, Morsch VM, Martins AF, et al. Effect of mercuric chloride intoxication and dimercaprol treatment on delta-aminolevulinate dehydratase from brain, liver and kidney of adult mice. Pharmacol Toxicol 1996;79:136–43.
[36] Nogueira CW, Borges VC, Zeni G, Rocha JB. Organochalcogens effects on delta-aminolevulinate dehydratase activity from human erythrocytic cells in vitro. Toxicology 2003;191:169–78.
[37] Abdalla FH, Cardoso A, Pereira LB, Schmatz R, Jamile FG, Naiara S, et al. Neuroprotective effect of quercetin in ectoenzymes and acetylcholinesterase activities in cerebral cortex synaptosomes of cadmium-exposed rats. Mol Cell Biochem 2013;381:1–8.
[38] Minami A, Takeda A, Nishibaba D, Takefuta S, Oku N. Cadmium toxicity in synaptic neurotransmission in the brain. Brain Res 2001;894:336–9.
[39] Shukla A, Shukla GS, Srimal RC. Cadmium-induced alterations in blood–brain barrier permeability and its possible correlation with decreased microvessel antioxidant potential in rat. Hum Exp Toxicol 1996;15:400–5.
[40] Galan A, Garcia-Bermejo L, Troyano A, Vilaboa NE, Fernandez C, de Blas E, et al. The role of intracellular oxidation in death induction (apoptosis and necrosis) in human promonocytic cells treated with stress inducers (cadmium, heat, X-rays). Eur J Cell Biol 2001;80:312–20.
[41] Lu J, Zheng YL, Luo L, Wu DM, Sun DX, Feng YJ. Quercetin reverses D-galactose induced neurotoxicity in mouse brain. Behav Brain Res 2006;171:251–60. http://dx.doi.org/10.1016/j.bbr.2006.03.043.
[42] Ezzati Nazhad Dolatabadi J, Mokhtarzadeh A, Ghareghoran SM, Dehghan G. Synthesis, characterization and antioxidant property of quercetin-Tb(III) complex. Adv Pharm Bull 2014;4:101–4. http://dx.doi.org/10.5681/apb.2014.016.
[43] Zalups RK, Ahmad S. Molecular handling of cadmium in transporting epithelia. Toxicol Appl Pharmacol 2003;186:163–88.
[44] Milton Prabu S, Muthumani M, Shagirtha K. Quercetin potentially attenuates cadmium induced oxidative stress mediated cardiotoxicity and dyslipidemia in rats. Eur Rev Med Pharmacol Sci 2013;17:582–95.
[45] Prabu SM, Shagirtha K, Renugadevi J. Amelioration of cadmium-induced oxidative stress, impairment in lipids and plasma lipoproteins by the combined treatment with quercetin and alpha-tocopherol in rats. J Food Sci 2010;75:T132–40. http://dx.doi.org/10.1111/j.1750-3841.2010.01757.x.
[46] Renugadevi J, Prabu SM. Quercetin protects against oxidative stress-related renal dysfunction by cadmium in rats. Exp Toxicol Pathol 2010;62:471–81. http://dx.doi.org/10.1016/j.etp.2009.06.006.
[47] Maciel RM, Costa MM, Martins DB, Franca RT, Schmatz R, Graca DL, et al. Antioxidant and anti-inflammatory effects of quercetin in functional and morphological alterations in streptozotocin-induced diabetic rats. Res Vet Sci 2013;95:389–97. http://dx.doi.org/10.1016/j.rvsc.2013.04.028.
[48] Berlese DB, Sauzem PD, Carati MC, Guerra GP, Stiegemeier JA, Mello CF, et al. Time-dependent modulation of inhibitory avoidance memory by spermidine in rats. Neurobiol Learn Mem 2005;83:48–53.
[49] Rubin MA, Boemo RL, Jurach A, Rojas DB, Zanolla GR, Obregon AD, et al. Intrahippocampal spermidine administration improves inhibitory avoidance performance in rats. Behav Pharmacol 2000;11:57–61.
[50] Frussa-Filho R, Barbosa-Junior H, Silva RH, Da Cunha C, Mello CF. Naltrexone potentiates the anxiolytic effects of chlordiazepoxide in rats exposed to novel environments. Psychopharmacology (Berl) 1999;147:168–73.
[51] Rubin MA, Albach CA, Berlese DB, Bonacorso HG, Bittencourt SR, Queiroz CM, et al. Anxiolytic-like effects of 4-phenyl-2-trichloromethyl-3H-1,5-benzodiazepine hydrogen sulfate in mice. Braz J Med Biol Res 2000;33:1069–73.
[52] Ellman GL, Courtney KD, Andres V, Feather-Stone Jr RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.
[53] Rocha JB, Emanuelli T, Pereira ME. Effects of early undernutrition on kinetic parameters of brain acetylcholinesterase from adult rats. Acta Neurobiol Exp (Wars) 1993;53:431–7.
[54] Wyse AT, Streck EL, Barros SV, Brusque AM, Zugno AI, Wajner M. Methylmalonate administration decreases Na^+,K^+-ATPase activity in cerebral cortex of rats. Neuroreport 2000;11:2331–4.
[55] Carvalho FB, Mello CF, Marisco PC, Tonello R, Girardi BA, Ferreira J, et al. Spermidine decreases $Na^{(+)},K^{(+)}$-ATPase activity through NMDA receptor and protein kinase G activation in the hippocampus of rats. Eur J Pharmacol 2012;684:79–86. http://dx.doi.org/10.1016/j.ejphar.2012.03.046.
[56] Fiske CH, Subbarow Y. The colorimetric determination of phosphorous. J Biol Chem 1925;66:375–400.
[57] Sassa S. Delta-aminolevulinic acid dehydratase assay. Enzyme 1982;28:133–45.
[58] Myhre O, Andersen JM, Aarnes H, Fonnum F. Evaluation of the probes 2′,7′-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation. Biochem Pharmacol 2003;65:1575–82.
[59] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351–8.
[60] Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, et al. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 1990;186:464–78.
[61] Swarup V, Srivastava AK, Padma MV, Rajeswari MR. Quantification of circulating plasma DNA in Friedreich's ataxia and spinocerebellar ataxia types 2 and 12. DNA Cell Biol 2011;30:389–94. http://dx.doi.org/10.1089/dna.2010.1165.
[62] Jacques-Silva MC, Nogueira CW, Broch LC, Flores EM, Rocha JB. Diphenyl diselenide and ascorbic acid changes deposition of selenium and ascorbic acid in liver and brain of mice. Pharmacol Toxicol 2001;88:119–25.
[63] Boyne AF, Ellman GL. A methodology for analysis of tissue sulfhydryl components. Anal Biochem 1972;46:639–53.
[64] Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82:70–7.
[65] Carlberg I, Mannervik B. Glutathione reductase. Methods Enzymol 1985;113:484–90.
[66] Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 1974;249:7130–9.
[67] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[68] Godt J, Scheidig F, Grosse-Siestrup C, Esche V, Brandenburg P, Reich A, et al. The toxicity of cadmium and resulting hazards for human health. J Occup Med Toxicol 2006;1:22. http://dx.doi.org/10.1186/1745-6673-1-22.
[69] Torres JL, Carreras A, Jimenez A, Brillas E, Torrelles X, Rius J, et al. Reducing power of simple polyphenols by electron-transfer reactions using a new stable radical of the PTM series, Tris(2,3,5,6-tetrachloro-4-nitrophenyl)methyl radical. J Org Chem 2007;72:3750–6. http://dx.doi.org/10.1021/jo062665a.
[70] Mendez-Armenta M, Rios C. Cadmium neurotoxicity. Environ Toxicol Pharmacol 2007;23:350–8. http://dx.doi.org/10.1016/j.etap.2006.11.009.
[71] Cahill L, Brioni J, Izquierdo I. Retrograde memory enhancement by diazepam: its relation to anterograde amnesia, and some clinical implications. Psychopharmacology (Berl) 1986;90:554–6.
[72] Belzung C, Griebel G. Measuring normal and pathological anxiety-like behaviour in mice: a review. Behav Brain Res 2001;125:141–9.
[73] Antonio MT, Corredor L, Leret ML. Study of the activity of several brain enzymes like markers of the neurotoxicity induced by perinatal exposure to lead and/or cadmium. Toxicol Lett 2003;143:331–40.
[74] Kumar A, Goyal R. Quercetin protects against acute immobilization stress-induced behaviors and biochemical alterations in mice. J Med Food 2008;11:469–73. http://dx.doi.org/10.1089/jmf.2006.0207.
[75] Bhutada P, Mundhada Y, Bansod K, Ubgade A, Quazi M, Umathe S, et al. Reversal by quercetin of corticotrophin releasing factor induced anxiety- and depression-like effect in mice. Prog Neuropsychopharmacol Biol Psychiatry 2010;34:955–60. http://dx.doi.org/10.1016/j.pnpbp.2010.04.025.
[76] Vissiennon C, Nieber K, Kelber O, Butterweck V. Route of administration determines the anxiolytic activity of the flavonols kaempferol, quercetin and myricetin—are they prodrugs? J Nutr Biochem 2012;23:733–40. http://dx.doi.org/10.1016/j.jnutbio.2011.03.017.
[77] Mesulam MM, Guillozet A, Shaw P, Levey A, Duysen EG, Lockridge O. Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyze acetylcholine. Neuroscience 2002;110:627–39.
[78] Fasitsas CD, Theocharis SE, Zoulas D, Chrissimou S, Deliconstantinos G. Time-dependent cadmium-neurotoxicity in rat brain synaptosomal plasma membranes. Comp Biochem Physiol C 1991;100:271–5.
[79] Carageorgiou H, Tzotzes V, Sideris A, Zarros A, Tsakiris S. Cadmium effects on brain acetylcholinesterase activity and antioxidant status of adult rats: modulation by zinc, calcium and L-cysteine co-administration. Basic Clin Pharmacol Toxicol 2005;97:320–4. http://dx.doi.org/10.1111/j.1742-7843.2005.pto_174.x.
[80] Casalino E, Sblano C, Landriscina C. Enzyme activity alteration by cadmium administration to rats: the possibility of iron involvement in lipid peroxidation. Arch Biochem Biophys 1997;346:171–9.
[81] Walker CH. An ecotoxicology perspective. Organic Pollutants; 2001.
[82] Tougu V, Kesvatera T. Role of ionic interactions in cholinesterase catalysis. Biochim Biophys Acta 1996;1298:12–30.
[83] Soreq H, Seidman S. Acetylcholinesterase—new roles for an old actor. Nat Rev Neurosci 2001;2:294–302. http://dx.doi.org/10.1038/35067589.
[84] Schmatz R, Mazzanti CM, Spanevello R, Stefanello N, Gutierres J, Correa M, et al. Resveratrol prevents memory deficits and the increase in acetylcholinesterase activity in streptozotocin-induced diabetic rats. Eur J Pharmacol 2009;610:42–8. http://dx.doi.org/10.1016/j.ejphar.2009.03.032.
[85] Gutierres JM, Carvalho FB, Rosa MM, Schmatz R, Rodrigues MV, Vieira JM, et al. Protective effect of α-tocopherol on memory deficits and Na^+,K^+-ATPase and acetylcholinesterase activities in rats with diet-induced hypercholesterolemia. Biomed Aging Pathol 2012;2:73–80.
[86] Fiorani M, De Sanctis R, Menghinello P, Cucchiarini L, Cellini B, Dacha M. Quercetin prevents glutathione depletion induced by dehydroascorbic acid in rabbit red blood cells. Free Radic Res 2001;34:639–48.
[87] Sato T, Tanaka K, Ohnishi Y, Teramoto T, Irifune M, Nishikawa T. Effects of steroid hormones on (Na^+,K^+)-ATPase activity inhibition-induced amnesia on the step-

through passive avoidance task in gonadectomized mice. Pharmacol Res 2004;49:151–9.
[88] Zhan H, Tada T, Nakazato F, Tanaka Y, Hongo K. Spatial learning transiently disturbed by intraventricular administration of ouabain. Neurol Res 2004;26:35–40. http://dx.doi.org/10.1179/016164104773026507.
[89] Rajanna B, Hobson M, Harris L, Ware L, Chetty CS. Effects of cadmium and mercury on $Na^{(+)}$-K^{+}, ATPase and uptake of 3H-dopamine in rat brain synaptosomes. Arch Int Physiol Biochim 1990;98:291–6.
[90] Chetty CS, Stewart TC, Cooper A, Rajanna B, Rajanna S. In vitro interaction of heavy metals with ouabain receptors in rat brain microsomes. Drug Chem Toxicol 1993;16:101–10. http://dx.doi.org/10.3109/01480549309038663.
[91] Stefanello FM, Ferreira AG, Pereira TC, da Cunha MJ, Bonan CD, Bogo MR, et al. Acute and chronic hypermethioninemia alter $Na^{+}K^{+}$-ATPase activity in rat hippocampus: prevention by antioxidants. Int J Dev Neurosci 2011;29:483–8. http://dx.doi.org/10.1016/j.ijdevneu.2011.02.001.
[92] Yu BP. Cellular defenses against damage from reactive oxygen species. Physiol Rev 1994;74:139–62.
[93] Santos FW, Zeni G, Rocha JB, do Nascimento PC, Marques MS, Nogueira CW. Efficacy of 2,3-dimercapto-1-propanesulfonic acid (DMPS) and diphenyl diselenide on cadmium induced testicular damage in mice. Food Chem Toxicol 2005;43:1723–30. http://dx.doi.org/10.1016/j.fct.2005.05.015.
[94] Rikans LE, Yamano T. Mechanisms of cadmium-mediated acute hepatotoxicity. J Biochem Mol Toxicol 2000;14:110–7.
[95] Lin YF, Aarts MG. The molecular mechanism of zinc and cadmium stress response in plants. Cell Mol Life Sci 2012;69:3187–206. http://dx.doi.org/10.1007/s00018-012-1089-z.
[96] Dalle-Donne I, Rossi R, Colombo R, Giustarini D, Milzani A. Biomarkers of oxidative damage in human disease. Clin Chem 2006;52:601–23. http://dx.doi.org/10.1373/clinchem.2005.061408.
[97] El-Demerdash FM, Yousef MI, Kedwany FS, Baghdadi HH. Cadmium-induced changes in lipid peroxidation, blood hematology, biochemical parameters and semen quality of male rats: protective role of vitamin E and beta-carotene. Food Chem Toxicol 2004;42:1563–71. http://dx.doi.org/10.1016/j.fct.2004.05.001.
[98] Krishnakumar N, Prabu SM, Sulfikkarali NK. Quercetin protects against cadmium-induced biochemical and structural changes in rat liver revealed by FT-IR spectroscopy. Biomed Prev Nutr 2012;2:179–85.
[99] Descarries L, Gisiger V, Steriade M. Diffuse transmission by acetylcholine in the CNS. Prog Neurobiol 1997;53:603–25.
[100] Watjen W, Beyersmann D. Cadmium-induced apoptosis in C6 glioma cells: influence of oxidative stress. Biometals 2004;17:65–78.
[101] Valverde M, Trejo C, Rojas E. Is the capacity of lead acetate and cadmium chloride to induce genotoxic damage due to direct DNA-metal interaction? Mutagenesis 2001;16:265–70.
[102] Hartwig A. Role of DNA repair inhibition in lead- and cadmium-induced genotoxicity: a review. Environ Health Perspect 1994;102(Suppl. 3):45–50.
[103] Lu SC. Glutathione synthesis. Biochim Biophys Acta 2013;1830:3143–53. http://dx.doi.org/10.1016/j.bbagen.2012.09.008.
[104] Toyama T, Shinkai Y, Yasutake A, Uchida K, Yamamoto M, Kumagai Y. Isothiocyanates reduce mercury accumulation via an Nrf2-dependent mechanism during exposure of mice to methylmercury. Environ Health Perspect 2011;119:1117–22. http://dx.doi.org/10.1289/ehp.1003123.
[105] Youdim KA, Qaiser MZ, Begley DJ, Rice-Evans CA, Abbott NJ. Flavonoid permeability across an in situ model of the blood–brain barrier. Free Radic Biol Med 2004;36:592–604. http://dx.doi.org/10.1016/j.freeradbiomed.2003.11.023.
[106] Pu F, Mishima K, Irie K, Motohashi K, Tanaka Y, Orito K, et al. Neuroprotective effects of quercetin and rutin on spatial memory impairment in an 8-arm radial maze task and neuronal death induced by repeated cerebral ischemia in rats. J Pharmacol Sci 2007;104:329–34.

3.2 Artigo 2

Efeito protetor da quercetina na atividade de ecto-enzimas, colinesterases e mieloperoxidase em linfócitos de ratos expostos ao cádmio

Protective effect of quercetin in ecto-enzymes, cholinesterases, and myeloperoxidase activities in the lymphocytes of rats exposed to cadmium

Fátima Husein Abdalla, Andréia Machado Cardoso, Roberta Schmatz,, Jamile Fabbrin Gonçalves, Jucimara Baldissarelli, Caroline Curry Martins, Daniela Zanini, Lizielle Souza de Oliveira, Pauline da Costa, Victor Camera Pimentel, Luciane Belmonte Pereira, Cibele Lima Lhamas, Maria Rosa Chitolina Schetinger, Vera Maria Morsch, Cinthia Melazzo Andrade Mazzanti

Publicado na revista "Molecular and Cellular Biochemistry"

Mol Cell Biochem
DOI 10.1007/s11010-014-2155-7

Protective effect of quercetin in ecto-enzymes, cholinesterases, and myeloperoxidase activities in the lymphocytes of rats exposed to cadmium

Fátima Husein Abdalla · Andréia Machado Cardoso · Roberta Schmatz · Jamile Fabbrin Gonçalves · Jucimara Baldissarelli · Caroline Curry Martins · Daniela Zanini · Lizielle Souza de Oliveira · Pauline da Costa · Victor Camera Pimentel · Luciane Belmonte Pereira · Cibele Lima Lhamas · Maria Rosa Chitolina Schetinger · Vera Maria Morsch · Cinthia Melazzo Andrade Mazzanti

Received: 27 January 2014 / Accepted: 14 July 2014
© Springer Science+Business Media New York 2014

Abstract The ex vivo and in vitro effects of quercetin on NTPDase, adenosine deaminase (ADA), and acetycholinesterase (AChE) activities in lymphocytes, as well as the effects of quercetin on butyrylcholinesterase (BChE) activity in serum and myeloperoxidase (MPO) activity in plasma were determined in rats. For the ex vivo experiment, animals were orally exposed to Cadmium (Cd) for 45 days. Animals were divided into eight groups: saline/ethanol, saline/Querc 5 mg/kg, saline/Querc 25 mg/kg, saline/Querc 50 mg/kg, Cd/ethanol, Cd/Querc 5 mg/kg, Cd/Querc 25 mg/kg, and Cd/Querc 50 mg/kg. The ex vivo data showed an increase in the ATP and ADP hydrolysis and ADA activity in Cd-exposed rats when compared to the control group. The treatment with quercetin 25 and 50 mg/kg prevented this increase in the ATP and ADP hydrolysis, while the treatment with quercetin 5, 25, and 50 mg/kg prevented the increase in the ADA activity. AChE, BChE, and MPO activities ex vivo presented an increase in the Cd-exposed group when compared to the control group, and the treatment with quercetin 5, 25, and 50 mg/kg prevented this increase caused by Cd exposure. The in vitro experiment showed that quercetin 5, 10, 25, or 50 μM decreased the ADA activity proportionally to the increase of the concentrations of quercetin when compared to the control group. Thus, we can suggest that the quercetin is able to modulate NTPDase, ADA, AChE, and MPO activities and contribute to maintain the levels of ATP, adenosine, and acetylcholine normal, respectively, exhibiting potent pro-inflammatory and anti-inflammatory actions.

Keywords Purinergic system · NTPDase · Cholinergic system · Cadmium · Antioxidant · Lymphocyte

Electronic supplementary material The online version of this article (doi:10.1007/s11010-014-2155-7) contains supplementary material, which is available to authorized users.

F. H. Abdalla (✉) · A. M. Cardoso · J. F. Gonçalves · J. Baldissarelli · C. C. Martins · D. Zanini · L. S. de Oliveira · P. da Costa · V. C. Pimentel · L. B. Pereira · M. R. C. Schetinger · V. M. Morsch
Programa de Pós Graduação em Ciências Biológicas: Bioquímica Toxicológica, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus Universitário, Camobi, Santa Maria, RS 97105-900, Brazil
e-mail: faha.biomed@hotmail.com

R. Schmatz
Instituto Federal de Educação, Ciência e Tecnologia do Triângulo Mineiro, Minas Gerais, CEP 38600-000, Brazil

C. L. Lhamas · C. M. A. 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
e-mail: cmelazzomazzanti@gmail.com

Introduction

Cadmium (Cd) is a heavy metal used in the manufacture of fertilizers, paints, plastics, and batteries, being also one of the major constituents of cigarette smoke [1]. Natural and anthropogenic sources of Cd may lead to the contamination of soils and to increased uptake of this metal by crops and vegetables grown for human consumption. Diet is the most important source of Cd exposure in the general population (non-occupational and nonsmoking) [2, 3]. Studies have reported that Cd exposure has been associated with several clinical manifestations including toxicity in the immune system [4, 5], where this metal is able to cause damage to humoral immune response, as well as cell-mediated immunity [6].

Published online: 27 July 2014

The immune system is essential to host defenses comprising an interactive network of lymphoid organs and immune cells [7]. The inflammatory process is complex and during the activation of the immune system, an interaction between various components occurs [8], including molecules of the purinergic and cholinergic systems that contribute to the regulation of the inflammatory process [9–11].

NTPDase (EC 3.6.1.5, CD39) is a member of the ecto-nucleoside triphosphate phosphohydrolase (E-NTPDase) family recognized as a marker of the lymphocyte activation [12]. NTPDase catalyzes the hydrolysis of extracellular nucleotide tri-and/or diphosphates (preferably ATP and ADP), and this sequential hydrolysis of nucleotide triphosphates generates ADP, AMP, and adenosine molecules involved in the regulation of immune defenses [13]. The extracellular ATP acts as pro-inflammatory and consequently as stimulation and proliferation of lymphocytes and cytokine release [11], whereas adenosine exhibits some potent anti-inflammatory and immunosuppressive action by inhibiting the proliferation of T cells and secretion of cytokines and migration of leukocytes across endothelial barriers [14]. The adenosine deaminase (ADA, E.C 3.5.4.4) enzyme participates in the degradation of purines by catalyzing the conversion of adenosine into inosine [15, 16]. The ADA activity is known to be increased in inflammatory diseases [17, 18].

Another important resource of regulation in the immune response is the cholinergic system. Immune cells possess a complete cholinergic system consisting of acetylcholine (ACh), muscarinic and nicotinic receptors, choline acetyltransferase, and acetylcholinesterase (AChE, E.C. 3.1.1.7) [10]. In the peripheral blood, the ACh circulating may regulate various physiological functions, including immune modulation [19]. When this molecule is found at high concentrations in blood, it acts as an anti-inflammatory agent [20]. However, the level of ACh is prevented by cholinesterase, which is able to hydrolyze ACh into choline and acetate [21]. Among the cholinesterases, AChE enzyme is present in neurons, lymphocytes, erythrocytes, ganglia of the autonomic nervous system, and motor plates, whereas the butyrylcholinesterase enzyme (BChE, E.C. 3.1.1.8) is present in serum, liver, glia, and pancreatic digestive tube walls [22].

Myeloperoxidase (MPO) is also an important molecule that contributes to the regulation of the inflammatory process. This molecule is a heme-enzyme released from leukocytes at the site of injury, and it reflects the activation of both neutrophils and lymphocytes. Several studies have demonstrated that the MPO from leukocytes is a classic inflammatory marker and has been used to aid the diagnosis of several diseases involved in the inflammatory process [23, 24].

Considering that Cd exposure can affect various mechanisms of the immune system, the administration of some anti-inflammatory agents such as flavonoid compounds in the Cd intoxication process may be important. Thus, there is increasing interest in the biological activities of plant extracts, such as flavonoids. These compounds are widely distributed in a variety of vegetables and fruits regularly consumed in our diet [25]. The average daily intake of flavonoids in the occidental diet is about 23 mg/day, and quercetin is one of the most abundant, representing about 60–75 % of the polyphenol ingestion [26]. Moreover, quercetin has been studied due to its wide therapeutic property that involves its antioxidant capacity, as well as antithrombotic and anti-inflammatory activities. It can also act on the processes of mitosis and cell apoptosis [27, 28].

Based on the above mentioned, the purpose of this study was to evaluate the ex vivo effects of quercetin at different doses on the NTPDase, ADA, and AChE activities in peripheral lymphocytes, as well as the BChE activity in serum and MPO activity in plasma of Cd-exposed rats. In addition, we also checked the in vitro effects of quercetin in order to evaluate the effect per se of this compound on the enzymes studied.

Materials and methods

Chemicals

Acetylthiocholine iodide, butyrylthiocholine iodide, 5,5′dithio-bis-2-nitrobenzoic acid (DTNB), tris (hydroxymethyl)-aminomethane GR, Coomassie brilliant blue G, Nucleotides, Trizma, 4-aminoantipyrine, quercetin, and cadmium chloride ($CdCl_2$) were obtained from Sigma Chemical Co (St. Louis, MO, USA). Ficoll-Histopaque (Lymphoprep^{TM}) was purchased from Nycomed Pharma (Oslo, Norway). All other reagents used in the experiments were of analytical grade and highest purity.

Animals

Adult male Wistar rats (70–90 days; 220-300 g) from the Central Animal House of the University Federal of Santa Maria (UFSM) were used in this experiment. 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 the Federal University of Santa Maria (protocol under number: 031/2011).

Ex vivo experimental procedure

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, since it is highly soluble in water [29]. Thus, in the present study rats were received by oral administration (gavage) Cd as $CdCl_2$ dissolved in saline (Cd 2.5 mg/kg) [30]. The choice of the doses of Cd was made based on previous works of our research group, in which Cd-exposed rats showed an increased Cd concentration in plasma, spleen, and thymus [31] and a small, but significant amount of Cd in brain structures, which was enough to cause brain injury [5]. On the other hand, the choice of the doses of quercetin 5, 25, and 50 mg/kg was made also based on previous works, which obtained beneficial results of this compound in rats [27, 32].

Animals were randomly divided into eight groups of 10–12 animals each: Control/Querc, control/Querc 5 mg/kg, control/Querc 25 mg/kg, control/Querc 50 mg/kg, Cd/control, Cd/Querc 5 mg/kg, Cd/Querc 25 mg/kg, and Cd/Querc 50 mg/kg.

Both solutions were administered five days a week for 45 days by oral gavage (1 mL/kg) between 9 and 11 a.m. Quercetin was administered 30 min after Cd. The solutions were freshly prepared and administered (1 mL/kg) between 9 and 11 a.m. After the treatment period, animals were submitted to euthanasia being previously anesthetized with halothane. The whole blood was collected in tubes containing EDTA as anticoagulant and the plasma, as well as lymphocytes were isolated for the subsequent enzymatic assay. Additionally, blood was collected without anticoagulant to serum separation.

It is important to note that controls for all ex vivo tests were performed to correct for vehicle (ethanol 25 %) interference. No differences between vehicle and control activities were observed (data not shown).

Experiments performed in vitro

To evaluate the effect of quercetin in nucleotide hydrolysis in the peripheral lymphocytes, an in vitro assay was performed using different concentrations of quercetin (1, 5, 10, 25, and 50 μM) diluted in methanol in the presence of ATP and ADP as substrate with the incubation medium as described below. The same concentrations of quercetin were used to evaluate ADA, AChE, BChE, and MPO activities. The choice of the concentrations of the quercetin for the in vitro experiment was made based on several in vitro studies that used similar ranges of concentrations and obtained satisfactory results [33, 34].

The final concentrations of methanol, when tested alone in the incubation medium did not affect the enzyme activities. All the other procedures for enzymatic assays were the same as those described below.

Preparation of samples and enzyme assays

Isolation of mononuclear cells from rat blood

The whole blood was collected in tubes containing EDTA as anticoagulant. Peripheral lymphocytes were isolated using Ficoll-Hypaque density gradients as described by Böyum [35]. Lymphocyte viability and integrity were confirmed by determining the percentage of cells, excluding 0.1 % trypan blue, and measuring lactate dehydrogenase (LDH) activity [36].

NTPDase and ADA activities in peripheral lymphocytes

After isolation of mononuclear cells, NTPDase activity was determined by measuring the amount of released inorganic phosphate using a colorimetric assay, according to the method of Leal et al. [37]. NTPDase activity was determined in a reaction medium containing 5 mM $CaCl_2$, 1,200 mM NaCl, 50 mM KCl, 600 mM glucose, and 500 mM Tris–HCl buffer, pH 8.0 in a final volume of 200 μL. Twenty microliters of intact lymphocytes suspended in saline solution was added to the reaction medium (01–02 mg/mL of protein) and pre-incubated for 10 min at 37 °C. The reaction was started by adding the substrate (ATP or ADP) at a final concentration of 2 mM and stopped with 200 μL of 10 % trichloroacetic acid (TCA) to provide a final concentration of 5 %. The samples were chilled on ice for 10 min before assaying for the release of inorganic phosphate (P_i) as described by Chan et al. [38], using malachite green as colorimetric reagent and KH_2PO_4 as standard. Controls with the addition of the enzyme preparation after the addition of TCA were used to correct for non-enzymatic hydrolysis of the substrate.

For the in vitro assay, variable quercetin concentrations were added to a final volume of 200 μL. All samples were run in triplicate and specific activity of NTPDase is reported as nmol P_i released/min/mg protein.

The ADA activity was assessed according to the colorimetric method of Giusti and Gakis [39], which is to quantify spectrophotometrically the amount of complexes of ammonia released per minute, from the degradation of adenosine. The activity of ADA was expressed in U/L. For the in vitro assay, the variable quercetin concentrations were added to a final volume of 525 μL to the reaction (37 °C) at the same time of the addition of the enzyme.

Cholinesterase activities in peripheral lymphocytes

The AChE activity in lymphocytes was determined by the colorimetric method described by Ellman et al. [40] modified by Fitzgerald and Costa [41]. The proteins of all

samples were adjusted to 0.1–0.2 mg/mL. Two hundred microliters of intact cells was added to a reaction mixture composed of 1.0 mM acetylthiocholine (AcSCh), 0.1 mM 5,50-dithio-bis-2-nitrobenzoic acid (DTNB), and 0.1 M phosphate buffer (pH 8.0). For the in vitro assay, the variable quercetin concentration was added to a final volume of 2 mL. Immediately before and after incubation for 30 min at 27 °C, the absorbance was read on a spectrophotometer at 412 nm. The specific activity of lymphocyte AChE was calculated from the quotient between lymphocyte AChE activity and protein content, and the results are expressed as μmol AcSCh/h/mg of protein.

The BChE enzymatic assay was determined in serum by a modification of the spectrophotometric method of Ellman et al. [40]. The reaction mixture (2 mL final volume) contained 100 mM potassium 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 during 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 (BSCh). For the in vitro assay, the variable quercetin concentration was added to a final volume of 2 mL. Immediately before and after incubation for 2 min at 25 °C, the absorbance was read on a spectrophotometer at 412 nm. All samples were run in duplicate or triplicate, and enzyme activity was expressed in μmol BuSCh/min/mg of protein.

MPO activity in plasma

MPO activity was measured in plasma from blood of rats collected with EDTA and followed by centrifugation at 1,800×*g* for 10 min. The MPO activity was analyzed spectrophotometrically by a modified peroxidase-coupled assay system involving phenol, 4-aminoantipyrine (AAP) and H_2O_2 [42]. Briefly, 390 μL of 2.5 mM AAP and 20 mM phenol were placed in each tube, followed by 450 μL of 1.7 mM H_2O_2. In the presence of H_2O_2 as oxidizing agent, MPO catalyzed the oxidative coupling of phenol and AAP yielding a colored product, quinoneimine, with a maximum absorbance at 500 nM. The millimolar absorbance coefficient for the quinoneimine was determined to be $\sum = 14 \pm 0.1$/mM/cm, close to the previously reported values [43]. For the in vitro assay, the variable quercetin concentrations were added to a final volume of 870 μL. Results were expressed in micromolar of quinoneimine produced at 30 min.

Protein determination

Protein was measured by the method of Bradford [44] using bovine serum albumin as standard.

Statistical analysis

Statistical analysis was done by the commercial SPSS package for Windows©. All data were expressed as mean ± S.E.M. Data of ex vivo experiments were analyzed statistically by two-way ANOVA, followed by Tukey's multiple range tests. Results of in vitro experiments were submitted to one-way ANOVA, followed by Tukey's multiple range tests. Differences were considered significant when the probability was $P < 0.05$.

Results

Ex vivo assay in the peripheral lymphocytes

NTPDase and ADA activities in the peripheral lymphocytes of Cd-exposed rats

The results of the present study demonstrate that the cascade of ecto-enzymes was altered in the Cd exposure rats and after treatment with quercetin. Figure 1 shows the results obtained for the NTPDase and ADA activities. Statistical analysis showed a significant Cd versus quercetin interaction [$F(2,34) = 5.26$; $P < 0.05$] for ATP hydrolysis. Post hoc comparisons revealed that NTPDase activity with ATP as substrate was significantly increased in the Cd-exposed group (88 %) when compared with the Control/Querc group ($P < 0.05$) (Fig. 1a). When quercetin 25 or 50 mg/kg was co-administrated with Cd, this flavonoid prevented this decrease in the NTPDase activity (46 and 53 % decrease, respectively) ($P < 0.05$). In addition, we observed a negative correlation between NTPDase activity with ATP as substrate and different doses of quercetin used in this experiment ($r = -0.7525$; $P < 0.0001$), indicating that higher doses of quercetin co-administered with Cd could lower the NTPDase activity with ATP as substrate (Table 1).

In relation to the NTPDase activity using ADP as substrate, we observed a significant Cd versus quercetin interaction [$F(2,34) = 6.79$; $P < 0.05$]. Post hoc comparisons revealed an increase in the NTPDase activity with ADP as substrate in Cd-exposed rats (92 %) when compared with the Control/Querc group (Fig. 1b). On the other hand, when the animals received co-treatment with Cd and quercetin 25 or 50 mg/kg, the decrease in the NTPDase activity was prevented (25 and 69 % decrease, respectively) ($P < 0.05$). We also observed a negative correlation between NTPDase activity with ADP as substrate and the different quercetin doses used in this experiment ($r = -0.8444$; $P < 0.0001$). Therefore, these results indicate that higher doses of quercetin co-administered with Cd may lower the NTPDase activity with ADP as substrate (Table 1).

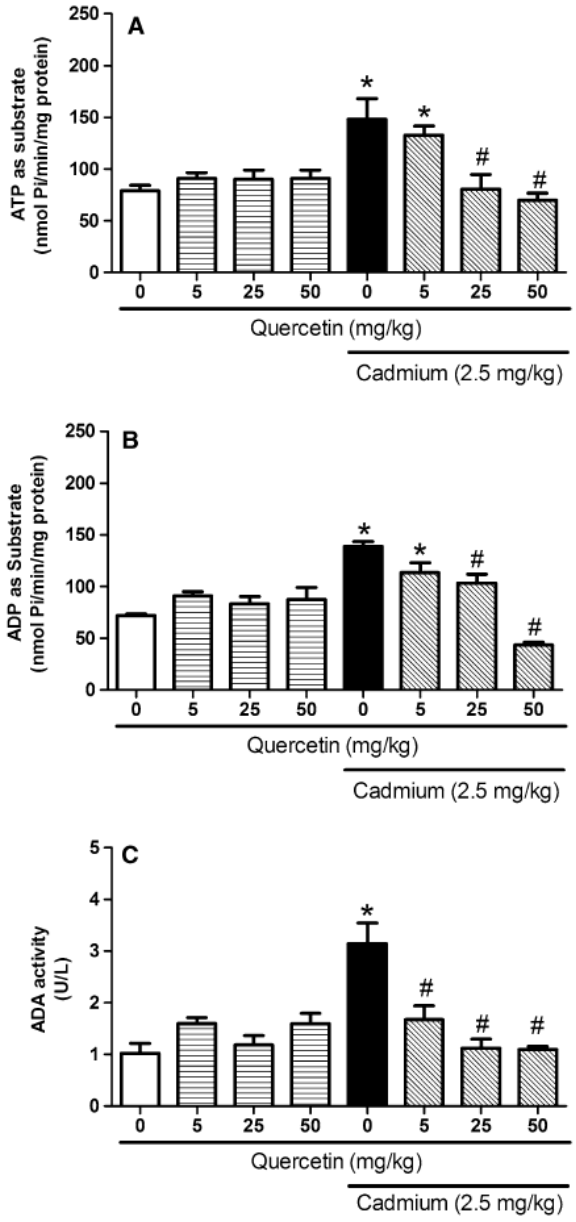


Fig. 1 ATP (**a**) and ADP (**b**) hydrolysis and ADA activity (**c**) ex vivo in peripheral lymphocytes of Cd-exposed rats and co-treated with different doses of quercetin (mg/kg). *Asterisk* indicates significant differences from the control group ($P < 0.05$). *Ash* indicates significant differences from the control Cd ($P < 0.05$). *Bars* represent mean ± S.E.M. ($n = 10$–12). Two-way ANOVA-Duncan's test ($P < 0.05$)

Table 1 Correlation between molecules that contribute to the regulation of the inflammatory process

Variables	Quercetin doses (from 0 to 50 mg/kg)	
	r value	*P* value
NTPDase activity with ATP as substrate	−0.7525	< 0.0001
NTPDase activity with ADP as substrate	−0.8444	< 0.0001
ADA activity	−0.6608	< 0.0015
AChE activity	−0.7180	< 0.0001
MPO activity	−0.6168	< 0.0029

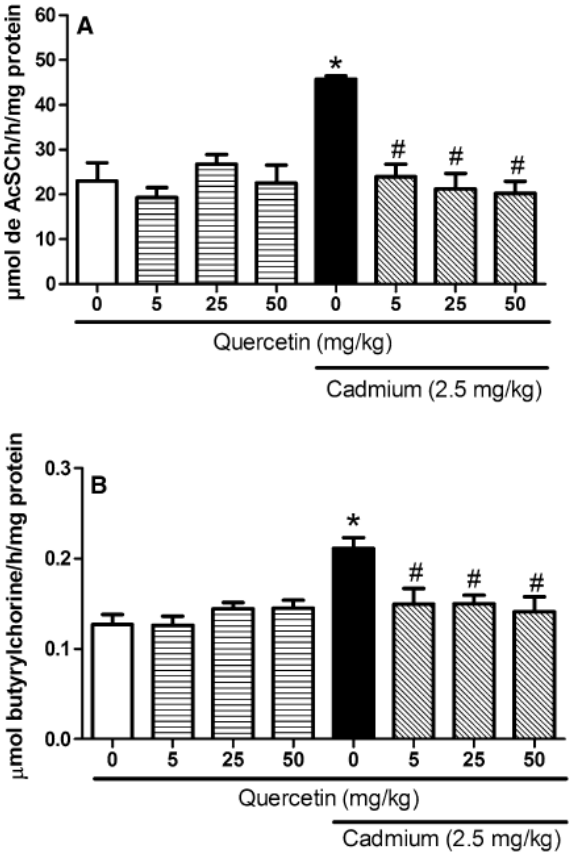


Fig. 2 AChE (**a**) and BChE (**b**) activities ex vivo in peripheral lymphocytes of Cd-exposed rats and co-treated with quercetin (mg/kg). *Asterisk* indicates significant differences from the control group ($P < 0.05$). *Ash* indicates significant differences from the control Cd ($P < 0.05$). *Bars* represent mean ± S.E.M. ($n = 10$–12). Two-way ANOVA-Duncan's test ($P < 0.05$)

The ADA activity in the peripheral lymphocytes showed a significant Cd versus quercetin interaction [$F(2,32) = 4.40$; $P < 0.05$]. Moreover, post hoc comparisons revealed that ADA activity was significantly increased in the Cd-exposed group (213 %) when compared with the Control/Querc group ($P < 0.05$) (Fig. 2c). However, when quercetin 5, 25,

or 50 mg/kg was co-administrated with Cd, the increase in ADA activity caused by this metal was prevented by treatment with this flavanoid (46, 64, and 65 % decrease, respectively) ($P < 0.05$). In addition, we observed a negative correlation between ADA activity and quercetin doses used in this experiment ($r = -0.6608$; $P < 0.0015$) indicating that higher doses of quercetin co-administered with Cd lowered the ADA activity (Table 1).

Cholinesterase activities in the peripheral lymphocytes of Cd-exposed rats

A significant Cd versus quercetin interaction [$F(2,32) = 7.35$; $P < 0.05$] for AChE activity was observed (Fig. 2a). Moreover, post hoc comparisons demonstrated that AChE activity in the peripheral lymphocytes was significantly increased in the Cd exposure (98 %) when compared with the Control/Querc group ($P < 0.05$). When the animals were co-treated with Cd and quercetin 5, 25, or 50 mg/kg, this effect was prevented by this flavanoid (47, 54, 56 % decrease, respectively) ($P < 0.05$). In addition, we observed a negative correlation between the AChE activity and quercetin doses used in this experiment ($r = -0.7180$; $P < 0.0001$) indicating that a higher doses of quercetin co-administered with Cd lowered the AChE activity (Table 1).

The effect of quercetin on the BChE activity from serum is presented in Fig. 2b. There was a significant Cd versus quercetin interaction [$F(2,32) = 6.30$; $P < 0.05$]. Post hoc comparisons demonstrated that BChE activity significantly increased in Cd exposure (66 %) when compared with the Control/Querc group ($P < 0.05$). However, the treatment with quercetin at the doses of 5, 25, or 50 mg/kg co-administrated with Cd prevented this effect caused by Cd exposure alone (29, 29, and 33 % decrease, respectively) ($P < 0.05$). It is important to point out that differently from the AChE activity, in the BChE activity, there was no correlation between the BChE activity and different doses of quercetin used in this experiment ($r = -0,3834$; $P = 0,0532$).

Myeloperoxidase activity in plasma of Cd-exposed rats

A significant Cd versus quercetin interaction [$F(2,30) = 5.32$; $P < 0.05$] for MPO activity was observed (Fig. 3). Post hoc comparisons demonstrated that the exposure to Cd increased the MPO activity (46 %) and the co-treatment Cd and quercetin 5, 25, or 50 mg/kg prevented this effect (41, 51, and 53 % decrease, respectively) ($P < 0.05$). In addition, we observed a negative correlation between MPO activity and the different quercetin doses used in this experiment ($r = 0.6168$; $P < 0.0029$), indicating that higher doses of quercetin co-administered with Cd lowered the MPO activity (Table 1).

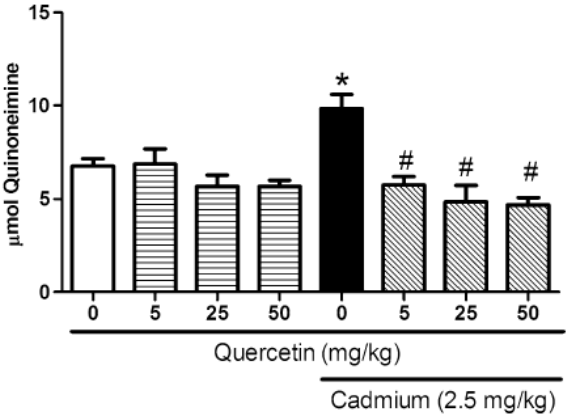


Fig. 3 MPO activity ex vivo in peripheral lymphocytes of Cd-exposed rats and co-treated with quercetin (mg/kg). *Asterisk* indicates significant differences from the control group ($P < 0.05$). *Ash* indicates significant differences from the control Cd ($P < 0.05$). *Bars* represent mean ± S.E.M. ($n = 10$–12). Two-way ANOVA-Duncan's test ($P < 0.05$)

In vitro effects of quercetin on NTPDase, ADA, and cholinesterase activities

The effect of quercetin on the NTPDase activity is presented in Fig. 4. When peripheral lymphocytes were incubated with 1, 5, 10, 25, and 50 µM of quercetin, no significant differences were observed in the NTPDase activity when ATP or ADP were used as substrate (Fig. 4a, b). On the other hand, the ADA activity in vitro showed a significant decrease (60, 49, 37, and 42 %, respectively) proportional to the increase of the concentrations of quercetin when compared to the control group (quercetin 0 µM) ($P < 0.05$) (Fig. 4c).

In relation to in vitro AChE, BChE, and MPO activities, no significant differences were observed (data not shown).

Discussion and conclusion

It has already been reported that the Cd-exposed rats presented an increase of Cd concentration in several tissues including in lymphoid organs [5, 45]. This fact could be a result of the long biological half life of Cd and mainly due to its low rate of excretion from the body [3]. In according to Lafuente et al. [6], Cd may cause changes in the immune system and inhibit the humoral and cellular response besides increasing significantly pro-inflammatory cytokines, including tumors necrosis factor-α (TNF-α), interleukin-1α (IL-1α), and interleukin-6 (IL-6). Moreover, the involvement of metal intoxication in modifying the cholinergic system has been proposed in studies performed in

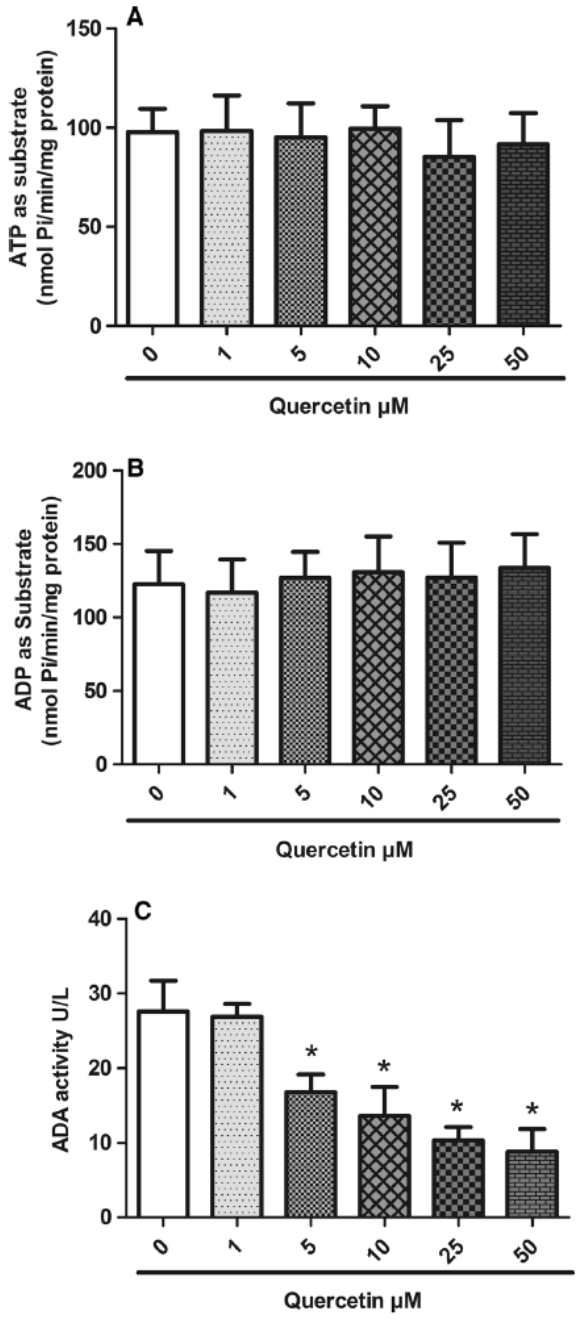


Fig. 4 In vitro effect of different concentrations of quercetin on ATP (**a**) and ADP (**b**) hydrolysis and ADA activity (**c**) in peripheral lymphocytes of rats. The *first bar* represents the control. The *other bars* represent different concentrations of quercetin (μM). ATP and ADP hydrolysis in lymphocytes was expressed as nmol Pi/min/mg of protein and ADA activity was expressed as U/L. Each *column* represents mean ± S.E.M. ($n = 5$). *Asterisk* indicates significant differences ($P < 0.05$) from the control group

our research group, showing that the enzymes of the cholinergic systems are altered in Cd intoxication [5, 31, 32] and in rats exposed to cigarette smoke, which has a significant amount of Cd [46].

Considering that the literature widely reports the involvement of heavy metals in changes in the immune system [6, 31], it is of great importance to evaluate the involvement of quercetin, which is an important flavonoid with anti-inflammatory and anti-carcinogenic activities [6, 27, 28] in orally Cd-exposed rats. It is important to note that the current study is the first one evaluating the role of quercetin in protecting peripheral NTPDase, ADA, AChE, BChE, and MPO activities in lymphocytes of Cd-exposed rats co-treated with quercetin. This way, this study was developed in order to contribute to the understanding of the involvement of Cd in the modulation of purinergic and cholinergic components of the immune system, as well as understanding the effect of quercetin in this condition.

In the present study, Cd exposure was strongly associated with an increase in lymphocytic NTPDases (hydrolyzing ATP and ADP) activities when compared to other groups. It is possible to infer that Cd exposure may be associated with a large quantity of ATP that is released by stressed cells in order to decrease the inflammatory state that is developed during this exposure [11, 47, 48]. However, the increase in enzyme activities found in our study may lead to a decrease in ATP levels in the extracellular medium contributing to the inflammatory process observed in Cd poisoning, since ATP is an important anti-inflammatory molecule.

In addition, our results showed that Cd exposure was also strongly associated with an increase in ADA activity in lymphocytes, which possibly indicates that a low concentration of adenosine is available in the extracellular medium. Adenosine is a molecule that has a role in suppressing effector cells, though A_2A receptor-mediated signaling, thus a low availability of this nucleoside may contribute to the inflammatory process that occurs due to Cd exposure. Taken together, these data suggest that an increased nucleotide hydrolysis and adenosine deamination in lymphocytes of Cd-exposed rats may contribute to the immune and inflammatory alterations in rats poisoning with this metal.

Interestingly, the co-administration of quercetin was able to abolish these undesirable effects caused by Cd exposure preventing the increase in the NTPDase and ADA activities. As already well reported in the literature, quercetin has antiinflammatory properties and this compound may have the ability to lower cellular damage [49–51]. Taken together these data, we can suggest that the

treatment with quercetin may cause a reduction in inflammatory processes caused by Cd exposure, since the inhibition of activities of these enzymes may result in an increase in ATP and adenosine concentrations in the extracellular medium, which has antiinflamatory effects. Thus, the modulation of ecto-enzymes activities by quercetin is likely to be one of the mechanisms through which this flavonoid exerts anti-inflammatory properties.

It is known that quercetin can chelate metals, such as Al and Cd, and consequently reduces its toxicity [52–54]. Consequently, the co-administration of quercetin in Cd-exposed rats found in our study could reduce the toxic effect of this metal and contribute to the reduction of inflammatory processes. Furthermore, the prevention of toxic effects of Cd by chelating effects of quercetin may contribute to prevent oxidative damage [25] in several cellular components and consequently alterations in enzymes linked the membranes such as NTPDase and ADA.

In addition, it is important to note that there was a negative correlation between NTPDase or ADA activities when different doses of quercetin were used in this experiment. This result reinforces that the treatment with quercetin 5, 25, or 50 mg/kg in Cd-exposed rats leads to lower NTPDase and ADA activities. Moreover, we observed that this diminution was more pronounced, when the quercetin doses were higher. Therefore, we believe that the protective effect of quercetin in the purinergic system is dose dependent. Taking this into account, we can suggest that this modulation may be very important in case of the accidental Cd exposure to maintain the extracellular concentration of ATP and adenosine, molecules that exhibit potent actions signaling the fine-tuning of inflammatory and immune responses.

Another important aspect to be discussed here is the effect of Cd on the cholinergic system. Several studies from our laboratory have demonstrated that the cholinergic system is also a signalization way that modulates the immunity through the action of neurotransmitter ACh, molecule that binds to nicotinic receptors in lymphocyte surfaces [31, 55, 56]. This neurotransmitter inhibits the proliferation of cytokines, serotonin, histamine, nitric oxide, lysosomal enzymes, prostaglandins, and leukotrienes, which are among the mediators of the inflammatory process [10, 57].

This way, in order to evaluate the effect of Cd on the cholinergic system, we investigated the AChE activity in lymphocytes and BChE activity in serum of Cd-exposed rats treated with quercetin at different doses. The present study showed that Cd exposure increased activities of these enzymes, which corroborates with other studies that have shown alterations in the AChE and BChE activities in various diseases and in intoxication processes [32, 56, 58]. On the other hand, a recent study developed by our research group showed a decrease in the activities of lymphocyte AChE, whole blood AChE and serum BChE of Cd-exposed rats (2 mg/kg) [31]. Moreover, El-Demerdash et al. [59] and Murugavel and Pari [45] showed an inhibition in both plasma and brain AChE activities of rats exposed to Cd. We believe that these controversial data may be due to different doses, vehicle, or by the time of exposure to the metal.

Taking into our results consideration, we suggest that the increase in the AChE and BChE activities observed in the present study occurs to due to inflammatory processes caused by Cd exposure. Moreover, an increase in the cholinesterase activities leads to a rapid degradation of ACh and consequently a decrease of concentration of this circulating molecule could decrease the anti-inflammatory activity promoted by ACh.

It is important to note that the treatment with quercetin prevented the increase caused by Cd exposure in the AChE and BChE activities. This indicates that the treatment with quercetin may cause a modulation in the cholinesterase activities, preventing the rapid degradation of ACh and maintaining the concentration of this molecule in potent anti-inflammatory actions. This action of quercetin can be considered extremely important in the prevention of the negative effects caused by accidental Cd exposure. In addition, we observed a negative correlation between the AChE activity and the different doses of quercetin, suggesting that higher doses of quercetin co-administered with Cd promote a lower AChE activity.

It is important to point out that some studies have reported that quercetin is able to bind the several important amino acid residues of both enzymes, AChE and BuChE, besides performing a number of hydrophobic interactions with these enzymes. Thus, it is plausible to suggest that this interaction compound-enzymes is one of the mechanisms by which the quercetin inhibits AChE and BChE activities found in our study [60].

To confirm the presence of an inflammatory condition in Cd exposure, we evaluated the level of classic inflammatory marker, MPO activity. Several studies have demonstrated that the release of MPO from leukocytes at the site of injury reflects the activation of both neutrophils and lymphocytes. MPO is a heme-enzyme produced by inflammatory mediators that catalyze the reaction of chloride ion with hydrogen peroxide superoxide to generate large amounts of hypochlorous acid (HOCl), a reactive oxygen species which reacts further to generate singlet oxygen and hydroxyl radical as powerful oxidizing and microbicidal agents [23].

In the present study, this inflammatory marker was increased after Cd exposure, indicating the presence of an inflammatory process. On the other hand, the treatment

with quercetin prevented the increase caused by Cd exposure in the MPO activity. Moreover, a negative correlation between MPO activity and different quercetin doses co-administrated with Cd was observed, showing that higher doses of quercetin co-administered with Cd are able to lower the MPO activity, which corroborates with several studies that have shown that flavonoids, such as quercetin, have anti-inflammatory effects [61, 62].

Reinforcing this line of reasoning, the next set of experiments was performed to verify the in vitro effects of the quercetin on NTPDase, 5-nucleotidase, ADA, AChE, BuChE, and MPO activities in lymphocytes. It is important to note that in vitro, we found that the NTPDase activity in lymphocytes, when ATP or ADP were used as substrate, presented no significant changes. On the other hand, ADA activity in vitro presented a decrease when quercetin was added to the reaction medium. These results are in accordance with Melzig [63] who showed that flavonoids such as quercetin can inhibit the ADA activity. These results suggest that quercetin per se promotes an increase in adenosine extracellular, playing an important anti-inflammatory and immunosuppressive action by inhibiting proliferation of T cells and secretion of cytokines [14, 64].

In conclusion, the results found in the present study demonstrate alterations in enzymes of the purinergic and cholinergic systems, as well as in the MPO activity in Cd-exposed rats, suggesting that these alterations may be due to the inflammation process caused by the exposure to this heavy metal. On the other hand, the treatment with quercetin was able to modulate the alterations caused by this metal probably by chelating effects of this compound or by inflammatory process regulation caused by Cd. These results support the suggestion that quercetin treatment was able to prevent the changes caused by accidental Cd exposure, maintaining normal levels of ATP, adenosine, ACh, and MPO molecules that exhibit potent anti-inflammatory and immunosuppressive actions. Thus, based on our results, we can suggest that the quercetin is a promise compound as adjuvant in the treatment of poisoning by Cd.

Acknowledgments This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), Coordenação e Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Federal University of Santa Maria, RS, Brazil, and the FINEP research grant "Rede Instituto Brasileiro de Neurociências (IBNet)", Instituto Nacional de Ciências Tecnológicas (INCT).

References

1. Godt J, Scheidig F, Grosse-Siestrup C, Esche V, Brandenburg P, Reich AGroneberg DA (2006) The toxicity of cadmium and resulting hazards for human health. J Occup Med Toxicol 1:22. doi:10.1186/1745-6673-1-22
2. Jarup L, Akesson A (2009) Current status of cadmium as an environmental health problem. Toxicol Appl Pharmacol 238:201–208. doi:10.1016/j.taap.2009.04.020
3. Jarup L, Berglund M, Elinder CG, Nordberg G, Vahter M (1998) Health effects of cadmium exposure: a review of the literature and a risk estimate. Scand J Work Environ Health 24(Suppl 1):1–51
4. Jarup L, Hellstrom L, Alfven T, Carlsson MD, Grubb A, Persson B, Pettersson C, Spang G, Schutz A, Elinder CG (2000) Low level exposure to cadmium and early kidney damage: the OSCAR study. Occup Environ Med 57:668–672
5. Goncalves JF, Fiorenza AM, Spanevello RM, Mazzanti CM, Bochi GV, Antes FG, Stefanello N, Rubin MA, Dressler VL, Morsch VM, Schetinger MR (2010) *N*-acetylcysteine prevents memory deficits, the decrease in acetylcholinesterase activity and oxidative stress in rats exposed to cadmium. Chem Biol Interact 186:53–60. doi:10.1016/j.cbi.2010.04.011
6. Lafuente A, Gonzalez-Carracedo A, Romero A, Esquifino AI (2003) Effect of cadmium on lymphocyte subsets distribution in thymus and spleen. J Physiol Biochem 59:43–48
7. Delves PJ, Roitt IM (2000) The immune system. Second of two parts. N Engl J Med 343:108–117. doi:10.1056/NEJM200007133430207
8. Rock KL, Hearn A, Chen CJ, Shi Y (2005) Natural endogenous adjuvants. Springer Semin Immunopathol 26:231–246. doi:10.1007/s00281-004-0173-3
9. Mancino G, Placido RDi, Virgilio F (2001) P2X7 receptors and apoptosis in tuberculosis infection. J Biol Regul Homeost Agents 15:286–293
10. Kawashima K, Fujii T (2003) The lymphocytic cholinergic system and its contribution to the regulation of immune activity. Life Sci 74:675–696
11. Bours MJ, Swennen EL, Di Virgilio F, Cronstein BN, Dagnelie PC (2006) Adenosine 5′-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. Pharmacol Ther 112:358–404. doi:10.1016/j.pharmthera.2005.04.013
12. Maliszewski CR, Delespesse GJ, Schoenborn MA, Armitage RJ, Fanslow WC, Nakajima T, Baker E, Sutherland GR, Poindexter K, Birks C et al (1994) The CD39 lymphoid cell activation antigen. Molecular cloning and structural characterization. J Immunol 153:3574–3583
13. Burch LHPicher M (2006) E-NTPDases in human airways: regulation and relevance for chronic lung diseases. Purinergic Signal 2:399–408. doi:10.1007/s11302-006-9001-7
14. Linden J (2006) New insights into the regulation of inflammation by adenosine. J Clin Invest 116:1835–1837. doi:10.1172/JCI29125
15. Zimmermann H (1992) 5′-Nucleotidase: molecular structure and functional aspects. Biochem J 285(Pt 2):345–365
16. Robson SC, Sevigny J, Zimmermann H (2006) The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. Purinergic Signal 2:409–430. doi:10.1007/s11302-006-9003-5
17. Conlon BA, Law WR (2004) Macrophages are a source of extracellular adenosine deaminase-2 during inflammatory responses. Clin Exp Immunol 138:14–20. doi:10.1111/j.1365-2249.2004.02591.x
18. Schetinger MR, Morsch VM, Bonan CD, Wyse AT (2007) NTPDase and 5′-nucleotidase activities in physiological and disease conditions: new perspectives for human health. BioFactors 31:77–98
19. Tayebati SK, El-Assouad D, Ricci A, Amenta F (2002) Immunochemical and immunocytochemical characterization of

cholinergic markers in human peripheral blood lymphocytes. J Neuroimmunol 132:147–155
20. de Almeida JP, Saldanha C (2010) Nonneuronal cholinergic system in human erythrocytes: biological role and clinical relevance. J Membr Biol 234:227–234. doi:10.1007/s00232-010-9250-9
21. Pavlov V, Xiao Y, Willner I (2005) Inhibition of the acetycholine esterase-stimulated growth of Au nanoparticles: nanotechnology-based sensing of nerve gases. Nano Lett 5:649–653. doi:10.1021/nl050054c
22. Inacio Lunkes G, Stefanello F, Sausen Lunkes D, Maria Morsch V, Schetinger MR, Goncalves JF (2006) Serum cholinesterase activity in diabetes and associated pathologies. Diabetes Res Clin Pract 72:28–32. doi:10.1016/j.diabres.2005.08.009
23. Hampton MB, Kettle AJ, Winterbourn CC (1998) Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood 92:3007–3017
24. Regasini LO, Vellosa JC, Silva DH, Furlan M, de Oliveira OM, Khalil NM, Brunetti IL, Young MC, Barreiro EJ, Bolzani VS (2008) Flavonols from *Pterogyne nitens* and their evaluation as myeloperoxidase inhibitors. Phytochemistry 69:1739-44. doi:10.1016/j.phytochem.2008.01.006
25. Molina MF, Sanchez-Reus I, Iglesias I, Benedi J (2003) Quercetin, a flavonoid antioxidant, prevents and protects against ethanol-induced oxidative stress in mouse liver. Biol Pharm Bull 26:1398–1402
26. Sampson L, Rimm E, Hollman PC, de Vries JH, Katan MB (2002) Flavonol and flavone intakes in US health professionals. J Am Diet Assoc 102:1414–1420
27. Choi EJ, Chee KM, Lee BH (2003) Anti- and prooxidant effects of chronic quercetin administration in rats. Eur J Pharmacol 482:281–285
28. Lu J, Zheng YL, Luo L, Wu DM, Sun DX, Feng YJ (2006) Quercetin reverses D-galactose induced neurotoxicity in mouse brain. Behav Brain Res 171:251–260. doi:10.1016/j.bbr.2006.03.043
29. Zalups RK, Ahmad S (2003) Molecular handling of cadmium in transporting epithelia. Toxicol Appl Pharmacol 186:163–188
30. Santos FW, Oro T, Zeni G, Rocha JB, do Nascimento PC, Nogueira CW (2004) Cadmium induced testicular damage and its response to administration of succimer and diphenyl diselenide in mice. Toxicol Lett 152:255–263. doi:10.1016/j.toxlet.2004.05.009
31. Goncalves JF, Duarte MM, Fiorenza AM, Spanevello RM, Mazzanti CM, Schmatz R, Bagatini MD, Antes FG, Costa P, Abdalla FH, Dressler VL, Morsch VM, Schetinger MR (2012) Hematological indices and activity of NTPDase and cholinesterase enzymes in rats exposed to cadmium and treated with *N*-acetylcysteine. Biometals 25:1195–1206. doi:10.1007/s10534-012-9582-2
32. Abdalla FH, Cardoso AM, Pereira LB, Schmatz R, Goncalves JF, Stefanello N, Fiorenza AM, Gutierres JM, Serres JD, Zanini D, Pimentel VC, Vieira JM, Schetinger MR, Morsch VM, Mazzanti CM (2013) Neuroprotective effect of quercetin in ectoenzymes and acetylcholinesterase activities in cerebral cortex synaptosomes of cadmium-exposed rats. Mol Cell Biochem 381:1–8. doi:10.1007/s11010-013-1659-x
33. Braganhol E, Tamajusuku AS, Bernardi A, Wink MR, Battastini AM (2007) Ecto-5′-nucleotidase/CD73 inhibition by quercetin in the human U138MG glioma cell line. Biochim Biophys Acta 1770:1352–1359. doi:10.1016/j.bbagen.2007.06.003
34. Rockenbach L, Bavaresco L, Fernandes Farias P, Cappellari AR, Barrios CH, Bueno Morrone FO, Battastini AM (2013) Alterations in the extracellular catabolism of nucleotides are involved in the antiproliferative effect of quercetin in human bladder cancer T24 cells. Urol Oncol 31:1204–1211. doi:10.1016/j.urolonc.2011.10.009
35. Boyum A (1968) Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand J Clin Lab Invest Suppl 97:77–89
36. Bergmeyer H (1983) Methods of enzymatic analysis. Verlag Chemie, Deerfiled Beach
37. Leal DB, Streher CA, Neu TN, Bittencourt FP, Leal CA, da Silva JE, Morsch VM, Schetinger MR (2005) Characterization of NTPDase (NTPDase1; ecto-apyrase; ecto-diphosphohydrolase; CD39; EC 3.6.1.5) activity in human lymphocytes. Biochim Biophys Acta 1721:9–15. doi:10.1016/j.bbagen.2004.09.006
38. Chan K, Delfert D, Junger KD. A direct colorimetric assay for Ca^{2+}-ATPase activity. Anal Biochem 1986;157:375-8
39. Giusti G, Gakis C (1971) Temperature conversion factors, activation energy, relative substrate specificity and optimum pH of adenosine deaminase from human serum and tissues. Enzyme 12:417–425
40. Ellman GL, Courtney KD, Andres V, Jr Feather-Stone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95
41. Fitzgerald BB, Costa LG (1993) Modulation of muscarinic receptors and acetylcholinesterase activity in lymphocytes and in brain areas following repeated organophosphate exposure in rats. Fundam Appl Toxicol 20:210–216
42. Metcalf J, Gallin WN, Nauseef R (1986) Root, laboratory manual of neutrophil function. Raven Press, New York
43. Kayyali US, Moore TB, Randall JC, Richardson RJ (1991) Neurotoxic esterase (NTE) assay: optimized conditions based on detergent-induced shifts in the phenol/4-aminoantipyrine chromophore spectrum. J Anal Toxicol 15:86–89
44. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
45. Murugavel PP, Pari L (2007) Diallyl tetrasulfide modulates the cadmium-induced impairment of membrane bound enzymes in rats. J Basic Clin Physiol Pharmacol 18:37–48
46. Thome GR, Mazzanti CM, Ahmed M, Correa M, Spanevello RM, Maldonado PA, Luchese C, Cargnelutti D, Morsch VM, Duarte MM, Fiorenza AM, Nogueira CW, De Bona KS, Moretto MB, Da Luz SC, Mazzanti A, Schetinger MR (2009) Activity of ectonucleotidases and adenosine deaminase in rats exposed to cigarette smoke. Inhal Toxicol 21:906–912. doi:10.1080/08958370802632267
47. Junger WG (2011) Immune cell regulation by autocrine purinergic signalling. Nat Rev Immunol 11:201–212. doi:10.1038/nri2938
48. Scheuplein F, Schwarz N, Adriouch S, Krebs C, Bannas P, Rissiek B, Seman M, Haag F, Koch-Nolte F (2009) NAD+ and ATP released from injured cells induce P2X7-dependent shedding of CD62L and externalization of phosphatidylserine by murine T cells. J Immunol 182:2898–2908. doi:10.4049/jimmunol.0801711
49. Nair MP, Mahajan S, Reynolds JL, Aalinkeel R, Nair H, Schwartz SA, Kandaswami C (2006) The flavonoid quercetin inhibits proinflammatory cytokine (tumor necrosis factor alpha) gene expression in normal peripheral blood mononuclear cells via modulation of the NF-kappa beta system. Clin Vaccine Immunol 13:319–328. doi:10.1128/CVI.13.3.319-328.2006
50. Chen JC, Ho FM, Pei-Dawn Lee C, Chen CP, Jeng KC, Hsu HB, Lee ST, Wen Tung W, Lin WW (2005) Inhibition of iNOS gene expression by quercetin is mediated by the inhibition of IkappaB kinase, nuclear factor-kappa B and STAT1, and depends on heme oxygenase-1 induction in mouse BV-2 microglia. Eur J Pharmacol 521:9–20. doi:10.1016/j.ejphar.2005.08.005

51. Cho SY, Park SJ, Kwon MJ, Jeong TS, Bok SH, Choi WY, Jeong WI, Ryu SY, Do SH, Lee CS, Song JC, Jeong KS (2003) Quercetin suppresses proinflammatory cytokines production through MAP kinases and NF-kappaB pathway in lipopolysaccharide-stimulated macrophage. Mol Cell Biochem 243:153–160
52. Marzena SM, Mateusz K (2012) Review article: flavonoids and their properties to form chelate complexes. Biotechnol Food Sci 76:35–41
53. Dehghan G, Khoshkam Z (2011) Chelation of toxic Tin(II) by quercetin: a spectroscopic study. Int Conf Life Sci Technol 3:1–3
54. Rajendran M, Ravichandran R, Devapiriam D (2012) Molecular modeling study of quercetin and their metal complexes. Int J Comput Appl 50:30–34
55. Jaques J, Rezer J, Ruchel J, Becker L, CS R, Luz S, Gutierres J, JF G, Morch V, Schetinger M, Leal D (2011) Lung and blood lymphocytes NTPDase and acetylcholinesterase activity in cigarette smoke-exposed rats treated with curcumin. Biomed Prevent Nutr 1:109–115
56. Kaizer RR, Gutierres JM, Schmatz R, Spanevello RM, Morsch VM, Schetinger MR, Rocha JB (2010) In vitro and in vivo interactions of aluminum on NTPDase and AChE activities in lymphocytes of rats. Cell Immunol 265:133–138. doi:10.1016/j.cellimm.2010.08.001
57. Nizri E, Hamra-Amitay Y, Sicsic C, Lavon I, Brenner T (2006) Anti-inflammatory properties of cholinergic up-regulation: a new role for acetylcholinesterase inhibitors. Neuropharmacology 50:540–547. doi:10.1016/j.neuropharm.2005.10.013
58. Mazzanti CM, Spanevello R, Ahmed M, Pereira LB, Goncalves JF, Correa M, Schmatz R, Stefanello N, Leal DB, Mazzanti A, Ramos AT, Martins TB, Danesi CC, Graca DL, Morsch VM, Schetinger MR (2009) Pre-treatment with ebselen and vitamin E modulate acetylcholinesterase activity: interaction with demyelinating agents. Int J Dev Neurosci 27:73–80. doi:10.1016/j.ijdevneu.2008.09.005
59. El-Demerdash FM, Yousef MI, Kedwany FS, Baghdadi HH (2004) Cadmium-induced changes in lipid peroxidation, blood hematology, biochemical parameters and semen quality of male rats: protective role of vitamin E and beta-carotene. Food Chem Toxicol 42:1563–1571. doi:10.1016/j.fct.2004.05.001
60. Loizzo MR, Tundis R, Menichini F (2008) Natural products and their derivatives as cholinesterase inhibitors in the treatment of neurodegenerative disorders: an update. Curr Med Chem 15:1209–1228
61. Manjeet KR, Ghosh B (1999) Quercetin inhibits LPS-induced nitric oxide and tumor necrosis factor-alpha production in murine macrophages. Int J Immunopharmacol 21:435–443
62. Geraets L, Moonen HJ, Brauers K, Wouters EF, Bast A, Hageman GJ (2007) Dietary flavones and flavonoles are inhibitors of poly(ADP-ribose)polymerase-1 in pulmonary epithelial cells. J Nutr 137:2190–2195
63. Melzig MF (1996) Inhibition of adenosine deaminase activity of aortic endothelial cells by selected flavonoids. Planta Med 62:20–21. doi:10.1055/s-2006-957788
64. Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, Chen JF, Enjyoji K, Linden J, Oukka M, Kuchroo VK, Strom TB, Robson SC (2007) Adenosine generation catalysed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. J Exp Med 204:1257–1265

4. DISCUSSÃO

O Cd é um metal pesado amplamente utilizado na fabricação de fertilizantes, tintas, plásticos, pilhas e baterias, sendo, também, um dos principais componentes da fumaça do cigarro (GODT et al., 2006). Produtos contendo Cd são frequentemente despejados junto com o lixo doméstico e, assim, contaminam o meio ambiente, especialmente se os resíduos são queimados. O Cd lançado no meio ambiente pode ser absorvido pelos tecidos animais e vegetais, contaminando o ecossistema terrestre e o aquático, entrando, desta forma, na cadeia alimentar (JARUP et al., 1998). Portanto, a dieta é considerada a principal fonte de exposição ao metal na população em geral (JARUP et al., 1998; JARUP e AKSSON, 2009).

Devido à meia vida biológica longa e à baixa taxa de excreção do Cd no organismo, o mesmo pode acumular-se em vários órgãos (SANTOS et al., 2004; SANTOS et al., 2005a,b; SANTOS et al., 2006; LUCHESE et al., 2007; BORGES et al., 2008; GONÇALVES et al., 2012). Neste contexto, ressalta-se que estudos realizados pelo nosso grupo de pesquisa corroboram com estes dados, indicando que a exposição ao Cd causa toxicidade a diferentes tecidos, podendo acumular-se em diferentes estruturas encefálicas, bem como no sistema periférico (GONÇALVES et al., 2012a,b; ABDALLA et al., 2013; GONÇALVES et al., 2013).

Entretanto, apesar da toxicologia do Cd ser amplamente estudada, ainda não esta bem esclarecido o mecanismo pelo qual o metal causa a toxicidade no SNC e sistema imunológico. Estudos sobre os efeitos crônicos do $CdCl_2$ relacionando a atividade das enzimas colinesterases, mieloperoxidase (MPO), ectonucleotidases, Na^+, K^+-ATPase e δ-ALA-D com a bioacumulação de Cd em diferentes estruturas encefálicas e com parâmetros do estresse oxidativo, comportamentais e de memória ainda não foram completamente registrados na literatura. Além disso, não é relatado o uso do flavonoide quercetina no tratamento deste tipo de intoxicação, interligando os parâmetros acima citados. Assim, estes dados podem nos fornecer uma melhor compreensão dos mecanismos pelos quais o Cd pode causar toxicidade ao SNC e SNP em animais experimentais. Adicionalmente, sabe-se que a utilização de compostos antioxidantes, como a quercetina, torna-se cada vez mais popular no tratamento de transtornos relacionados ao dano oxidativo, sendo, desta forma, considerados agentes terapêuticos promissores.

Desta maneira, este é o primeiro trabalho no qual investigou-se os efeitos da exposição crônica do $CdCl_2$ sobre o perfil oxidativo, a atividade das enzimas AChE, Na^+, K^+-ATPase e δ-ALA-D no encéfalo de ratos, bem como o envolvimento da atividade destas enzimas com parâmetros comportamentais. Além disso, investigou-se, neste estudo, a atividade das enzimas dos sistemas purinérgico e colinérgico nos linfócitos, a atividade da BuChE no soro e da MPO no plasma de animais expostos ao $CdCl_2$. Foi analisado, ainda, o possível efeito protetor da quercetina, composto com ação antioxidante, antiinflamatória e neuroprotetora, através da análise dos mesmos parâmetros citados acima em animais submetidos a exposição do $CdCl_2$ e tratados com a quercetina em diferentes doses.

Dentre os resultados obtidos no presente trabalho destaca-se, primeiramente, que, de fato, o Cd é capaz de atravessar a barreira hematoencefálica, pois, embora a quantidade de Cd acumulada nas diferentes estruturas encefálicas estudadas (córtex cerebral, cerebelo, estriado, hipocampo e hipotálamo) tenha sido baixa, ela ainda foi significativamente maior que o controle e suficiente para causar possíveis danos cerebrais. Contudo, o tratamento concomitante da quercetina nos animais expostos ao Cd foi ineficiente em diminuir estes níveis, sugerindo que os prejuízos ocasionados pelo Cd, e que foram revertidos ou amenizados pela quercetina, tenham sido, provavelmente, devido à sua atividade antioxidante e não à sua atividade como quelante, uma vez que essa molécula não removeu o Cd dos tecidos.

Tendo em vista os prejuízos das funções cognitivas observadas em animais experimentais expostos ao Cd, foi investigado o efeito da exposição ao metal, bem como do tratamento com a quercetina em diferentes doses sobre a aprendizagem e memória, através da tarefa de esquiva inibitória, e o comportamento do tipo ansiogênico, através da tarefa do labirinto em cruz elevada. Além disso, para identificar possíveis alterações que possam influenciar no desempenho dos testes comportamentais, foi realizado o teste do campo aberto, que avalia a capacidade motora e exploratória do animal, e o teste de sensibilidade ao choque.

Observou-se um decréscimo na latência dos ratos expostos ao Cd quando submetidos a essa tarefa, sugerindo prejuízos na aprendizagem e memória. Estes dados estão de acordo com um recente estudo realizado pelo nosso grupo de pesquisa onde animais expostos ao $CdCl_2$ (1, 5 ou 25 mg/kg) e a tubérculos de

batata contendo Cd, também apresentaram alteração na aprendizagem e memória (GONÇALVES et al., 2012). Contudo, é importante salientar que, quando os animais expostos ao Cd foram submetidos ao tratamento concomitante com a quercetina, a latência de descida da plataforma do teste de esquiva inibitória foi similar àquela encontrada em ratos controles. Estes dados sugerem que o tratamento com a quercetina foi capaz de prevenir o déficit de aprendizagem e memória nos animais expostos ao metal. Os resultados do presente estudo corroboram com recentes pesquisas realizadas pelo nosso grupo de pesquisa onde o tratamento com compostos naturais, como a N-acetilcisteína e a quercetina, previnem a neurotoxicidade causada pela a exposição ao Cd, sugerindo o papel antioxidante dos compostos (GONÇALVES et al., 2010; ABDALLA et al., 2013).

É importante ressaltar que, após o teste de esquiva inibitória, os ratos foram submetidos ao teste do campo aberto e de sensibilidade ao choque. No teste do campo aberto, observou-se que os ratos expostos ao Cd e os tratados somente com a quercetina não apresentaram alterações no número de cruzamento ou levantada. Além disso, no teste de sensibilidade ao choque, os ratos expostos ao Cd não apresentaram sensibilidade nas patas quando avaliado os limiares de recuar e saltar da plataforma. Assim, estes resultados excluem a possibilidade que a atividade locomotora ou da sensibilidade do animal possam ter influenciado nos testes de aprendizagem e memória.

Em relação ao comportamento do tipo ansiogênico, avaliado pelo teste do labirinto em cruz elevada, observou-se que os ratos expostos ao Cd apresentaram um comportamento do tipo ansiogênico. Interessantemente, a quercetina apresentou propriedades ansiolíticas sobre o efeito do Cd. De fato, vários estudos relataram as propriedades ansiogênicas do Cd; Gonçalves et al., (2012b) observaram que ratos expostos a 25 mg/kg de Cd apresentaram um aumento na ansiedade e diminuição da memória, sugerindo o envolvimento do sistema colinérgico e da enzima Na^+, K^+-ATPase, visto que esses animais apresentaram uma atividade aumentada da AChE e diminuição na atividade Na^+, K^+-ATPase em diferentes estruturas encefálicas. Leret et al. (2003) também observaram um comportamento do tipo ansiogênico em ratos co-expostos ao Cd (10 mg/L) e ao Pb (300 mg/L), sugerindo que este efeito 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. Por outro lado, Minetti e Reale (2005) demonstraram que fetos expostos pré-natalmente a 0,6 mg/kg de Cd apresentaram menor ansiedade. É importante ressaltar que a dose de Cd utilizada por Minetti e Reale (2005) é menor se comparada à dose utilizada no presente trabalho.

Um importante resultado observado no presente estudo foi o efeito ansiolítico da quercetina nos ratos expostos ao Cd. Vários estudos têm relatado as propriedades ansiolíticas deste flavonoide em outros modelos experimentais (KUMAR e GOYAL, 2008; BHUTADA et al., 2010; VISSIENNON et al., 2012). Outrossim, diferentes mecanismos são sugeridos para explicar os efeitos ansiolíticos da quercetina, como a modulação dos sistemas gabaérgicos e serotonérgicos, neurotransmossores envolvidos com a ansiedade e depressão (FRUSSA-FILHO, et al., 1999).

De acordo com os resultados obtidos no presente trabalho, sugere-se que as alterações na ansiedade e memória observada nos animais expostos ao Cd e tratados com a quercetina possa estar associada a possíveis alterações no sistema colinérgico, assim como com a atividade da enzima Na^+, K^+-ATPase, visto que o neurotransmissor ACh tem um papel importante em muitas funções no SNC (SCARR, 2011), atuando, principalmente, nos processos de aprendizagem e memória, além do controle locomotor e do fluxo sanguíneo cerebral (KIEHN, 2006; DEIANA et al., 2011; HAVEKES et al., 2011; HUT e VAN DER ZEE, 2011; KLINKENBERG et al., 2011). É importante ressaltar, também, que a enzima Na^+, K^+-ATPase desempenha um papel importante na neurotransmissão sináptica através da manutenção do gradiente eletrolítico de íons de Na^+e K^+ e do volume de pH celular (LINGREL, 1992; LINGREL et al., 1994a; LINGREL et al., 1994b).

Como forma de comprovar o possível envolvimento do sistema colinérgico com as alterações observadas no comportamento de animais expostos ao Cd, no presente estudo, avaliou-se a atividade da enzima AChE em diferentes estruturas encefálicas (córtex cerebral, hipocampo, hipotalamo, cerebelo e estriado), uma vez que a enzima AChE hidrolisa o neurotransmissor ACh em muitos tecidos, modulando a concentração do mesmo na sinapse colinérgica (APPLEYARD, 1994). Observou-se que a exposição ao Cd reduziu a atividade da enzima AChE no córtex cerebral e no hipocampo. Em compensação, uma ativação da enzima foi observada no hipotálamo dos animais expostos ao Cd. Sugere-se que uma inibição da atividade

da AChE, causado pela exposição ao metal, pode levar a uma diminuição na hidrólise da ACh na sinapse neuronal e, consequentemente, um aumento do neurotransmissor na fenda sináptica, podendo causar uma superestimulação dos receptores colinérgicos e possíveis efeitos tóxicos. Por outro lado, a ativação da AChE poderia levar a um aumento na degradação do neurotransmissor ACh e, consequentemente, causar uma redução no estimulo dos receptores deste neurotransmissor, causando efeitos indesejáveis sobre a neurotransmissão colinérgica e um declínio cognitivo.

É importante observar que, em outro estudo realizado pelo nosso grupo de pesquisa, uma ativação da AChE foi observada no sinaptossoma do córtex cerebral de ratos expostos à mesma dose de Cd e com o mesmo tempo de exposição (ABDALLA et al., 2013). Acredita-se que estas diferenças de resultados encontrados para a atividade da AChE, no homogeneizado do córtex em sinaptossomas da mesma estrutura, possam ser explicadas devido às diversas formas moleculares da enzima AChE no organismo, a forma globular e a assimétrica, já que a forma globular apresenta-se solúveis ou ancoradas à membrana, enquanto que as formas assimétricas estão incluídas na matriz extracelular (ALDUNATE et al., 2004).

Em contraste como resultados encontrados, Gonçalves et al. (2010) mostram uma diminuição na atividade da AChE no hipocampo, cerebelo e hipotálamo, enquanto que nenhuma alteração na atividade da enzima foi observada no sinaptossomas de córtex cerebral e estriado de ratos expostos ao Cd (2 mg/kg). Neste caso, é importante salientar que a dose do Cd e período experimental de exposição a este metal utilizado por Gonçalves et al. (2010) foi menor do que o utilizado no presente estudo. Além disso, é importante ressaltar que os resultados descritos na literatura relacionados aos efeitos do Cd na atividade da AChE são controversos (CARAGEORGIOU et al., 2004; LUCHESE et al., 2007). Alguns estudos mostraram que o Cd pode influenciar a atividade de AChE de uma maneira diferente dependendo do período da administração do metal (FASITSAS et al., 1991; CARAGEORGIOU et al., 2004; CARAGEORGIOU et al., 2005). Tendo em vista estes resultados encontrados na literatura, em relação à atividade da AChE, é plausível sugerir que a alteração na atividade dessa enzima pode depender da via de administração, forma química, e, ainda, duração e dose de exposição do Cd.

Em relação à atividade da Na^+, K^+-ATPase verificou-se uma diminuição na atividade da enzima no córtex cerebral, hipocampo e hipotálamo de ratos expostos ao Cd. Acredita-se que essa inibição causada pela exposição ao Cd seja devido à competição do 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 exposição ao Cd pode aumentar a produção de radicais livres, os quais podem reagir com os lipídios de membrana, e causar desestruturação da bicamada lipídica com consequente alteração na atividade de enzimas de membrana como a AChE e a Na^+, K^+-ATPase (KAKO et al., 1988; MATTÉ et al., 2006). Uma vez que a Na^+, K^+-ATPase desempenha uma função crucial na comunicação dos sinais extracelulares com o meio intracelular nos tecidos neuronais, e que a sua inibição prejudica os processos de aprendizado e memória, supõem-se que a alteração na atividade das enzimas AChE e Na^+, K^+-ATPase em algumas estruturas encefálicas poderia estar envolvida com o prejuízo cognitivo observado em animais expostos ao Cd no presente estudo (ANTONIO et al., 2002; SATO et al., 2004; ZHAN et al., 2004).

Interessantemente, a quercetina foi capaz de prevenir os efeitos indesejáveis causados pela exposição ao Cd. O tratamento com a quercetina, concomitante a exposição ao metal, impediu alterações na atividade das enzimas AChE e Na^+, K^+-ATPase em diferentes estruturas encefálicas. Desta maneira, considerando o potencial antioxidante da quercetina, é plausivel sugerir que o tratamento com este flavonoide preveniu o estresse oxidativo induzido pela exposição ao Cd, como observado, neste trabalho, pela prevenção no aumento da lipoperoxidação. Consequentemente, o tratamento com a quercetina modulou os níveis de ACh no SNC melhorando a neurotransmissão colinérgica e a cognição. O tratamento com a quercetina também preveniu modificações do estado conformacional da Na^+, K^+-ATPase, mantendo, desta forma, o potencial de repouso na membrana neuronal.

Outro aspecto importante a ser observado neste estudo, é a diminuição na atividade da δ-ALA-D no encéfalo de animais expostos ao Cd. A δ-ALA-D é uma metaloenzima que requer Zn^{2+}para atingir a sua atividade catalítica máxima. Portanto, um dos mecanismos sugeridos para a inibição da enzima pela exposição ao Cd é o deslocamento do íon Zn^{2+} causado pelo aumento do metal no encéfalo

(RODRIGUES et al., 1996; GALAN et al., 2001). Esta inibição na atividade δ-ALA-D pode prejudicar a via biossintética do heme (SASSA, 1982) e conduzir a um acúmulo do seu substrato, o ALA, que pode ser auto-oxidado e levar a um aumento na produção de espécies reativas de oxigênio (ERO) no SNC (EMANUELLI et al., 1996).

A utilização de compostos com propiedades quelantes ou antioxidantes tem sido amplamente proposta como uma terapia alternativa no tratamento da intoxicação ao Cd (LUCHESE etal., 2007; GONÇALVES et al., 2012b; ABDALLA et al., 2013), uma vez que compostos com estas propiedades atuam através do aumento da excreção de elementos tóxicos, impedindo a ligação a células alvo e, consequentemente, reduzindo a toxicidade ao organismo (SANTOS et al., 2005). Ressalta-se que estudos realizados pelo nosso grupo de pesquisa relatam a capacidade dos compostos naturais, tais como quercetina e N-acetilcisteína, em prevenir danos causados pela exposição ao Cd no SNC de animais (GONÇALVES et al., 2010; ABDALLA et al., 2013). Neste trabalho, entretanto, o tratamento com o flavonoide quercetina não foi capaz de prevenir a inibição da atividade da enzima δ-ALA-D. Sugere-se, assim, que os prejuízos ocasionados pela exposição ao Cd e que foram revertidos ou amenizados pela quercetina tenham sido devido a sua atividade antioxidante.

Tendo em vista a propriedade pró-oxidante do Cd, e na tentativa de melhor compreender os efeitos indesejáveis causados pela exposição ao metal, como as alterações comportamentais e enzimáticas observadas no presente estudo, investigou-se, também, os efeitos da exposição ao Cd em diferentes parâmetros do estresse oxidativo. Supõem-se que estes dados poderiam esclarecer melhor o mecanismo possível pelo qual a quercetina exerce seus efeitos neuroprotetores neste modelo experimental.

O presente trabalho confirmou que, de fato, houve um aumento na produção de EROs nos animais expostos ao Cd, e, junto com este aumento na produção de EROs, observou-se, também, um aumento na lipoperoxidação, na oxidação de proteínas e nos níveis de DNA dupla fita, no córtex cerebral, hipocampo e hipotálamo dos animais expostos ao Cd. Estes resultados sugerem que a diminuição na atividade da enzima δ-ALA-D em ratos expostos Cd possa estar intimamente associada com o aumento na produção de EROs, devido ao acúmulo do seu

substrato, ALA. Consequentemente, este aumento na produção de EROs induziu a um estresse oxidativo, levando ao dano em lipídeos e proteínas, fato este que pode induzir à alterações nas funções das membranas, causando a apoptose celular, bem como necrose, de vários tecidos, incluindo o encéfalo (MENDEZ-ARMENTA et al., 2003; EL-DEMERDASH et al., 2004; KRISHNAKUMAR et al., 2012). Além disso, é importante notar que as enzimas AChE e Na^+, K^+-ATPase são importantes proteínas presentes nas membranas biológicas e, desta maneira, o aumento na lipoperoxidação e na oxidação de proteínas pode ser um fator decisivo na modificação do estado conformacional dessas moléculas (DESCARRIES et al., 1997). Estes resultados nos permitem inferir que o estresse oxidativo pode explicar as mudanças enzimáticas observadas neste estudo, as quais, consequentemente, podem causar uma diminuição da memória e um aumento no comportamento do tipo ansiogênico observado nos animais expostos ao Cd.

Além do aumento na lipoperoxidação e do dano às proteínas, observou-se um aumento nos níveis de DNA dupla fita em diferentes estruturas encefálicas dos animais expostos ao metal. O Cd pode causar um aumento no dano ao DNA indiretamente devido à interação das EROs produzidas pela exposição ao metal ou, ainda, devido à capacidade de diminuição do reparo do DNA (WATJEN e BEYERSMANN, 2004; VALVERDE et al., 2001). Outro importante resultado verificado é a relação dos danos oxidativos, observados nos animais expostos ao Cd, com o sistema antioxidante enzimático e não enzimático, como a atividade da glutationa redutase (GR), os níveis dos tióis totais (T-SH) de glutationa reduzida (GSH) e a atividade da glutationa S-transferase (GST). Paralelamente ao aumento na produção de EROs, da lipoperoxidação, oxidação de proteínas e os níveis DNA dupla fita no córtex cerebral, hipocampo e hipotálamo dos animais expostos Cd, também houve alterações no sistema antioxidante destes tecidos, incluindo uma diminuição na atividade da enzima GR e nos níveis de T-SH e GSH, bem como um aumento na atividade da enzima GST.

A GSH é um antioxidante de proteção central e é considerada a primeira linha de defesa contra a geração de EROs e o dano oxidativo no tecido cerebral. Faz parte de um sistema de defesa antioxidante não enzimático, podendo eliminar, diretamente, os radicais livres ou, ainda, agir como um substrato para a glutationa peroxidase e GST na eliminação do peróxido de hidrogênio (LU, 2013). Assim,

propõe-se que a depleção nos níveis de GSH, de T-SH e a diminuição na atividade da enzima GR observadas nos animais expostos ao Cd possam ser um efeito compensatório devido a maior demanda do sistema de defesa, a fim de eliminar as EROs e produtos da lipoperoxidação e oxidação das proteínas. Além disso, a diminuição da GR pode ser causada diretamente pelo Cd, que pode ligar-se a grupos -SH de enzimas, causar modificações nas cadeias de aminoácidos, através da oxidação dos mesmos, e, assim, alterar a estrutura enzimática, levando a uma diminuição da atividade ou inativando a enzima GR. Em contrapartida, verificou-se um aumento na atividade de GST em animais expostos ao metal. Sobre este ponto, sugere-se que, sob condições de estresse oxidativo, como na exposição ao Cd, a produção excessiva de ERO induz a um aumento na atividade da GST, uma vez que esta enzima tem por finalidade metabolizar os produtos tóxicos da peroxidação lipídica, da oxidação de proteínas, e dos danos ao DNA dentre outras moléculas (TOYAMA etal., 2011).

Tendo em vista os resultados obtidos neste trabalho, em relação aos parâmetros do estresse oxidativo, avalia-se que o mecanismo de toxicidade do Cd no encéfalo pode estar relacionado com a diferença na vulnerabibidade das estruturas encefálicas ao dano oxidativo, uma vez que, embora ambas as estruturas encefálicas tenham apresentado um aumento significativo nos níveis de Cd, nos animais expostos ao metal, não foi observado alterações nos parâmetros do estresse oxidativo no cerebelo e estriado nesses animais. Observou-se alterações relacionadas ao estresse oxidativo e na atividade de enzimas AChE, Na^{+}-ATPase somente no córtex, hipocampo e hipotálamo de animais expostos ao metal.

Os benefícios dos flavonóides no SNC estão bem definidos na literatura e acredita-se que o efeito neuroprotetor ocorra, principalmente, devido às propriedades antioxidantes destes compostos (FIORANI et al., 2001; GONÇALVES et al., 2010; ABDALLA et al., 2013). Ressalta-se, ainda, que vários estudos já relataram a presença da quercetina no encéfalo de ratos suplementados com a mesma, demonstrando a capacidade deste composto em atravessar a BHE para exercer seus efeitos neuroprotetores (YOUDIN et al., 2004). Interessantemente, notou-se, no presente estudo, que a co-administração da quercetina nos animais expostos ao Cd foi capaz de impedir totalmente ou parcialmente os efeitos indesejáveis causados pela exposição ao metal. Os efeitos benéficos da quercetina

podem ocorrer devido as propriedades antioxidantes e pela sua propriedade quelante (EZZATI NAZHADDOLATABADI et al., 2014). Assim, a quercetina pode diminuir a produção de EROs, a lipoperoxidação, a carbonilação de proteínas e os danos ao DNA, aumentando as defesas antioxidantes do organismo e prevenindo a alteração na atividade ou na conformação de importantes enzimas. Corroborando com estes resultados, vários trabalhos relataram que a quercetina diminuiu a lipoperoxidação e aumentou a atividade das enzimas SOD e CAT, diminuindo o esgotamento da glutationa e atenuando os danos neuronais, como a morte dos neurônios do hipocampo, resultando, consequentemente, na melhora da aprendizagem e da memória dos animais (PU et al., 2007; KRISHNAKUMAR et al., 2012; FIORANI etal., 2001). Essas observações reforçam o potencial antioxidante da quercetina no encéfalo e nos permitem sugerir que o possível mecanismo para os efeitos neuroprotetores deste flavonoide em animais expostos ao Cd é através da modulação do sistema antioxidante, prevenindo os danos causados pelo estresse oxidativo. Portanto, acredita-se que a quercetina, administrada por via oral, é um forte coadjuvante no tratamento de possível envenenamento por metais como o Cd.

Para obter-se uma melhor compreensão dos efeitos do $CdCl_2$ e da quercetina sobre o sistema periférico, decidiu-se avaliar os efeitos do $CdCl_2$ sobre a atividade da enzima AChE em linfócitos, bem como sobre a atividade da BuChE em soro e a MPO no plasma. Verificou-se, também, os efeitos do $CdCl_2$ e da quercetina sobre a atividade da enzima NTPDase e ADA de linfócitos e o efeito *in vitro* da quercetina nos parâmetros acima mencionados.

Em relação à atividade da enzima NTPDase e da ADA em linfócitos de animais expostos ao Cd, notou-se um aumento na atividade das enzimas quando comparado com o grupo controle. A partir desse resultado, pode-se inferir que o aumento na atividade da NTPDase seja um efeito compensatório devido ao provável aumento dos níveis de ATP na circulação, para que ocorra uma maior ativação dos linfócitos com a consequente liberação de mediadores inflamatórios (JUNGER, 2011; KANTHI et al., 2014). Apesar dos níveis aumentados de ATP, que estão sendo liberados no meio extracelular, a adenosina, que esta sendo formada e que poderia estar exercendo suas ações anti-inflamatórias, está sendo hidrolisada e inativada pelo aumento na atividade da enzima ADA (SCHENK et al., 2008; JUNGER, 2011). É pertinente citar, também, que a quercetina foi capaz de prevenir os efeitos

negativos causados pelo Cd na atividade de enzimas do sistema purinérgico. Desta maneira, tendo em vista que as propriedades anti-inflamatórias da quercetina já estão bem estabelecidas na literatura, sugere-se que o tratamento concomitante da quercetina em animais expostos ao Cd pode levar à uma redução nos processos inflamatórios causados pela exposição ao metal, e, consequentemente, resultar na inibição da atividade enzimática e nos níveis de ATP e adenosina no meio extracelular.

Em relação à atividade da AChE em linfócitos e BuChE no soro dos animais expostos ao Cd, observou-se um aumento na atividade em ambas enzimas. Estes resultados nos permitem sugerir que níveis diminuídos de ACh estão disponíveis na circulação devido ao aumento na atividade das colinesterases periféricas. Portanto, uma vez que a ACh é uma molécula com ações anti-inflamatórias (DAS, 2007), uma diminuição na sua concentração pode evidenciar o possível quadro inflamatório causado pela exposição ao metal. Diferentes resultados foram encontrados em recentes trabalhos realizados pelo nosso grupo de pesquisa, onde verificou-se uma diminuição na atividade da BuChE no soro e da AChE em sangue total e linfócitos de animais expostos a 2mg/kg de $CdCl_2$ (GONÇALVES et al., 2012). Sendo assim, 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.

É importante notar que o tratamento com quercetina preveniu o aumento na atividade das enzimas AChE e BuChE causado por exposição de Cd. O provável mecanismo de inibição da AChE e BuChE é através da interação enzima – composto, uma vez que a quercetina pode se ligar a resíduos de aninoácidos além de realizar interações hidrofóbicas com ambas enzimas (LOIZZO et al., 2008). Deste modo, estes resultados nos permitem inferir que, através de uma modulação enzimatica na atividade de enzimas do sistema colinérgico, a quercetina diminui a degradação da ACh, mantendo níveis normais desta molécula com potente ação anti-inflamatória no meio extracelular.

Para confirmar a presença de uma condição inflamatória em animais expostos ao Cd, bem como o possível potencial anti-inflamatório da quercetina nestas condições, a atividade da enzima MPO, que é um marcador inflamatório

clássico, liberado a partir de leucócitos ativados, foi avaliada. Os resultados encontrados neste estudo mostraram um aumento na atividade da MPO em animais expostos ao Cd, confirmando a presença de um processo inflamatório causado pela exposição ao metal. Por outro lado, o tratamento com a quercetina impediu o aumento na atividade da MPO causado pela exposição ao Cd, fato que ocorre, provavelmente, pela diminuição do processo inflamatório.

É importante salientar que foi visualizado uma correlação negativa entre a atividade das enzimas estudadas, NTPDase, ADA, AChE, BuChE ou MPO, e as diferentes concentrações de quercetina (5, 25 ou 50 mg/kg) co-administradas com o Cd. Destaca-se que o efeito da quercetina no sistema periférico sobre a atividade destas enzimas é dose dependente.

Analisou-se, também, o efeito da quercetina *in vitro* sobre a atividade da NTPDase, ADA, AChE, BuChE e MPO. Os resultados demonstraram que não houve alteração significativa na atividade da enzima NTPDase utilizando ATP ou ADP como substrato em linfócitos periférico. Todavia, uma diminuição na atividade da ADA em linfócitos periféricos foi observada. Estes resultados corroboram com Melzig (1996), que relatou a capacidade de flavonoides, dentre eles a quercetina, em inibir a atividade da enzima ADA. Assim, sugere-se que a quercetina *in vitro*, *per se* pode promover um aumento da molécula de adenosina no meio extracelular, exercendo uma importante ação anti-inflamatória, bem como imunossupressora, através da inibição da proliferação de células T e secreção de citocinas (LINDEN, 2006; DEAGLIO et al., 2007).

Contudo, este estudo permitiu desvendar, em parte, os mecanismos relacionados aos processos de toxicidade do Cd no SNC e SNP. Observou-se que, apesar das controvérsias em relação aos mecanismos propostos pelos quais o Cd poderia causar alterações na atividade de importantes enzimas para a neurotransmissão e para parâmetros comportamentais, é evidente que a exposição ao metal é prejudicial para as funções cerebrais e, consequentemente, para o comportamento animal, em aspectos relacionados à memória e ansiedade. Os dados do presente trabalho sugerem que os danos ao comportamento exibidos pelos animais intoxicados pelo Cd podem estar intimamente associados ao estresse oxidativo e à alteração na enzima α-ALA-D, que induz alterações nas atividades das enzimas AChE e Na^+, K^+-ATPase em diferentes estruturas encefálicas. Além disso,

verificou-se que o Cd afeta sistematicamente o organismo dos animais, alterando a atividade das enzimas NTPDase, AChE, BuChE e MPO de diferentes tipos celulares. É importante ressaltar a importância dos achados relacionados à capacidade antioxidante e anti-inflamatória da quercetina em prevenir vários efeitos danosos causados pelo Cd, sugerindo que este composto possa ser considerado, após estudos adicionais, um importante aliado em terapias contra a intoxicação por esse metal.

5. CONCLUSÕES

- A exposição ao Cd levou a um aumento no comportamento de ansiedade e déficit de aprendizagem e memória dos ratos, o que, provavelmente, resulta de uma concentração aumentada do Cd no encéfalo, e de alterações na atividade das enzimas AChE e Na^+,K^+-ATPase em diferentes estruturas cerebrais devido à exposição a esse metal. O co-tratamento com a quercetina não foi capaz de reduzir os níveis de Cd nas diferentes estruturas encefálicas mas previniu os efeitos indesejáveis causados pela exposição ao Cd na memória e no comportamento do tipo ansiogênico.

- O Cd foi capaz de atravessar a barreira hematoencefálica e acumular-se em diferentes estruturas encefálicas, como o córtex cerebral, cerebelo, estriado, hipocampo e hipotálamo. A quercetina foi ineficiente em diminuir os níveis de Cd nas diferentes estruturas, sugerindo que os prejuízos ocasionados pelo Cd, e que foram revertidos ou amenizados pela quercetina, tenham sido, provavelmente, devido à sua atividade antioxidante e não à sua atividade como quelante, uma vez que a quercetina não foi capaz de mudar as concentrações de Cd encontradas no encéfalo.

- A redução na atividade da AChE poderia levar à mudança na concentração do neurotransmissor na fenda sináptica e, consequentemente, causar efeitos indesejáveis na neurotransmissão. Sugere-se que a alteração na atividade da Na^+,K^+-ATPase encefálicas poderia estar envolvida com o prejuízo cognitivo observado em animais expostos ao Cd. A desestruturação da bicamada lipídica ou o aumento da oxidação de proteínas, causadas pelo aumento na produção de EROs em animais expostos ao Cd, pode ser um dos prováveis mecanismos pelo qual o Cd induz alterações na atividade dessas enzimas de membrana.

- Verificou-se uma diminuição na atividade da δ-ALA-D no encéfalo de animais expostos ao Cd. A inibição desta enzima pode prejudicar a síntese do heme, além de acumular o substrato da mesma, o ALA, que pode aumentar a produção de EROs através da sua oxidação.

- A exposição ao Cd causou um aumento na produção das EROs nos ratos devido, provavelmente, às propriedades pro-oxidantes do metal, bem como pelo acúmulo do substrato da enzima δ-ALA-D. O desequilíbrio entre a produção de EROs e o sistema antioxidante levou ao desenvolvimento do estresse oxidativo, que induziu, consequentemente, um aumento de danos nos lipídeos, proteínas e DNA.

- Apesar do co-tratamento com a quercetina em animais expostos ao Cd não prevenir as alterações na atividade da enzima α-ALA-D, verificou-se que o flavonoide, em ambas as doses utilizadas no estudo, foi eficaz em diminuir a produção de EROs, bem como reduzir os danos causados pelo estresse oxidativo nos animais expostos ao Cd. Consequentemente, com a diminuição da lipoperoxidação e a oxidação de proteínas, a quercetina preveniu alterações em importantes enzimas presentes na membrana biológica, como a AChE e Na^+,K^+-ATPase. Portanto, pode-se sugerir que o tratamento com a quercetina modulou a concentração do neurotransmissor ACh na fenda sináptica, e manteve o potencial de repouso na membrana neuronal, prevenindo o deficit de aprendizagem e memória, além de diminuir efeito ansiogênico da exposição ao metal.

- O tratamento com a quercetina modulou a atividade das enzimas do sistema purinérgico devido às propriedades anti-inflamatórias, sugerindo que, tratamento concomitante da quercetina em animais expostos ao Cd, leva a uma redução nos processos inflamatórios causados pela exposição ao metal, e, consequentemente, resulta na modulação na atividade enzimática, bem como nos níveis de ATP e adenosina no meio extra-celular.

- O aumento na atividade da AChE nos linfócitos e BuChE no soro em ratos expostos ao Cd permite-nos inferir que níveis diminuídos de ACh estão disponíveis na circulação, evidenciando um possível quadro inflamatório causado pela exposição ao metal. Em adição, o aumento na atividade da MPO nestes animais confirma o processo inflamatório desenvolvido pela exposição ao Cd. Em ratos co-tratados com a quercetina, o flavonoide preveniu o aumento na atividade

dessas enzimas, provavelmente, através da diminuição do processo inflamatório ou pela interação entre a enzima e o composto.

- A quercetina *in vitro* não alterou a atividade da NTPDase. Entretanto, a atividade da ADA apresentou-se diminuída em linfócitos periféricos, sugerindo uma capacidade da quercetina em aumentar, *per se,* os níveis de adenosina no meio extracelular.

6. PERSPECTIVAS

Este trabalho forneceu subsídios importantes para melhor esclarecer a relação entre as enzimas dos sistemas purinérgico e colinérgico na intoxicação com o Cd no SNC e SNP, bem como o papel protetor da quercetina nestas condições.

Neste sentido, para melhor esclarecer o real mecanismo de toxicidade do Cd e da prevenção da quercetina sobre o sistema colinérgico e purinérgico aponta-se como foco de futuros trabalhos, o seguinte:

1. Avaliar os efeitos do $CdCl_2$ e da quercetina sobre a expressão gênica das enzimas já estudadas nessa tese com a finalidade de obter um maior esclarecimento sobre os mecanismos pelos quais esse metal atua sobre essas proteínas.

2. Quantificar os níveis de nucleotídeos e nucleosídeos de adenina bem como da ACh no SNC e sistema periférico de animais expostos ao $CdCl_2$ e tratados com quercetina.

3. Determinar o possível efeito protetor da quercetina em relação à intoxicação por $CdCl_2$ através da verificação da concentração de Cd em diferentes tecidos como, no plasma, baço e timo em ratos expostos ao CdCl2 e tratados com quercetina.

4. Avaliar o possível efeito protetor da quercetina em relação à intoxicação por $CdCl_2$ através de dados hematológicos e também, da dosagem de interleucinas em ratos expostos ao $CdCl_2$ e tratados com quercetina.

5. Avaliar no sistema periférico os possíveis mecanismos moleculares envolvidos na modulação dos sistemas colinérgico e purinérgico de ratos expostos ao $CdCl_2$ e tratados com quercetina.

REFERÊNCIAS

ABDALLA, F.H. et al., Neuroprotective effect of quercetin in ectoenzymes and acetylcholinesterase activities in cerebral cortex synaptosomes of cadmium-exposed rats. **Molecular and cellular biochemistry,** v.381, p.1-8, 2013.

AGENCY FOR TOXIC SUBSTANCES AND DISEASE REGISTRY – ATSDR. **Toxicological profile for cadmium.** Atlanta: ATSDR, 347 p, 1997.

AHERNE, S.A.; O'BRIEN, N.M. Dietary flavonols: chemistry, food content, and metabolism. **Nutrition**, v.18, p.75-81, 2002.

AHLENSTIEL, T. et al. Bioflavonoids attenuate renal proximal tubular cell injury during cold preservation in Euro-Collins and University of Wisconsin solutions. **Kidney Int**., v.63, p.554-563, 2003.

ALDUNATE, R. et al. Structural and functional organization of synaptic acetylcholinesterase. **Brain Research Reviews**, v. 47, p. 405-410, 2004.

ALLOWAY, B. J.; STEINNES, E. **Anthropogenic additions of cadmium to soils.** In: McLAUGHLIN, M. J.; SING, B. R. (Org.). Cadmium in soils and plants. Dordrecht: Kluwer Academic Publishers, 98p, 1999.

ANGLADE, P.; LARABI-GODINOT, Y. Historical landmarks in the histochemistry of the cholinergic synapse: perspectives for future researches. **Biomedical Research,** v. 31(1), p. 1-12, 2010.

ANICH, M. et al. A transverso-cori apyrase activity and changes in mtabolites Turing germination and tuberization of Solanum tuberosum. **Phytochemistry**, v.29, p.1411-1415, 1990.

ANTONIO, M. T.; LÓPEZ, N.; LERET, M. L. Pb and Cd poisoning during development alters cerebellar and striatal function in rats. **Toxicology,** v. 176, n. 1-2, p. 59–66, 2002.

ANTONIO, M.T.; CORREDOR, L.; LERET, M.L. Study of the activity of several brain enzymes like markers of the neurotoxicity induced by perinatal exposure to lead and/or cadmium. **Toxicol. Lett,** v.143, p.331-340, 2003.

APPLEYARD, M.E. Non-cholinergic functions of acetylcholinesterase. **Biochem Soc Trans** v. 22, p. 749-755, 1994.

ARAÚJO, M. C. et al. Enzymes that hydrolyze nucleotides in platelets from breast cancer patients. **Biochim Biophys Acta,** v. 1740, n. 3, p. 421–6, 2005.

ARIAS, H. R. Binding sites for exogenous non-competitive inhibitors of the nicotinic acetylcholine receptor. **Biochim Biophys Acta,** v. 1376, n. 2, p. 173-220, 1998.

ARUOMA, O. I. et al. The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. **Free Rad Biol Med,** v. 6, n. 6, p. 593–7, 1989.

AZEVEDO, F. A; CHASIN, A. A. M. **Metais: gerenciamento da toxicidade.** 1ª Edição, Editora Atheneu, São Paulo, 554p., 2003.

BALZ, D. et al. In vitro effects of L-arginine and guanidino compounds on NTPDase 1 and 5'-nucleotidase activities from rat brain synaptosomes. **International Journal of Developmental Neuroscience**, v.21, p.75-82, 2003.

BATTASTINI, A.M.O. et al. Characterization of an ATP diphosphohydrolase (E.C. 3.6.1.5) in synaptosomes from cerebral cortex of adult rats. **Neurochemical Research,** v.16, p.1303-1310, 1991.

BECHARA, E.J.H. et al. A free radical hypothesis of lead poisoning and inborn porphyrias associated with 5-aminolevulinic acid overload. **Quim. Nova**, v. 16, p. 385-392.1993.

BENAVIDES, M. P.; GALLEGO, S. M.; TOMARO, M. L. Cadmium toxicity in plants. **Braz J Plant Physiol,** v. 17, n. 1, p. 21-34, 2005.

BERNARD, A. Cadmium and its adverse effects on human health. **Indian J. Med. Res.** v.128, p.557-564, 2008.

BERNARD, A.; LAUWERYS, R. Cadmium in human population. **Experientia**, v.40, p.143-152, 1984.

BHATIA, A.; JAIN, M. Spinacia oleracea L. protects against gamma radiations: A study on glutathione and lipid peroxidation in mouse liver. **Phytomedicine**, v.11, p.607-615, 2004.

BHUTADA, P. et al. Reversal by quercetin of corticotrophin releasing factor induced anxiety- and depression-like effect in mice. **Prog Neuropsychopharmacol Biol Psychiatry,** v. 34, p.955-960, 2010.

BIGONNESSE, F. et al. Cloning and characterization of mouse triphosphate diphosphohydrolase 8. **Biochemistry,** v.43, p.5511-5519, 2004.

BOECK, A. T. et al. Naturally occurring mutation, Asp70His, in human butyrylcholinesterase. **Ann Clin Biochem,** v. 39, Pt 2, p. 154–6, 2002.

BORGES L.P. et al. Oral administration of diphenyl disselenide protects against cadmium-induced liver damage in rats. **Chemico-Biological Interactions**, v.171, p.15-25, 2008.

BORGES, V. C. et al. Effect of diphenyl diselenide, diphenyl ditelluride and ebselen on cerebral Na+, K+-ATPase activity in rats. **Toxicology,** v. 215, n. 3, p. 191–7, 2005.

BOURNE, Y. et al. Structural insights into conformational flexibility at the peripheral site and within the active center gorge of AChE. **Chemico-Biological Interactions**, v.15, p.159-165, 2005.

BOURS, M.J. et al. Adenosine 50-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. **Pharmacol Ther,** v. 112, p. 358–404, 2006.

BRAVO, L. Polyphenols: Chemistry, Dietary Sources, Metabolism and Nutritional Significance. Nutr. Rev, v.56, p.317-333, 1998.

BURNSTOCK, G. **Purinergic signaling**. British Journal of Pharmacology, v.147, p.172-181, 2006.

CALABRESE, V.; BATES, T. E.; STELLA, A. M. G. NO synthase and Nodependent signal pathways in brain aging and neurodegenerative disorders: the role of oxidant/antioxidant balance. **Neurochem Res,** v. 25, n. 9-10, p. 1315–41, Oct 2000.

CAO H, et al. Quercetin exerts anti-melanoma activities and inhibits STAT3 signaling. **Biochemical Pharmacology,** v. 87, p. 424-434, 2014.

CARAGEORGIOU, H. et al. Cadmium effects on brain acetylcholinesterase activity and antioxidant status of adult rats: modulation by zinc, calcium and L-cysteine co-administration co-administration. **Basic and Clinical Pharmacology and Toxicology**, v.97, p.320-324, 2005.

CARAGEORGIOU, H. et al. In vivo and in vitro effects of cadmium on adult rat brain total antioxidant status, acetylcholinesterase, (Na^+, K^+)-ATPase and Mg^{2+}- ATPase activities: protection by L-cysteine. **Basic and Clinical Pharmacology and Toxicology**, v.94, p.112-118, 2004.

CASALINO, E. et al. Cadmium dependent enzyme activity alteration is not imputable to lipid peroxidation. **Arch. Biochem. Biophys**, v. 383, p. 288 – 295. 2000.

CASALINO, E.; SBLANO, C.; LANDRISCINA, C. Enzyme activity alteration by cadmium administration to rats: the possibility of iron involvement in lipid peroxidation. **Archives of Biochemistry and Biophysics**, v.346, p.171-179, 1997.

CHATONNET, A.; LOCKRIDGE, O. Comparison of butyrylcholinesterase and acetylcholinesterase. **Biochem J,** v. 260, n. 3, p. 625–34, 1989.

CHERIAN, M.G.; GOYER, R.A.; VALBERG, L.S. Gastrointestinal absorption and organ distribution of oral cadmium chloride and cadmium-metallotionein in mice. **Journal of Toxicology and Environmental Health**, v.4, p.861-868, 1978.

CHETTY, C. S. et al. The effects of cadmium in vitro on adenosine triphosphatase system and protection by thiol reagents in rat brain microsomes. **Arch Environ Contam Toxicol,** v. 22, n. 4, p. 456–8, 1992.

CHETTY, C.S. et al. In vitro interaction of heavy metals with ouabain receptors in rat brain microsomes. **Drug and chemical toxicology,** v. 16, p. 101-110, 1992.

CHINEMBIRI, T.N., et al. Review of Natural Compounds for Potential Skin Cancer Treatment. **Molecules,** v. 19, p. 11679-11721, 2014.

CHO S.Y. et al. Quercetin suppresses proinflammatory cytokines production through MAP kinases and NF-κB pathway in lipopolysaccharide-stimulated macrophage. **Molecular and Cellular Biochemistry,** v. 243, p.15-160, 2003.

CHOI E.J.; CHEE K.M.; LEE B.H. Anti- and prooxidant effects of chronic quercetin administration in rats. **European Journal of Pharmacology**, v.482, p.281-285, 2003.

COKUGRAS, A.N. Butyrylcholenesterase: Structure and physiological importance. **Turkish Journal of Biochemistry**, v.28, p.54-61, 2003.

COLGAN, S. et al. Physiological roles for ecto-5'-nucleotiase (CD73). **Purinergic Signalling**. v.2, p.351-360, 2006.

COOK, N.C.; SAMMAN, S.; Flavonoids-chemistry, metabolism, cardioprotective effects, and dietary sources-review. **The Journal of Nutrition Biochemistry**, v.7, p.66-76, 1996.

CRESPY, V. et al. Part of quercetin absorbed in the small intestine is conjugated and further secreted in the intestinal lumen. Am. J. Physiol, v.277, p.120-126, 1999.

CUNHA, R.; RIBEIRO, J.A. ATP as a presynaptic modulator. **Life Sciences**, v.68, p.119-137, 2000.

CUNHA, R.A. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different role, different sources and different receptors. **Neurochemistry International**, v.38, p.107-125, 2001.

DAHM, K. C. S. et al. In vitro exposure of heavy metals on nucleotide and cholinesterase activities from the digestive gland of Helix aspersa. **Comp Biochem Physiol,** v. 143, n. 3, p. 316-20, 2006.

DAS, U. N. Acetylcholinesterase and butyrylcholinesterase as possible markers of low-grade systemic inflammation. **Med Sci Monit,** v. 13, n. 12, p. 214-21, 2007.

DEAGLIO, S. et al. Adenosine generation catalysed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. **J Exp Med,** v. 204, p. 1257–1265, 2007.

DEIANA, S.; PLATT, B.; RIEDEL, G. The cholinergic system and spatial learning. **Behavioural brain research,** v. 221, p. 389-411, 2011.
DESCARRIES, L., GISIGER, V., STERIADE, M. Diffuse transmission by acetylcholine in the CNS. **Prog Neurobiol,** v. 53, p. 603–25, 1997.

DI VIRGILIO, F. et al. Nucleotide receptors: an emerging family of regulatory molecules in blood cells. **Blood**, v.97, p.587-600, 2001.

DOS REIS, E.A. et al. Arginine administration inhibits hippocampal Na(+),K(+)-ATPase activity and impairs retention of an inhibitory avoidance task in rats. **Brain Res,** v. 951, p. 151-157, 2002.

DUNWIDDIE, T.; MASINO, S. The role and regulation of adenosine in the central nervous system. **Annual Review of Neuroscience**, v.24, p.31-55, 2001.

EL-DEMERDASH, F.M. et al. Cadmium-induced changes in lipid peroxidation, blood hematology, biochemical parameters and semen quality of male rats: protective role of vitamin E and beta-carotene. **Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association**, v. 42, p. 1563-1571, 2004.

EMANUELLI, T. et al. Effect of mercuric chloride intoxication and dimercaprol treatment on delta-aminolevulinate dehydratase from brain, liver and kidney of adult mice. **Pharmacology & toxicology,** v. 79, p. 136-143, 1996.

EZZATI NAZHAD DOLATABADI, J. et al. Synthesis, Characterization and Antioxidant Property of Quercetin-Tb(III) Complex. **Advanced pharmaceutical bulletin,** v. 4, p. 101-104, 2014.

FANG, Y.Z., YANG, S., WU, G. Free radicals, antioxidants, and nutrition. **Nutrition,** v. 18, p. 872-879, 2002.

FASITSAS, C.D. et al. Time-dependent cadmium-neurotoxicity in rat brain synaptosomal plasma membranes. **Comp Biochem Physiol C,** v. 100, p. 271-275, 1991.

FIORANI, M. et al. Quercetin prevents glutathione depletion induced by dehydroascorbic acid in rabbit red blood cells. **Free Radic Res,** v. 34, p. 639-648, 2001.

FOX, S. Nutritional factors that may influence bioavailability of cadmium. **J Environ Quality,** v.17, p.175-80, 1988.

FRASSETO, S.; DIAS, R.D.D.; SARKIS, J.F.F. Inhibition and kinetics alterations by excess of free ATP, ADP, of the ATP diphosphohydrolase activity (e.c. 3.6.1.5) from rat blood platelets. **Biochemistry and Molecular Biology International**, v.35, p.499-506, 1995.

FREDHOLM, B.B. et al. Adenosine and brain functions. **International Review of Neurobiology,** v.63, p.191-270, 2005.

FREDHOLM, B.B. et al. International union of pharmacology. XXV. Nomenclature and classification of adenosine receptors. **Pharmacological Reviews**, v.52, p.527-552, 2001.

FRUSSA-FILHO, R. et al. Naltrexone potentiates the anxiolytic effects of chlordiazepoxide in rats exposed to novel environments. **Psychopharmacology (Berl),** v. 147, p. 168-173, 1999.

FUJISAWA, H. et al. Movement of radioactive calcium in brain slices and influences on it of protoveratrine, ouabain, potassium chloride and cocaine. **Jpn J Pharmacol,** v. 15, p. 327-334, 1965.

GALAN, A. et al. The role of intracellular oxidation in death induction (apoptosis and necrosis) in human promonocytic cells treated with stress inducers (cadmium, heat, X-rays). **Eur J Cell Biol,** v. 80, p. 312-320, 2001.

GESSI, S. et al. Adenosine and lymphocyte regulation. **Purinergic Signal,** v. 3, n. 1-3, p. 109-16, 2007.

GIBSON, K. D.; NEUBERGER, A.; SCOTT, J. J. The purification and properties of delta-aminolevulinic acid dehydratase. **Biochem. J.**, v. 61, p. 618-628. 1955.

GODT. J. et al. The toxicity of cadmium and resulting hazards for human health. **J Occup Med Toxicol,** v. 1, p. 22, 2006.

GOERING, P.L. Lead–protein interactions as a basis for lead toxicity. **Neurotoxicology**, v. 14, p. 45–60. 1993

GONÇALVES J.F. et al. N-acetylcysteine prevents memory deficits, the decrease in acetylcholinesterase activity and oxidative stress in rats exposed to cadmium. **Chemico-Biological Interactions**, v.186, p.53-60, 2010.

GONCALVES, J.F. et al. Behavior and brain enzymatic changes after long-term intoxication with cadmium salt or contaminated potatoes. **Food Chem Toxicol,** v. 50, p. 3709–3718, 2012.

GONCALVES, J.F. et al. Hematological indices and activity of NTPDase and cholinesterase enzymes in rats exposed to cadmium and treated with N-acetylcysteine. **Biometals**: an international journal on the role of metal ions in biology, biochemistry, and medicine, v. 25, p. 1195-1206, 2012a.

GONCALVES, J.F. et al. Hematological indices and activity of NTPDase and cholinesterase enzymes in rats exposed to cadmium and treated with Nacetylcysteine. **Biometals,** v. 25:1195–1206, 2012b.

GONCALVES, J.F. et al. NTPDase and 5'-nucleotidase activities from synaptosomes and platelets of rats exposed to cadmium and treated with N-acetylcysteine.

International journal of developmental neuroscience: the official journal of the International Society for Developmental Neuroscience, v. 31, p. 69-74, 2013.

GRINTHAL, A.; GUIDOTTI, G. Dynamic motions of CD39 transmembrane domains regulate and are regulated by the enzymatic active site. **Biochemistry**, v.43, p.13849-13858, 2006.

GUGLER, R.; LESCHIK, M.; DENGLER, H.J. Disposition of quercetin in man after single oral and intravenous doses. **Eur J Clin Pharmacol,** v. 9, p. 229-34, 1975.

HA, H.J. et al. Quercetin attenuates oxygen-glucose deprivation- and excitotoxin-induced neurotoxicity in primary cortical cell cultures. **Biol Pharm Bull**, v.26, p.544-546, 2003.

HALLIWELL, B.; GUTTERIDGE, J. M. C. **Free radicals in Biology and Medicine**. Oxford: Clarendon Press, 4th, 2007.

HAMPTON, M.B.; KETTLE, A.J.; WINTERBOURN, C.C. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. **Blood,** v. 92, p. 3007–3017, 1998.

HANSSON, M.; OLSSON, I.; NAUSEEF, W.M. Biosynthesis, processing and sorting of human myeloperoxidase. **Archives of Biochemistry and Biophysics**, v. 445, p. 214-224, 2006.

HARWOOD M, et al. A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. **Food Chem Toxicol,** v. 45, p. 2179-205, 2007.

HASSOUN, E.A.; STOHS, S.J. Cadmium-induced production of superoxide anion and nitric oxide, DNA single strand breaks and lactate dehydrogenase leakage in J774A.1 cell cultures. **Toxicology**, v.112, p.219-226, 1996.

HAVEKES, R.; ABEL, T.; VAN DER ZEE, E.A. The cholinergic system and neostriatal memory functions. **Behav Brain Res,** v. 221, p. 412-423, 2011.

HAVSTEEN, B.N. The biochemistry and medical significance of the flavonoids. **Pharmacol Therapeut**, v.96, p.67-202, 2002.

HERTOG, M.G. et al. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands**. Nutr. Cancer**, v.20, p.21-29, 1993.

HEXUM, T.D. Studies on the reaction catalysed by transport (Na, K). Adenosine triphosphatase-I. Effect of divalent metals. **Biochem. Pharmacol,** v. 23, p. 3441–3447, 1974.

HOLLMAN, P.C.H.; KATAN, M.B. Health effects and bioavailability of dietary flavonols. **Free Radic. Res**, v.31, p.75-80S, 1999.

HOLLOWAY, J.R.; THOR, D.H. Socialmemory deficits in adultmale rats exposed to cadmium in infancy. **Neurotoxicol Teratol,** v.10, p. 193–197, 1988.

HUNSUCKER, S.; MITCHELL, B.; SPYCHALA, J. The 5'-nucleotidase as regulators of nucleotide and drug metabolism. **Pharmacology and Therapeutics**, v.107, p.1-30, 2005.

HUT, R.A.; VAN DER ZEE, E.A. The cholinergic system, circadian rhythmicity, and time memory. **Behavioural brain research,** 221, p. 466-480, 2011.

ILLES, P.; RIBEIRO, A. Molecular physiology of P2 receptors in the central nervous system. **Europen Journal of Pharmacology**, v.483, p.5-7, 2004.

JAFFE, E. K.; VOLIN, M.; MYERS, C. B. 5-Chloro [1,4-^{13}C] levulinic acid modification of mammalian and bacterial porphobilinogen synthase suggests an active site containing two Zn(II). **Biochemistry**, v. 33, p. 11554-11562. 1994.

JARUP L. et al. Health effects of cadmium exposure–a review of the literature and a risk estimate, **Scand J Work Environ Health**, v.24, p.1-52, 1998.

JARUP, L. Hazards of heavy metal contamination. **British medical bulletin,** v. 68, p.167-182, 2003.

JÄRUP, L.; AKESSON, A. Current status of cadmium as an environmental health problem. **Toxicol Appl Pharmacol**, v. 238, n. 3, p. 201–8, 2009.

JOHNSON, G.; MOORE, S.W. The adhesion function on acetylcholinesterase is located at peripheral anionic site. **Biochemical and Biophysical Research Communicatins**, v.258, p.758-762, 1999.

JONES, M.M.; CHERIAN, M.G. The search for chelate antagonists for chronic cadmium intoxication. **Toxicology**, v.6, p.1-25, 1990.

JÓSÊ A.S. et al. Sensitivity of young rats to nicotine exposure: Physiological and biochemical parameters. **Ecotoxicol Environ Saf**, v.72, p. 242-7, 2009.

JUNGER, W.G. Immune cell regulation by autocrine purinergic signalling. **Nat Rev Immunol,** v.11, p.201–212, 2011.

KABATA-PENDIAS, A.; PENDIAS, H. **Trace elements in soil and plants**. Boca Raton: CRC Press Inc, 148p, 2001.

KAHRAMAN, A. et al. Protective effect of quercetina on renal ischemia/reperfusion injury in rats. **J. Nephrol.**, v.16, p. 219-224, 2003.

KAIZER, R.R. et al. The effect of aluminium on NTPDase and 5'-nucleotidase activities from rat synaptosomes and platelets. **International Journal of Developmental Neuroscience**, v.25, p.381-386, 2007.

KAKO, K. et al. Depression of membrane-bound Na+-K+-ATPase activity induced by free radicals and by ischemia of kidney. **Am J Physiol**, v. 254, n. 2, p. C330-7, 1988.

KANG, S.S., et al. Neuroprotective effects of naturally occurring biflavonoids. Bioorg **Med Chem Lett**, v.15, p.3588-3591, 2005.

KANTHI, Y.M.; SUTTON, N.R.; PINSKY, D.J. CD39: interface between vascular thrombosis and inflammation. **Current Atherosclerosis Report,** v. 16 (7), p. 425, 2014.

KAWASHIMA, K.; FUJII, T. Extraneural cholinergic system in lymphocytes. Pharmacological and Terapeutics, v.86, p.29-48, 2000.

KIEHN, O. Locomotor circuits in the mammalian spinal cord. **Annual review of neuroscience,** v.29, p.279-306, 2006.

KLINKENBERG, I.; SAMBETH, A.; BLOKLAND, A. Acetylcholine and attention. **Behav Brain Res**, v. 221, p. 430-42, 2011.

KRISHNAKUMAR, N.; PRABU, S.M.; SULFIKKARALI, N.K. Quercetin protects against cadmium-induced biochemical and structural changes in rat liver revealed by FT-IR spectroscopy. **Biomedicine & Preventive Nutrition**, v.2, p.179-185, 2012.

KUMAR, A.; GOYAL, R. Quercetin protects against acute immobilization stress-induced behaviors and biochemical alterations in mice. **J Med Food,** v.11, p. 469-473, 2008.

KUMAR, R.; AGARWAL, A.K.; SETH, P.K. Oxidative stress-mediated neurotoxicity of cadmium. **Toxicol. Lett,** v.89, p.65-69, 1996.

KUTTY, K. M. Biological function of cholinesterase. **Clin Biochem,** v. 13, n. 6, p. 239-43, 1980.

LAFUENTE, A. et al. Effect of cadmium on lymphocyte subsets distribution in thymus and spleen. **J Physiol Biochem,** v. 59, p. 43–48, 2003.

LANGSTON, H. et al. Secretion of IL-2 and IFN-y, but not IL-4, by antigen - specific T cells requires extracellular ATP. **J Immunol,** v. 170, n. 6, p. 2962-70, 2003.

LAVOIE, E.G. et al. Cloning and characterization of mouse nucleoside triphosphate diphosphohydrolase 3. Biochemical Pharmacology, v.67, p.1917-1926, 2004.

LEAL, D.B.R. et al. Characterization of NTPDase (NTPDase 1; ecto-apyrase; ecto-diphosphohydrolase; CD39; E.C. 3.6.1.5) activity in human lymphocytes. **Biochemica et Biophysica Acta**, v.1721, p.9-11, 2005.

LEES, G.J. et al. The neurotoxicity of ouabain, a sodium– potassium ATPase inhibitor, in the rat hippocampus. **Neurosci Lett,** v. 62, p. 120-159, 1990.

LI, S.; STYS, P.K. Na(+)-K(+)-ATPase inhibition and depolarization induce glutamate release via reverse Na(+)-dependent transport in spinal cord white matter. **Neuroscience.** v.7:675-83, 2001.

LINDEN, J. New insights into the regulation of inflammation by adenosine. **J Clin Invest** v. 116, p. 1835–1837, 2006.

LINGREL, J.B. et al. Na,K-ATPase: isoform structure, function, and expression. J Bioenerg **Biomembr,** v. 24, p. 263-270, 1992a.

LINGREL, J.B. et al. Na,K-ATPase: structure-function studies. **Ren Physiol Biochem,** v. 17, p. 198–200, 1992b.

LINGREL, J.B. et al. Structure-function studies of the Na,K-ATPase. **Kidney Int Suppl** v. 44, p. 32-39, 1994a.

LINGREL, J.B. et al. Na,K-ATPase: structure-function studies. **Renal physiology and biochemistry,** v. 17, p. 198-200, 1994b.

LOIZZO, M.R.; TUNDIS, R.; MENICHINI, F. Natural products and their derivatives as cholinesterase inhibitors in the treatment of neurodegenerative disorders: an update. **Curr Med Chem,** v. 15, p.1209–1228, 2008.

LÓPEZ, E. et al. induces reactive oxygen species generation and lipid peroxidation in cortical neurons in culture, Free Radic. **Biol. Med,** v.40, p.940-951, 2006.

LU, S.C. Glutathione synthesis. **Biochimica et biophysica acta**, v. 1830, p. 3143-3153, 2013.

LUCHESE, C. et al. Cadmium inhibits δ-aminolevulinate dehydratase from rat lung in vitro: interaction with chelating and antioxidant agents. **Chem Biol Interact**, v. 165, n. 2, p; 127–37, 2007.

LUNKES, G. I. et al. Enzymes that hydrolyze adenine nucleotides in diabetes and associated pathologies. **Thromb Res,** v. 109, n. 4, p. 189–94, 2003.

MALDONADO, P. A. et al. Nucleotide degrading enzymes in platelets from uterine cervical neoplasia patients treated with conization or radiotherapy. **Biomed Pharmacother,** v. 64, n. 7, p. 499-504, 2010.

MALMSJO, M.; EDVINSSON, L.; ERLINGUE, D. P2X receptors counteract the vasodilatory effects of endothelium derived hyperpolarising factor**. European Journal of Pharmacology**, v.390, p.173-180, 2000.

MANACH, C. et al. Quercetin is recovered in human plasma as conjugated derivates which retain antioxidant properties. **FEBS Lett**, v.426, p.331-336, 1998.

MANDEL, S.A., et al. Multifunctional activities of green tea catechins in neuroprotection. Modulation of cell survival genes, iron-dependent oxidative stress and PKC signaling pathway**. Neurosignals**, v.14, p. 46-60, 2005.

MASSOULIÉ, J. et al. Molecular and cellular biology of cholinesterase. **Progress in Neurobiology,** v.41, p.31-41, 1993.

MATOS, J.J.A. et al. Characterization of an ATP diphosphohydrolase (apyrase, E.C. 3.6.1.5) activity in Trichomonas vaginalis**. International Journal for Parasitology**, v.31, p.770-775, 2001.

MATTÉ, C. et al. Folic acid pretreatment prevents the reduction of Na(+),K(+)-ATPase and butyrylcholinesterase activities in rats subjected to acute hyperhomocysteinemia. **Int J Dev Neurosci**, v. 24, n. 1, p. 3-8, 2006.

McTIERNAN, C. et al. Brain cDNA clone for human cholinesterase. **Proc Natl Acad Sci USA,** v. 84, n. 19, p. 6682-6, 1987.

MELZIG, M.F. Inhibition of adenosine deaminase activity of aortic endothelial cells by selected flavonoids. **Planta Med**, v. 62, p.20–21, 1996.

MÉNDEZ-ARMENTA M. et al. Histopathological alterations in brain regions of rats after perinatal combined treatment with cadmium and dexamethazone, **Toxicology,** v.161, p.189-199, 2001.

MENDEZ-ARMENTA, M. et al. Brain regional lipid peroxidation and metallothionein levels of developing rats exposed to cadmium and dexamethasone. **Toxicology letters,** v. 144, p. 151-157, 2003.

MÉNDEZ-ARMENTA M.; RÍOS C. Cadmium neurotoxicity, **Environ. Toxicol. Appl. Pharmacol,** v.23, p.350-358, 2007.

MESULAM, M.M. et al. Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyze acetylcholine. **Neuroscience**, v. 110, p. 627-39, 2002.

MIDDLETON, E.; KANDASWAMI, C.; THEOHARIDES, T.C. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. **Pharmacol. Rev**, v.52, p.673-751, 2000.

MINETTI, A.; REALE, C. A. Sensorimotor developmental delays and lower anxiety in rats prenatally exposed to cadmium. **J Appl Toxicol**, v. 26, n. 1, p. 35–41, 2006.

MORSELT, A.F. A cell biological approach using chronic cadmium exposure in the animal model as a paradigm case. **Toxicology,** v.70, p.1-132, 1991

MOSELEY, A.E. et al. Deficiency in Na,K-ATPase alpha isoform genes alters spatial learning, motor activity, and anxiety in mice. **J Neurosci** v. 27, p. 616-626, 2007.

MUROTA, K.; TERAO, J. Antioxidative flavonoid quercetin: implications of its intestinal absorption and metabolism. **Arch. Biochem. Bioph,** v.417, p.12-17, 2003.

NAIDU, P.S.; SINGH, A.; KULKARNI, S.K. Quercetin, a bioflavonoid, attenuates haloperidol-induced orofacial dyskinesia. **Neuropharmacology**. v.44, p.1100-1106, 2003.

NATIONAL TOXICOLOGY PROGRAM. Toxicology and Carcinogenesis studies of Quercetin (CAS NO. 117-39-5) in F344/N Rats (Feed Studies). u.s. depart" of health and human services, **Nat Toxicol Program,** p.171, 1992.

NIJVELDT, R.J et al. Flavonoids: a review of probable mechanisms of action and potential applications. **Am. J. Clin. Nutr., Bethesda**, v.74, p.418-425, 2001.

NOMIYAMA, K.; NOMIYAMA, H. Critical concentration of “unbound” cadmium in the rabbit renal cortex. **Experientia**, v.42, p.149, 1986.

NORDBERG, G. F. Historical perspectives on cadmium toxicology. **Toxicol Appl Pharmacol,** v. 238, n. 3, p. 192–200, 2009.

NORDBERG, G.F; KJELLSTROM T.; NORDBERG M. Gnetics and metabolism. In: FRIBERG, L.; ELINDER, C.G.; KJELLSTROM, T.; NORDBERG, G.F.; editors. Cadmium and health: a toxicological and epidemiological appraisal, v. I (Exposure, dose, and metabolism). **Boca Raton (FL): CRC Press**, p.103-78, 1985

NUNES-TAVARES, N. et al. Inhibition of acetylcholinesterase from electrophorus eletricus (L.) by tricycic antidepressants. **The International Journal of Biochemistry and Cell Biology,** v.34, p.1071-1079, 2002.

ORESKOVIC, K.; KUNEC-VAJIC, E. Pseudocholinesterase in alloxan-diabetic rats. **Res Commun Chem Pathol Pharmacol,** v. 78, n. 1, p. 117-20, 1992.

ORRENIUS, S., NICOTERA, P. The calcium ion and cell death. **J. Neural. Trans.** v.43, p.1-11, 1994.

OSPAR Commission. Cadmium. **Hazardous Substances Series**, 58p., 2002

PATRICK, G. L. **Medicinal Chemistry.** 2nd ed., Oxford, pp. 432-82, 2001.

PEREIRA, B. et al. 5-Aminolevulinic acidinduced alterations of oxidative metabolism in sedentary and exercise-trained rats. **J. Appl. Physiol**., v. 72, p. 226-230. 1992.

PIERPOINT, W.S. Flavonoids in the human diet. **Prog. Clin. Biol. Res**., v.213, p.125-140, 1986.

PRADO, M.A.M. et al. Regulation of acetylcholine synthesis and storage. **Neurochemistry International**, v.41, p.291-299, 2002.

PRAKASH, N.J.; FONTANA, J.; HENKIN, R.I. Effect of transitional metal ions on (Na+K+)-ATPase activity and the uptake of norepinephrine and choline by synaptosomes. **Life Sci,** v. 12, p. 249–269, 1973.

PRIOR, R.L. Fruits and vegetables in the prevention of cellular oxidative damage. **Am J Clin Nutr**, v.78, p.570S-578S, 2003.

PRODY, C. A. et al. Isolation and characterization of full-length cDNA clones coding for cholinesterase from fetal human tissues. **Proc Natl Acad Sci USA,** v. 84, n. 11, p.3555-9, 1987.

PROVIAS, J.P. Cadmium encephalopathy: a report with elemental analysis and pathological findings. **Acta Neuropathol,** v. 88, p. 583–586, 1994.

PU, F. et al. Neuroprotective effects of quercetin and rutin on spatial memory impairment in an 8- arm radial maze task and neuronal death induced by repeated cerebral ischemia in rats. **J Pharmacol Sci,** v.104, p.329-334, 2007.

PURVES, D. et al. **Neurociências.** 2ed. Porto Alegre: Artmed, 728p., 2005.

RAJANNA, B. Effects of chronic treatment with cadmium on ATPases, uptake of catecholamines, and lipid peroxidation in rat brain synaptosomes. **Ecotoxicol Environ Saf.** v.20, p.36-41, 1990.

RAKONCZAY, Z. et al. Peripheral cholinergic disturbances in Alzheimer's disease. **Chemico-Biological Interactions**, v.157, p.233-238, 2005.

RANG, H. P. et al. Dependência e abuso de fármacos. In: RANG, H. P.; DALE, M. M.; RITTER, J. M.; MOORE, P. K. (Ed). **Farmacologia. Elsevier,** São Paulo, Brasil, pp. 674-95, 2004.

RAO, A. A.; SRIDHAR, G. R.; DAS, U. N. Elevated butyrylcholinesterase and acetylcholinesterase may predict the development of type 2 diabetes mellitus and Alzheimer's disease. **Med Hypotheses,** v. 69, n. 6, p. 1272-6, 2007.

RATHBONE, M. et al. Trophi effects of purines in neuron and glial cells**. Progress in Neurobiology,** v.59, p.663-690, 1999.

REGASINI, L.O. et al. Flavonols from Pterogyne nitens and their evaluation as myeloperoxidase inhibitors. **Phytochemistry,** v. 69, p. 1739-44, 2008.

RICE-EVANS, C.A. et al. The relative antioxidant activities of plant derived polyphenolic flavonoids. **Free Radical Research**, v.22, p.375-383, 1995.

RIBEIRO, J.A.; SEBASTIÃO, A.M.; MENDONÇA, A. Adenosine receptors in the nervous system: pathological implications. **Progress in Neurobiology**, v.68, p.377-392, 2003.

RICO, E.P. et al. ATP and ADP hydrolysis in brain membranes of zebrafish (Danio renio). **Life Sciences**, v.73, p.2071-2082, 2003.

RIKANS, L.E.; YAMANO, T. Mechanisms of cadmium mediated-acute hepatotoxicity. **Journal of Biochemical and Molecular Toxicology**, v.14. p.110-117, 2000.

ROBSON, S.C.; SÉVIGNY, J.; ZIMMERMANN, H. The E-NTPDase family of ectonucleotidases: structure, function, relationships and pathophysiology significance. **Purinergic Signalling**, v.2, p.409-430, 2006.

ROCHA, J.B. et al. Effect of mercuric chloride and lead acetate treatment during the second stage of rapid post-natal brain growth on the behavioral response to chlorpromazine and on delta-ALA-D activity inweaning rats. **Toxicol Lett,** v. 50, p. 125-143, 2001.

RODRIGUES, A.L. et al. Delta-aminolevulinic acid dehydratase activity in weanling and adult rats exposed to lead acetate. **Bulletin of environmental contamination and toxicology.** v. 57, p. 47-53, 1996.

SANTOS, F. W. et al. Cadmium induced testicular damage and its response to administration of succimer and diphenyl diselenide in mice. **Toxicol Lett,** v. 152, n. 3, p. 255-63, 2004.

SANTOS, F.W. et al. Efficacy of 2,3-dimercapto-1-propanesulfonic acid (DMPS) and diphenyl diselenide on cadmium induced testicular damage in mice. Food and Chemical Toxicology**, v.43, p.1723-1730, 2005a.**

SANTOS, F.W. et al. Diphenyl diselenide reverses cadmium-induced oxidative damage on mice tissues. **Chemico-Biological Interactions**, v.151, p.159-165, 2005b.

SANTOS F.W. et al. Sub-chronic administration of diphenyl disselenide potentiates cadmium-induced testicular damage in mice. **Reproductive Toxicology**, v.22, p.546-550, 2006.

SARKAR, S.; DAS, N. Mannosylated liposomal flavonoid in combating agerelated ischemia-reperfusion induced oxidative damage in rat brain. **Mech Ageing Dev**. v.127, p.391-397, 2006.

SARTER, M.; PARIKH, V. Choline transporters cholinergic transmission and cognition. **Nature Reviews**, v.6, p.48-56, 2005.

SASSA, S. Delta-aminolevulinic acid dehydratase assay. **Enzyme.** v. 28, p.133-145, 1982.

SASSA, S.; FUJITA, H.; KAPPAS, A. Genetic and chemical influences on heme biosynthesis. In: A. Kotyk, J. Skoda, V. Paces and V. Kostka (Eds.), **Highlights of Modern Biochemistry, VSP, Utrecht,** v. 1, p. 329-338. 1989.

SATO, T. et al. Effects of steroid hormones on (Na+, K+)-ATPase activity inhibition-induced amnesia on the step-through passive avoidance task in gonadectomized mice. **Pharmacol Res.** v. 49, p. 151-159, 2004.

SCALBERT, A.; WILLIAMSON, G. Dietary intake and bioavailability of polyphenols. **J Nutr,** v.130, p.2073S-2085S, 2000.

SCARR, E. Muscarinic Receptors: Their Roles in Disorders of the Central Nervous System and Potential as Therapeutic Targets. **CNS Neurosci Ther**. 2011.

SCHENK, U. et al. Purinergic control of T cell activation by ATP released through pannexin-1 hemichannels. **Science Signaling**, v. 1(39), p.1 a 6, 2008.

SCHETINGER, M.R.C. et al. ATP and ADP hydrolysis in fish, chicken and rat synaptosomes**. Comparative Biochemistry and Physiology**, v.128, p.731-741, 2001.

SCHMATZ, R. et al. Effects of resveratrol on nucleotide degrading enzymes in streptozotocin-induced diabetic rats. **Life Sci,** v.84, p.345-350b, 2009a

SCHMATZ, R. et al. Resveratrol prevents memory deficits and the increase in acetylcholinesterase activity in streptozotocin-induced diabetic rats. **European Journal of Pharmacology**, v. 610, p.42-48, 2009b.

SENGER, M.R. et al. In vitro effect of zinc and cadmium on acetylcholinesterase and ectonucleotidase activities in zebrafish (Danio rerio) brain. **Toxicol In Vitro,** v. 20, n. 6, p. 954-8, 2006.

SILMAN, I.; SUSSMAN, J.L. Acetylcholinesterase: “classical” and “non-classical” functions and pharmacology. **Current Opinion in Pharmacology**, v.5, p.293-302, 2005.

SOREQ, H.; SEIDMAN, S. Acetylcholine-news roles for an old actor. **Nature Reviews. Neuroscience**, v.2, p. 294-302, 2001.

SPANEVELLO, R. M. et al. Activities of the enzymes that hydrolyze adenine nucleotides in platelets from multiple sclerosis patients. **J Neurol,** v. 257, n. 1, p. 24-30, 2010a.

SPENCER, J.P.E. et al. Intracellular metabolism and bioactivity of quercetin and its in vivo metabolites. **Biochem**. J. 372, 173-81, 2003.

TAVARES, M.T; CARVALHO, M.F. Avaliação de exposição de população humana a metais pesados no ambiente: Exemplos do recôncavo Baiano. **Química Nova,** v.15, 1992.

TAYEBATI, S.K.; EL-ASSOUAD, D.; RICCI, A.; AMENTA, F. Immunochemical and immunocytochemical characterization of cholinergic markers in human peripheral blood lymphocytes. **J Neuroimmunol,** v. 132, p. 147–155, 2002.

TAYLOR, P.; BROWN, J.H. Acetylcholine. In: Siegel, G.J., Agranof, B.W., Albers, R.W. & Molinoff, P.B. (Ed.). Basic Neurochemistry: **Molecular, cellular and Medical Aspects. Lippincott-Raven Publishers, Philadelphia,** U.S.A, p. 214-242, 1999.

TERCARIOL, S.G.; ALMEIDA, A.A.; GODINHO, A.F Cadmium and exposure to stress increase aggressive behavior. **Environ Toxicol Pharmacol,** v. 32, p. 40–45, 2011.

TOHYAMA, C.; SHAIKH, Z.A. Metallothionein in plasma and urine of cadmium-exposed rats determined by a single anti-body radioimunoassay. **Fundamental and Applied Toxicology**, v. 1, p. 1-7, 1981.

TORRES, B. B. **Nutrição e esporte uma abordagem bioquímica.** Departamento de Bioquímica, Instituto de Química, USP, 2003.

TOYAMA, T. et al. Isothiocyanates reduce mercury accumulation via an Nrf2-dependent mechanism during exposure of mice to methylmercury. **Environmental health perspectives** v. 119, p.1117-1122, 2011.

VALVERDE, M.; TREJO, C.; ROJAS, E. Is the capacity of lead acetate and cadmium chloride to induce genotoxic damage due to direct DNA-metal interaction? **Mutagenesis** v. 16, p. 265-270, 2001.

VAN DER ZEE, E. A., LUITEN, P. G. Muscarinic acetylcholine receptors in the hippocampus, neocortex and amygdala: A review of immunocytochemical localization in relation to learning and memory. **Prog Neurobiol,** v. 58, n. 5, p. 409-71, 1999.

VASCONCELOS, E.G. et al.Characterization and localization of an ATP diphosphohydrolase on the external surface of the tegument of the tegument of Schistosoma mansoni. **Molecular Biochemistry Parasitology**, v.58, p.205-214, 1993.

VISSIENNON, C. et al. Route of administration determines the anxiolytic activity of the flavonols kaempferol, quercetin and myricetin--are they prodrugs? **J Nutr Biochem** v. 23, p. 733-740, 2012.

VORHOFF, T. et al.Cloning and characterization of ecto-nucleotidase NTPDase 3 from rat brain: predicted secondary structure and relation to members of the E-NTPDase family and action. **Purinergic Signalling**, v.1, p.259-270, 2004.

WANG, T.; GUIOTTI, G.; Widespread expression of ecto-apyrase (C39) in the central nervous system. Brain Research, v.790, p.318-322, 1998.

WANG, A. Simultaneous etermination of paracetamol and caffeine in human plasma by LC-ESI-MS. **Chromatografia**. v.67, p.281-285, 2007.

WATJEN, W.; BEYERSMANN, D. Cadmium-induced apoptosis in C6 glioma cells: influence of oxidative stress. **Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine** v. 17, p. 65-78, 2004.

WHO, World Health Organization. 1980. Recommended Health-Based Limits in Occupational Exposure to Heavy Metals. **Tech. Rep.** Ser. 647. Geneva: WHO. p.116.

WHO. Environmental Health Criteria, Cadmium. 1st ed. **World Health Organization**, Geneva, Switzerland, v.134. 1992.

WILLIANS, R.J.; SPENCER, J.P.E.; RICE-EVANS, C. Free Radical**. Biol Med**, v.36, v.838-849, 2004.

YAO H.W. et al. Inhibitory effect of leflunomide on hepatic fibrosis induced by Ccl_4 in rats. **Acta Pharmacol** Sin, v.25, p.915-920, 2004.

YEGUTKIN G. Nucleotide and nucleoside conservating ectoenzymes: important modulators of purinergic cascade. **Biochim Biophy Acta**, v.1783, p.673-694, 2008.

YOUDIM, K.A. et al. Flavonoid permeability across an in situ model of the blood-brain barrier. **Free Radic Biol Med** v. 36, p. 592-604, 2004.

YOUDIM, M.B.H.; RIEDERER, P. Iron in the brain, normal and pathological.conditions. In: SMITH, B.; ADELMAN, G. (Eds.), Encyclopedia of Neuroscience. Elsevier, Amsterdam (in press). ARUOMA, O.I.; BAHORUN, T.; JEN, L.S. (2003) Neuroprotection by bioactive components in medicinal and food plant extracts. **Mutat Res,** v.544, p.203-215, 2003.

ZARROS A., KALOPOTA K., TSAKIRIS S., BALLIE G.S. Can acetylcholinesterase activity be considered as a reliable biomarker for the assessment of cadmium-induced neurotoxicity? **Food Chem Toxicol.** v. 56, p. 406–410, 2013.

ZHAN H. et al. Spatial learning transiently disturbed by intraventricular administration of ouabain. **Neurol Res** v. 26, p. 35-40, 2004.

ZHANG, X.M. et al. Apoptosis of murine melanoma B16-BL6 cells induced by quercetin targeting mitochondria, inhibiting expression of PKC-alpha and translocating PKC-delta. **Cancer Chemother Pharmacol,** v. 3, p. 251-62, 2005.

ZIMMERMANN, H. Biochemistry, localization, and functional roles of ecto-nucleotidases in the nervous system. **Progress in Neurobiology**, v.49, p.589-618, 1996.

ZIMMERMANN, H. et al. New insights into molecular structure and structure and function of ectonucleotidases in the nervous system. **Neurochemistry International,** v.32, p.421-425, 1998.

ZIMMERMANN, H. Ectonucleotidases: some recent developments and a note on nomenclature**. Drug Development Reseach**, v. 52, p. 44-56, 2001.

ZIMMERMANN, H. et al. Ecto-nucleotidases, molecular properties and functional impact. **Anales de La Real Academia Nacional de Farmacia**, v.73, p.537-566, 2007.

ANEXOS

ANEXO A – Carta de Aprovação pelo Comitê Interno de Ética em Experimentação Animal - UFSM

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMITÊ INTERNO DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL-UFSM

CARTA DE APROVAÇÃO

O Comitê Interno de Ética em Experimentação Animal-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Avaliação da atividade de enzimas que degradam nucleotídeos de adenina e ésteres de colina e estudo do perfil oxidativo em ratos expostos ao cádmio e tratados com quercetina"

Numero do Parecer: 031/2011

Pesquisador Responsável: Cinthia Melazzo de Andrade Mazzanti

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

Os membros da CIETEA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DA REUNIÃO DE APROVAÇÃO:

Santa Maria, 13 de Junho de 2011.

Marta Lizandra do Rêgo Leal
Coordenadora do Comitê Interno de Ética em Experimentação Animal-UFSM

Comitê Interno de Ética em Experimentação Animal - UFSM - Av. Roraima, 1000 – Prédio da Reitoria - 2º andar - Campus Universitário 97105-900 – Santa Maria – RS - - Tel: 0 xx 55 3220 9362