PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

FACULDADE DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

STEFÂNIA KONRAD RICHETTI

AVALIAÇÃO DOS EFEITOS DE COMPOSTOS POLIFENÓLICOS EM PARÂMETROS BIOQUÍMICOS E NO TRATAMENTO DE DÉFICITS COGNITIVOS ASSOCIADOS À ADMINISTRAÇÃO DE ESCOPOLAMINA EM PEIXE ZEBRA (*Danio rerio*)

Orientadora: Prof. Dra. Carla Denise Bonan

PORTO ALEGRE – RS

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Dissertação apresentada como requisito para a obtenção do grau de Mestre pelo Programa de Pós-Graduação em Biologia Celular e Molecular da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul.

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Da minha aldeia vejo quanto da terra se pode ver no Universo...

Por isso a minha aldeia é tão grande como outra qualquer

Porque eu sou do tamanho do que vejo

E não do tamanho da minha altura...

(Alberto Caeiro)

RESUMO

O peixe zebra é um dos modelos vertebrados mais importantes para estudos de genética, biologia do desenvolvimento, neurofisiologia e biomedicina, sendo utilizado como um modelo de doenças humanas e para triagem de novas drogas, tais como fármacos para o tratamento da doença de Alzheimer. A doença de Alzheimer é uma doença neurodegenerativa que se caracteriza pela deposição de placas amilóides, desenvolvimento de emaranhados de neurofibrilas, inflamação e perda neuronal em diversas partes do cérebro que contribuem para os déficits cognitivos característicos da doença. O sistema colinérgico, o principal sistema envolvido nesta doença, apresenta a acetilcolina (ACh) como neurotransmissor e está fortemente relacionado à processos de aprendizado e formação da memória. Além do sistema colinérgico, outros sistemas, como o sistema purinérgico, estão envolvidos na patologia da doença de Alzheimer. Os nucleosídeos e nucleotídeos derivados de purinas exercem um papel de moléculas sinalizadoras extracelulares em vários tecidos, através dos receptores purinérgicos. O ATP tem seus níveis extracelulares controlados por enzimas da família das ectonucleotidases, entre elas as ectonucleosídeo trifosfato difosfoidrolases (E-NTPDases) e a ecto-5´-nucleotidase, que realizam a degradação de nucleotídeos púricos até adenosina, um nucleosídeo neuromodulador da homeostase celular. Os polifenóis, os quais são compostos derivados de plantas, podem atuar como moduladores da sinalização purinérgica e colinérgica, além de apresentarem efeitos antioxidantes, com ausência de efeitos colaterais severos, apresentando um grande potencial como tratamento para a doença de Alzheimer. Portanto, o objetivo deste estudo foi avaliar o potencial neuroprotetor dos polifenóis quercetina e rutina na prevenção dos déficits cognitivos causados pela escopolamina, um antagonista colinérgico muito utilizado para testes de novos fármacos facilitadores da capacidade cognitiva, bem como seus efeitos sobre as enzimas responsáveis pela modulação dos níveis dos neurotransmissores ATP e acetilcolina. Os resultados obtidos demonstram que a administração intraperitonial de quercetina ou rutina (50 mg/kg) em peixe zebra previniu o déficit cognitivo causado pela exposição à escopolamina (200 μM), como demonstrado pelo aumento da latência para cruzar para o lado escuro do aparato da tarefa de esquiva inibitória. A exposição a estes compostos não promoveu alterações na locomoção dos animais. Além disso, foi observado que o tratamento com rutina seguido de exposição à água inibiu a hidrólise de acetilcolina enquanto que o tratamento com rutina seguido pela exposição à escopolamina diminuiu a hidrólise de ATP. Com relação aos efeitos da quercetina, esta inibiu a hidrólise de AMP quando sua administração foi seguida pela exposição à água ou escopolamina. Portanto, estes resultados demonstram que os polifenóis podem apresentar potencial protetor com relação ao prejuízo cognitivo induzido pela escopolamina. Além disso, nossos resultados demonstram que rutina e quercetina *per se* são capazes de modular os níveis de acetilcolina, ATP e adenosina em encéfalo de peixe zebra. Estes resultados são promissores em relação à possibilidade de terapia preventiva a ser realizada ao longo da vida, visando a não ocorrência de declínio cognitivo associado ao envelhecimento.

Palavras chaves: rutina, quercetina, peixe zebra, nucleosídeo trifosfato difosfoidrolase, ecto-5´-nucleotidase, acetilcolinesterase, memória.

ABSTRACT

The zebrafish is one of the most important vertebrate models for studying genetics, developmental biology, neurophysiology, and biomedicine, and it is used as a model of human diseases and for the development of new therapeutic drugs, including drugs for Alzheimer's disease treatment. Alzheimer's disease is a neurodegenerative disease characterized by amyloid plaques deposition, development of neurofibrillary tangles, inflammation and neuronal loss in different parts of the brain that contribute to the cognitive impairment characteristic of the disease. The cholinergic system, the main system involved in this disease, presents acetylcholine (ACh) as a neurotransmitter and is strongly related to processes of learning and memory formation. Besides the cholinergic system, other neurotransmitter systems, such as the purinergic system are involved in Alzheimer's pathology. The purine-derived nucleosides and nucleotides display a role as extracellular signaling molecules in various tissues via purinergic receptors. ATP has its extracellular levels controlled by a group of enzymes called ectonucleotidases, which includes ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) and ecto-5'-nucleotidase, which carry out the degradation of purine nucleotides to adenosine, a nucleoside neuromodulator of cellular homeostasis. Polyphenols, compounds derived from plants, can act as modulators of cholinergic and purinergic signaling, present known antioxidant effects and no severe side effects, showing great potential as a treatment for Alzheimer's disease. Therefore, the aim of this study was to evaluate the potential neuroprotective role of polyphenols quercetin and rutin in the prevention of cognitive impairment caused by scopolamine, a cholinergic antagonist widely used for testing new drugs that facilitate cognitive abilities, as well as to analyze their effects on the enzymes responsible for modulation of neurotransmitters levels, such as ATP and acetylcholine. Our results have shown that administration of quercetin or rutin intraperitoneal (50 mg/kg) in zebrafish prevents cognitive deficits caused by exposure to scopolamine (200 μM), as demonstrated by increased latency to cross to the dark side of the inhibitory avoidance apparatus. None of the compounds in which the animals were exposed are able to alter the locomotor activity of the animals. Moreover, it has been observed that treatment with rutin followed by water exposure inhibited acetylcholine hydrolysis whereas the treatment with rutin followed by scopolamine exposure reduced ATP hydrolysis. Regarding the effects of quercetin, it has inhibited the AMP hydrolysis when its administration was followed by water or scopolamine exposure. Therefore, these results have demonstrated that polyphenols may display protective potential on to the cognitive impairment induced by scopolamine. Moreover, our findings have shown that rutin and quercetin *per se* are able to modulate the levels of acetylcholine, ATP, and adenosine in zebrafish brain. These results are promising regarding the possibility of preventive therapy to be carried throughout lifespan to avoid the occurrence of cognitive decline associated with aging.

Keywords: rutin, quercetin, zebrafish, nucleoside triphosphate diphosphohydrolase, ecto-5´-nucleotidase, acetylcholinesterase, memory.

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CAPÍTULO 1 - INTRODUÇÃO E OBJETIVOS

1. INTRODUÇÃO

1.1. PEIXE ZEBRA

O peixe zebra, *Danio rerio* ou *zebrafish* (Hamilton 1822), pertence à família dos peixes de água-doce Cyprinidae, que corresponde à família de vertebrados mais rica em número de espécies (Nelson, 1994), sendo as espécies do gênero *Danio* distribuídas ao longo do sul e sudeste asiático. O nome *Danio* é derivado da palavra Bengali "*dhani"*, que significa "do campo de arroz" (Talwar e Jhingran, 1991). Este gênero é caracterizado pelo seu pequeno tamanho (até 12 cm de comprimento) e um distinto padrão de coloração baseado na alternância de linhas horizontais claras e escuras. O *Danio rerio* raramente excede o tamanho padrão de 4 cm da ponta do focinho até a origem da nadadeira caudal. O padrão de coloração compreende três tipos de células pigmentares, ou cromatóforos: os melanóforos azul-escuros, os xantóforos dourados e os iridóforos iridescentes (Parichy, 2006). Assim como na maioria dos teleósteos, os melanóforos podem ser concentrados ou dispersados em resposta aos estímulos, sendo úteis na camuflagem, em resposta às alterações na luminosidade (Guo, 2004), além de serem úteis para a demonstração de agressividade, medo e outras situações comportamentais (Kondo *et al*., 2009; Larson *et al.,* 2006; Price *et al*., 2008). Os machos e as fêmeas apresentam coloração semelhante, embora os machos apresentem nadadeiras anais maiores e com coloração mais amarelada em relação às das fêmeas (Schilling, 2002). O *Danio rerio* é uma espécie onívora, se alimentando principalmente de zooplancton e insetos, além de fitoplancton, algas

filamentosas, ovos de invertebrados, aracnídeos e detritos (Engeszer *et al*., 2007; Spence *et a*l., 2007 e 2008).

O peixe zebra é um dos modelos vertebrados mais importantes para estudos de genética, biologia do desenvolvimento (Brittijn *et al*., 2009), neurofisiologia e biomedicina (Amsterdam e Hopkins, 2006; Rubinstein, 2003). Esta espécie apresenta um grande número de atributos que a torna atrativa para o desenvolvimento de pesquisas. É um peixe pequeno permitindo que grandes quantidades deste animal sejam mantidas facilmente com a necessidade de pouco espaço e sem altos custos de manutenção em laboratório. Em relação à sua reprodução, esta ocorre ao longo de todo o ano, e cada fêmea pode se reproduzir a cada 2-3 dias e uma única postura pode conter várias centenas de ovos. O fato desta espécie apresentar fecundação e reprodução externas é uma característica ainda mais interessante do ponto de vista da utilização deste modelo para estudos de biologia do desenvolvimento. O peixe zebra apresenta ovos relativamente grandes em relação a outros peixes (0,7 mm no momento da fertilização) e estes são transparentes, permitindo o acompanhamento e a observação em tempo real da divisão celular e formação de um novo organismo. Além da sua importância em estudos ontogenéticos, o peixe zebra vem ganhando importância nas pesquisas biomédicas (Dooley e Zon, 2000; Shin e Fishman, 2002), particularmente como um modelo de doenças humanas (Guyon *et al*., 2006; Ingham, 2009), doenças neurodegenerativas (Best e Alderton, 2008; Kishi *et al*., 2009; Sager *et al*., 2010), para testes com novas substâncias terapêuticas (Crawford *et al*., 2008; Mandrekar e Thalur, 2009; Rihel *et al*., 2010; Zon e Peterson, 2005), compreensão da evolução do genoma vertebrado, estudos teratológicos e neurociências (Ivetac *et al*., 2000; Morris, 2009). Como possui a sua

genética bem caracterizada, o estudo do genoma do peixe zebra pode servir como um complemento funcional para o projeto genoma humano, o qual produz enormes quantidades de sequências, mas carece de informações funcionais para a maioria dos genes identificados (Dooley e Zon, 2000). Além disso, os genes deste teleósteo são evolutivamente conservados e apresentam um alto grau de similaridade com os genes humanos e de camundongo (Barbazuk *et al*., 2000; Lieschke e Currie, 2000). Em relação às pesquisas de doenças humanas, o peixe zebra tem vantagem em relação a outros modelos animais, como drosófilas, pois devido ao fato de ser um vertebrado, estudos realizados neste modelo são mais comparáveis aos humanos do que modelos invertebrados (Barbazuk *et al*., 2000; Postlethwait *et al*., 2000). Além disto, o peixe zebra ainda apresenta certa vantagem em relação a animais modelos mamíferos como camundongos, pela maior facilidade de manipulações embriológicas neste animal (Zhang e Leung, 2010). Atualmente, mais de 400 laboratórios utilizam o peixe zebra como modelo animal (www.zfin.org). Há um crescente interesse em utilizá-lo como ferramenta para estudos sobre o ciclo celular (Eimon e Ashkenasi, 2010), estudos bioquímicos (Taylor *et al*., 2004), farmacológicos (Chakraborty *et al*., 2009; Eimon e Rubinstein, 2009; Goldsmith, 2004; Rubinstein, 2006; Vogt *et al*., 2010; Yang *et al*., 2009), e comportamentais, visando a compreensão da base genética do comportamento (Gerlai, 2003; Guo, 2004; Miklosi e Andrew, 2006; Mueller e Neuhauss, 2010; Rihel *et al*., 2010) e para estudos toxicológicos (Hill *et al*., 2005). Para tais pesquisas, a utilização deste modelo que absorve os componentes diretamente da água pelas suas brânquias e os acumula em diferentes tecidos, principalmente no sistema nervoso central (SNC) (Grosell

e Wood, 2002) vem ganhando importância significativa (Froehlicher *et al*., 2009; Yang *et al*., 2009).

Muitos estudos já foram realizados visando a caracterização de sistemas de neurotransmissão no peixe zebra, resultando na detecção dos sistemas glutamatérgico (Edwards e Michel, 2002; Rico *et al*., 2010), colinérgico (Behra *et al*., 2002; Clemente *et al*., 2004), dopaminérgico (Boehmler *et al*, 2004; Kastenhuber *et al*., 2010; Schweitzer e Driever, 2009), serotoninérgico (Bencan *et al*., 2009; Gabriel *et al*., 2009; Rink e Guo, 2004), gabaérgico (Kim *et al*., 2004) e purinérgico (Boehmler *et al*., 2009; Kucenas *et al*., 2003; Rico *et al*., 2003; Young, 2010), além da caracterização de algumas enzimas e receptores envolvidos nestes sistemas.

1.2. SISTEMA PURINÉRGICO

A sinalização intercelular mediada por purinas está presente desde cedo na evolução e, portanto, é uma rota amplamente distribuída para comunicação célula-célula (Burnstock, 2008). As purinas parecem ser os mensageiros químicos mais primitivos e dispersos em animais e plantas (Burnstock e Verkhratsky, 2009). O conceito de transmissão purinérgica foi proposto por Geoffrey Burnstock em 1972 após a observação da ação do ATP como transmissor em nervos inibitórios não adrenérgicos, não colinérgicos em *Taenia coli* de roedores (Burnstock, 1972).

Os nucleosídeos e nucleotídeos derivados de purinas exercem um papel de moléculas sinalizadoras extracelulares em vários tecidos, através dos receptores purinérgicos (Abbraccio *et al*., 2009; Burnstock e Knight, 2004). Diversas evidências têm

demonstrado o importante papel desempenhado por essas moléculas, entre elas o ATP e a adenosina, no SNC (Burnstock, 2009a e 2009b; Dunwiddie e Masino, 2001; Franco, 2009; Ralevic e Burnstock, 1998). O ATP é reconhecido como um neurotransmissor, pois é sintetizado e armazenado em terminais sinápticos e liberado após estímulo destes terminais. O ATP pode ser co-liberado juntamente com vários neurotransmissores, como a acetilcolina, glutamato, dopamina, noradrenalina, serotonina e ácido γ-amino butírico (GABA) (Burnstock, 1999; Di Iorio *et al*., 1998; Zimmerman, 2008). Pode atuar tanto como um transmissor ou como um co-transmissor, na maioria dos nervos no SNC e periférico (Burnstock, 2007).

A transmissão de sinal via ATP desempenha papéis complexos, tais como a neurotransmissão excitatória ou inibitória, além de atuar como um fator trófico com efeitos a longo prazo sobre a proliferação celular, crescimento e desenvolvimento, em doenças e citotoxicidade (Abbracchio e Burnstock, 1998). O ATP pode ser liberado nos terminais pré- e pós-sinápticos. Esta liberação pode ocorrer tanto a partir de células saudáveis, como um mecanismo fisiológico ou em resposta à danos celulares, como hipóxia, injúrias e deformação celular (Burnstock, 2008). O ATP age através de receptores purinérgicos específicos do tipo P2, divididos em dois grupos de acordo com a base do mecanismo de ação, farmacologia e clonagem molecular: o primeiro grupo é constituído pelos receptores P2X (Skaper *et al*., 2010; Surprenatant e North, 2009), os quais são ionotrópicos (Ralevic e Burnstock, 1998) e o segundo grupo é constituído pelos receptores metabotrópicos P2Y, acoplados a uma proteína G. Estas duas classes de receptores apresentam uma ampla distribuição nos tecidos e sistemas, tais como sistema nervoso, vascular e cardíaco (Burnstock, 2004). Os sete receptores do tipo P2X são

ligados a canais iônicos e quando ativados resultam na abertura de um poro na membrana celular que permite a passagem de Na^{+}, K^{+}, Ca^{+2}. Estes receptores interagem com vários receptores ionotrópicos, incluindo receptores colinérgicos nicotínicos, receptores $GABA_A$, e receptores NMDA. Os mecanismos para estas interações parecem ser mediados pela fosforilação destes receptores através da ação de cinases ativadas por Ca^{+2} intracelular (Pankratov *et al*., 2009). Os oito receptores do tipo P2Y podem ser divididos em dois subgrupos, de acordo com a similaridade filogenética e de acordo com a especificidade de proteínas G aos quais são ligados. O primeiro grupo contempla os receptores $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ e $P2Y_{11}$, os quais utilizam proteínas $G_q\backslash G_{11}$ para ativar a rota de liberação de Ca^{+2} através da ação da fosfolipase C. O segundo grupo, composto pelos receptores $P2Y_{12}$, $P2Y_{13}$ e $P2Y_{14}$, por sua vez, liga-se quase que exclusivamente a proteínas $G_{i/o}$, que quando ativadas inibem a adenilil ciclase e modulam canais iônicos. A clonagem e caracterização molecular dos receptores P2X do peixe zebra já foram realizadas (Appelbaum *et al*., 2007; Diaz-Hernandez *et al*., 2002; Low *et al*., 2008; Young, 2010). A subfamília P2X possui nove membros, sendo destes seis ortólogos a genes dos receptores P2X de mamíferos, dois parálogos e um gene que ainda precisa ser devidamente classificado (Kucenas *et al*., 2003 e 2009). Os subtipos dos receptores P2X do peixe zebra contêm resíduos altamente conservados, os quais são encontrados nas subunidades de mamíferos. Até o momento, na família de receptores P2Y foram identificadas oito proteínas funcionais (Ralevic e Burnstock, 1998), e somente os receptores P2Y1 foram identificados em trombócitos de peixe zebra (Gregory e Jagadeeswaran, 2002).

Uma vez liberado no espaço extracelular, o ATP pode ser metabolizado pela ação de ecto-enzimas que fazem a conversão deste nucleotídeo até adenosina (Robson *et al*., 2006; Zimmermann, 2001). A concentração extracelular de adenosina é um fator determinante dos efeitos neuromoduladores desta molécula.

A adenosina é um metabólito constituinte de todas as células (Cunha, 2001) que desenvolve diversos papéis chave no organismo. Esta molécula está envolvida na síntese de ácidos nucléicos, metabolismo de aminoácidos e modulação do estado metabólico da célula. A adenosina não é considerada um neurotransmissor clássico, como o ATP, pelo fato de não ser armazenada em vesículas, não ser liberada por exocitose e não atuar predominantemente em sinapses (Fredholm, 2003). A adenosina pode ser sintetizada intracelularmente pela clivagem da S-adenosil-homocisteína pela enzima S-adenosil-homocisteína hidrolase ou pela degradação do nucleosídeo monofosfatado AMP pela enzima 5´-nucleotidase e extracelularmente por uma cascata enzimática de degradação de nucleotídeos, realizada pelas ectonucleotidases, a qual inclui uma ecto-5'-nucleotidase (Colgan *et al*., 2006).

A ação da adenosina se dá através da ativação de receptores purinérgicos (Sebastião e Ribeiro, 2009), do tipo P1 acoplados à proteínas G (Trincavelli *et al*., 2010). Estes receptores são divididos em 4 subtipos de acordo com suas características, como estrutura molecular, distribuição tecidual e afinidade pelo seu ligante. São eles: o receptor A_1, A_{2A}, A_{2B} e A_3. Os receptores A_1 e A_3 se ligam à família das proteínas $G_{i/o}$, responsáveis pela inibição da produção do segundo mensageiro AMP cíclico (AMPc) (Borea *et al*., 2009; Dhalla *et al*., 2009). Os receptores A_{2A} e A_{2B} estimulam a produção de AMPc via ativação de proteínas G_s (Brown e Short, 2008). A porção N-terminal do

receptor está voltada para o meio extracelular e a porção C-terminal está voltada para o lado citosólico da membrana plasmática (Ralevic e Burnstock, 1998). Recentemente, foi demonstrado a presença de duas formas de receptores A_{2A} (adora2a.1 e adora2a.2) e uma forma de receptor A_{2B} em embriões de peixe zebra (Boehmler *et al*., 2009).

A adenosina desempenha dois papéis, atuando como uma molécula que mantém a homeostase no meio intracelular e como um neuromodulador do sistema nervoso. No SNC, age modulando a liberação de neurotransmissores, as respostas pós-sinápticas e a ação de outros sistemas (Cunha, 2001 e 2005), além de proteger o organismo em caso de isquemia e injúrias (Fredholm, 2003; Stone *et al*., 2009). Devido a este papel neuromodulador, a adenosina está envolvida na regulação de importantes mecanismos no SNC, como inflamação (Di Virgílio *et al*., 2009; Haskó *et al*., 2008), estados de ansiedade (El Yacoubi *et al*., 2000), sono (Porkka-Heiskanen, 1999), cognição e memória (Ribeiro *et al*., 2003). Além disso, este nucleosídeo apresenta especial importância nos estudos de patofisiologias, como na doença de Parkinson e na esquizofrenia (Lara *et al*., 2001), tendo os seus receptores como possíveis alvos para o desenvolvimento de fármacos (Fredholm, 2010). Recentemente, antagonistas de receptor adenosinérgico A_{2A} receberam uma elevada atenção por se tratarem de novas estratégias para prevenir ou retardar o desenvolvimento de doenças neurodegenerativas, como Parkinson (Cunha *et al*, 2008; Jenner *et al*., 2010; Schwartzchild *et al*., 2006). Também foi observado o papel de antagonistas destes receptores em prevenir o déficit cognitivo associado à doença de Alzheimer (Arendash *et al*., 2006; Cunha *et al*., 2008; Dall´Igna *et al*., 2007), como foi observado após a utilização do antagonista cafeína, que demonstrou uma atenuação do declínio da memória em idosos (Ritchie *et al*., 2007; Takahashi *et al*., 2008) e também

uma redução no risco do desenvolvimento da doença de Alzheimer (Maia e de Mendonça, 2002; Stone *et al*., 2009).

1.3. ECTONUCLEOTIDASES

Os nucleotídeos extracelulares podem atuar como moléculas sinalizadoras que precisam ter o seu sinal inativado após a sua ação. A inativação deste sinal e a manutenção das concentrações dos nucleotídeos pode se dar pela hidrólise extracelular dos mesmos. Os nucleotídeos são hidrolisados por uma cascata que resulta na formação do respectivo nucleosídeo e fosfato livre. As enzimas que catalisam esta reação são as ectonucleotidases. As ectonucleotidases estão ancoradas na membrana celular, possuindo seu sítio ativo voltado para o meio extracelular ou estão presentes na forma solúvel no meio intersticial (Schetinger *et al*., 2007). Diversas famílias de enzimas constituem o grupo das ectonucleotidases constituído por quatro famílias de enzimas: Ecto-nucleosídeo trifosfato difosfoidrolases (E-NTPDases), Ecto-nucleotídeo pirofosfatase/fosfodiesterase (E-NPPs), fosfatases alcalinas e Ecto 5'-nucleotidases (E- 5´NT) (Robson *et al*., 2006) (Figura1) . Neste estudo, daremos ênfase às E-NTPDases e Ecto-5´nucleotidases.

As E-NTPDases podem hidrolisar nucleosídeos-5´trifosfatados e nucleosídeos-5´difosfatados e apresentam membros em vertebrados, invertebrados, plantas, leveduras e protozoários (revisado em Zimmermann, 2001). Os membros desta família diferem em relação à preferência pelo substrato, neste caso nucleosídeos 5´-tri e nucleosídeos 5´-difosfatados, que vão ser hidrolisados. Os membros da família das NTPDases são codificados por oito genes diferentes, chamados de genes *entpd*. Dos oito membros

descritos até o momento, quatro estão localizados na membrana celular com o sitio ativo voltado para o meio extracelular (NTPDases 1, 2, 3 e 8). As NTPDases 5 e 6 se localizam intracelularmente, porém são secretadas após expressão heteróloga. As NTPDases 4 e 7 apresentam localização intracelular com o sítio ativo voltado para o lúmen de organelas citoplasmáticas (Robson *et al*., 2006; Zimmermann, 2000).

A Ecto-5´-NT é uma enzima que desfosforila nucleosídeos monofosfatados não cíclicos (Bianchi e Spychala, 2003), através da hidrólise da ligação fosfodiéster de 5´-ribonucleotídeos, levando à formação do correspondente ribonucleosídeo e fosfato. A principal função em animais é a hidrólise de AMP até adenosina (Zimmermann, 2000). As Ecto-5´-NT apresentam uma ampla distribuição tecidual e fazem parte da cascata para finalizar a ação de nucleotídeos como ATP e moléculas sinalizadoras que agem em receptores P2X e P2Y, sendo a principal enzima responsável pela formação extracelular de adenosina (Zimmermann, 2000).

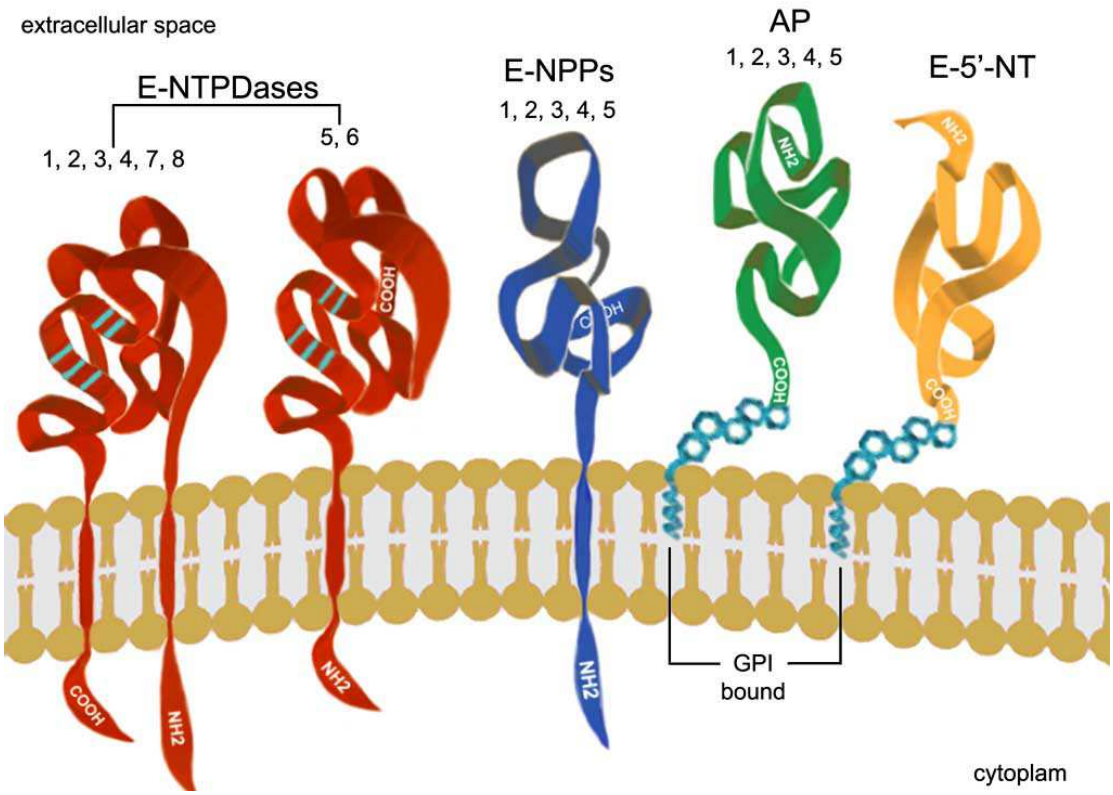


Figura 1: Topografia de membrana das ectonucleotidases (Cognato e Bonan, 2010)

No peixe zebra, estudos demonstraram a presença de uma NTPDase e uma Ecto-5´-NT em membranas cerebrais. Estas duas enzimas foram caracterizadas como cátion-dependentes, apresentando atividade máxima à temperatura de 37 °C, pH ótimo entre 7,2 e 8,0, Km na faixa do micromolar e uma ampla especificidade por outros nucleotídeos (Rico *et al*., 2003; Senger *et al*., 2004). Estudos do nosso laboratório identificaram a presença de três isoformas diferentes de NTPDase 2 (NTPDase2mv, NTPDase2mg, NTPDase2mq) em encéfalo de peixe zebra (Rico *et al*., 2006) e duas isoformas de NTPDase5 (NTPDase5ms e NTPDase5me) (Rosemberg *et al*., 2010). A co-localização da NTPDases 1 e 2 já foi descrita em células de retina de peixe zebra (Ricatti *et al*., 2009)

e a NTPDase 3 foi caracterizada em neurônios sensoriais desta espécie (Appelbaum *et al*., 2007).

1.4. SISTEMA COLINÉRGICO

O sistema colinérgico já foi muito estudado, visto que a acetilcolina foi o primeiro neurotransmissor identificado durante a primeira metade do século vinte, a partir de estudos de Henry Dale, Otto Loewi, Feldberg e outros pesquisadores (Anglade e Larabi-godinot, 2010; Wessler *et al*., 1999). O sistema colinérgico pode modular funções cognitivas de maneira eficiente no cérebro (Hasselmo e Giocomo, 2006; Karczmar, 2010), agindo em receptores metabotrópicos e ionotrópicos. A acetilcolina (ACh) é sintetizada nas extremidades dos axônios colinérgicos. A colina, um dos precursores da ACh é captada do meio extracelular pela ação de transportadores específicos. Outra importante fonte de acetilcolina é a quebra de fosfatidilcolina. A ACh é sintetizada pela ação da enzima colina-acetiltransferase (ChAT), responsável por transferir um grupamento acetil da acetilcoenzima A para a colina (Oda, 1999). O passo limitante na formação de acetilcolina é o transporte de acetil coenzima A, oriunda da membrana interna da mitocôndria após a via metabólica de transformação da glicose em piruvato. Após a síntese, a ACh é transportada dentro de vesículas para os terminais dos axônios colinérgicos, onde é armazenada (Amenta e Tayebati, 2008; Ferguson e Blakely, 2004). Quando liberada no meio extracelular a acetilcolina age em receptores específicos ionotrópicos nicotínicos e metabotrópicos muscarínicos. Os receptores nicotínicos

(nAChRs) quando ativados, permitem a passagem de íons, sendo responsáveis pelo aumento do influxo de íons como Na^{+}, K^{+} e Ca^{+2} (Dani e Bertrand, 2007). Os receptores nicotínicos estão envolvidos em mecanismos de recompensa no sistema nervoso central, isto explica em grande parte o mecanismo do uso de tabaco e nicotina (Adinoff, *et al*., 2010; Changeux, 2010; Picciotto *et al*., 1998; Sherva *et al*., 2010).

Os receptores muscarínicos (mAChRs) se associam à proteínas G e consistem em cinco tipos diferentes de receptores (M1-M5). Assim como os nAChRs, um único neurônio colinérgico pode expressar mais de um tipo de subtipo de mAChR. Muitas evidências relacionam os receptores colinérgicos muscarínicos com processos de aprendizado e memória, entre elas a observação de déficits cognitivos em ratos *knockout* para o gene do receptor M1 (Anagnostaras *et al*., 2003). Os receptores M5 são pouco expressos no cérebro e, até o momento, seu papel fisiológico não foi claramente estabelecido, porém foi atribuída a este receptor a dilatação induzida por ACh das veias cerebrais que podem ser importantes para o desenvolvimento da doença de Alzheimer. Os receptores muscarínicos M1, M3 e M5 se ligam às proteínas Gq e a sua ativação mobiliza Ca^{+2} intracelular. Através de proteínas Gq/11, estes mAChRs ativam a fosfolipase C e por consequência a proteína quinase C, a qual inicia a liberação de Ca^{+2} intracelular, o que resulta em um aumento da excitabilidade neuronal. Os receptores M2 e M4 ativam os canais iônicos de K^{+} da membrana plasmática através de proteínas Gi, resultando em uma hiperpolarização, culminando em efeitos inibitórios na atividade neuronal. A ativação destes receptores também gera a inibição da liberação de neurotransmissores e a inibição da adenilato ciclase (Brown e Sihra, 2008).

No espaço extracelular, a acetilcolinesterase (AChE) é a enzima responsável pela degradação de acetilcolina, eliminando os efeitos desencadeados por esta molécula. A AChE é sintetizada no retículo endoplasmático, processada e transportada para o meio extracelular pela presença de um peptídeo sinal na região N-terminal. A AChE, já descrita em peixe zebra, é codificada por um único gene neste animal, porém várias formas moleculares são observadas (monômeros, dímeros, trímeros e tetrâmeros), resultado da ocorrência de splicing alternativo nos éxons da região C-terminal (Massoulié *et al*., 2008).

1.5. DOENÇA DE ALZHEIMER

A doença de Alzheimer é uma doença neurodegenerativa progressiva com ocorrência principalmente em pessoas idosas (Francis *et al*., 1999), sendo o envelhecimento um fator que contribui para o desenvolvimento da patologia. Esta doença acomete 18 milhões de pessoas no mundo, podendo chegar a 34 milhões em 2025, segundo a *Alzheimer´s Society* (Mount e Downton, 2006). Tem causa multifatorial, com uma combinação complexa de componentes genéticos e não genéticos (De Strooper, 2010; Haberland, 2010; Mondragón-Rodríguez, *et al*., 2010; Ramassamy, 2006; Singh *et al*., 2008). Entre os componentes genéticos, a forma herdada da doença representa apenas uma pequena fração (< 5%) dos casos, enquanto a forma não-genética ou esporádica corresponde à maioria dos casos (Singh *et al*., 2008). Em relação à forma herdada da doença, foram identificadas mutações em três genes, que parecem contribuir para o desenvolvimento da doença. Embora estas mutações ocorram em diferentes genes, elas

compartilham a mesma rota bioquímica: a produção alterada de peptídeo β-amilóide (Aβ) que leva à morte neuronal e demência (Singh *et al*., 2008). Em nível celular, fatores como inflamação, toxicidade glutamatérgica, ativação de rotas apoptóticas, elevação do óxido nítrico e estresse oxidativo parecem estar envolvidos (Crews e Masliah, 2010; Ramassamy, 2006; Reddy, 2007).

A doença de Alzheimer resulta da neurodegeneração caracterizada pela deposição de placas amilóides (Chen *et al*., 2008; Ramassamy, 2006), desenvolvimento de emaranhados de neurofibrilas, (Blennow *et al*., 2006; Selkoe, 2001) inflamação e perda neuronal em diversas partes do cérebro, principalmente no prosencéfalo e hipocampo (Janas *et al*., 2005; Sambamurti *et al*., 2002). A deposição de placas amilóides, formada principalmente por fragmentos β-amilóides resulta de deficiências no metabolismo desta proteína, enquanto a formação dos emaranhados de neurofibrilas está relacionado com a hiperfosforilação da proteína TAU (Bertrand *et al*., 2010; Cotman, 1997; Gustaw-Rothenberg *et al*., 2010; Kovacech *et al*., 2010; Siemers *et al*., 2010; Zetterberg *et al*., 2010).

Embora muitos sistemas de neurotransmissão, como glutamatérgico, serotoninérgico e catecolaminérgico estejam envolvidos nesta patologia, a degeneração e déficits funcionais no sistema colinérgico parecem estar fortemente relacionados com os sintomas da doença de Alzheimer (Giacobini, 2003; Kim *et al*., 2007). Alguns autores, como Han *et al*. (2007), chegam a definir o sistema colinérgico como o principal sistema desencadeador dos sintomas desta doença.

A redução na expressão ou atividade da AChE resulta em um aumentado tônus colinérgico no cérebro, com concomitante regulação na expressão dos receptores

colinérgicos muscarínicos e nicotínicos (Fisher, 2008; Guan, 2008; Kihara e Shimohama, 2004; Poulin *et al.*, 2010). No caso de pacientes com a doença de Alzheimer, é observada uma redução da atividade colinérgica (Mufson *et al*., 2008), através da perda de neurônios colinérgicos e suas projeções corticais e da redução nos níveis disponíveis deste neurotransmissor na fenda sináptica, o que significa que a molécula acetilcolina não consegue se ligar aos seus receptores e desencadear suas ações fisiológicas. Esta redução nos níveis da acetilcolina é resultado da redução da atividade da enzima responsável pela produção deste neurotransmissor e um aumento na atividade da AChE, ocasionando uma maior degradação de acetilcolina (Kim *et al*., 2008). Isto desencadeia os principais sintomas da doença, que são a perda de memória e progressivo prejuízo cognitivo (Bartus *et al*., 1982; Mattson, 2004; Mondragón-Rodríguez *et al*., 2010; Roberson e Mucke, 2006). O aumento do tônus colinérgico através da inibição da atividade da AChE corresponde à principal terapia atualmente utilizada para o tratamento desta doença (Alcaro *et al*., 2010; Darreh-Shori e Soininen, 2010; Desmarais e Gauthier, 2010; Grill e Cummings, 2010; Nieoullon, 2010; Pepeu e Giovannini, 2009).

O sistema purinérgico também está relacionado com esta doença. Foi observada uma expressão elevada dos receptores purinérgicos $P2X_7$ em cérebro de pacientes e modelos animais desta doença (McLarnon *et al*., 2006). A estimulação destes receptores em macrófagos e microglia de humanos resultou em um aumento das lesões degenerativas observadas na doença de Alzheimer (Rampe *et al*., 2004). Os receptores $P2Y_1$ são encontrados em estruturas características desta patologia como emaranhados de neurofibrilas e placas neuríticas (McLarnon *et al*., 2006). Além disso, anormalidades na transmissão de sinal desencadeada por ATP e mediada por Ca^{+2} foram observadas em

pacientes com a doença de Alzheimer (McLarnon *et al*., 2006). Estudos demonstraram uma redução da expressão dos receptores de adenosina A_1 no hipocampo de pacientes com esta doença e um acúmulo destes receptores em estruturas neurodegenerativas, nas quais são responsáveis pela fosforilação da proteína TAU (Angulo *et al*., 2003).

Atualmente, poucos medicamentos para o tratamento da doença de Alzheimer estão disponíveis (Cítron, 2010; Grill e Cummings, 2010). A hipótese colinérgica desta doença foi a base utilizada para o desenvolvimento de tratamentos pré-sinápticos, sinápticos e pós-sinápticos, formulados de maneira a manter e facilitar a atividade do sistema colinérgico (Han *et al*., 2007; Kim *et al*., 2008). Atualmente, os medicamentos disponíveis atuam inibindo a AChE, para aumentar os níveis deste nucleotídeo na fenda sináptica (Kang *et al*., 2005). Os medicamentos disponíveis para pacientes são a rivastigmina, tacrina, galantamina, fisostigmina e donezepil (Kim *et al*., 2008; Shigeta e Homma, 2001). Estes medicamentos apresentam efeitos colaterais gastrointestinais e hepatotóxicos muito severos (Coelho e Birks, 2001; Heydorn, 1997). Portanto, o desenvolvimento de novos medicamentos que não apresentem estes efeitos colaterais é necessário e se torna urgente (Colombres *et al*., 2004; Lee *et al*., 2003). Os polifenóis, moléculas encontradas na natureza, podem atuar como inibidores da acetilcolinesterase, além de apresentarem efeitos antioxidantes, com ausência de efeitos colaterais severos. Portanto, apresentam um grande potencial como tratamento para esta doença (Ji e Zhang, 2006). Vários estudos já demostraram a ação neuroprotetora de diversos polifenóis (Chen *et al*., 2008; Kang *et al*., 2005; Lee *et al*., 2003; Warda *et al*., 2002; Youdim *et al*., 2002).

1.5.1. Escopolamina

A escopolamina é um alcalóide que ocorre na natureza, e foi primeiramente isolada de plantas como *Atropa belladona* L., *Datura stramponium* L. e *Hyosyamus niger* L. (Zhang *et al*., 2008) É utilizada no tratamento de disfunções do SNC, como náusea, doença de Parkinson e dependência por opióides (Xiang *et al*., 2006). Seu mecanismo de ação envolve principalmente o bloqueio dos receptores muscarínicos colinérgicos, agindo como um antagonista competitivo (Brouilette *et al*., 2007; Hulme *et al*., 2003), porém apresentando efeitos também em outros sistemas de neurotransmissão. Ela reduz os efeitos da acetilcolina ao se ligar aos receptores muscarínicos sem gerar a despolarização (Ebert e Kirsh, 1998). Ela penetra facilmente a barreira hemato-encefálica e apresenta uma ação prejudicial em relação à cognição, formação e armazenamento da memória em ratos, primatas e humanos saudáveis (Spinelli *et al*., 2006). Portanto, a escopolamina vem sendo amplamente utilizada desde 1974 como um modelo animal de indução de déficits cognitivos e perda de memória, simulando um modelo de demência a até mesmo mimetizando os principais sintomas da doença de Alzheimer (Deiana *et al*., 2009; Drachman e Leavitt, 1974; Kim *et al*., 2008).

Esta molécula é muito utilizada para a avaliação da potencialidade de novas drogas anticolinérgicas e dos efeitos de drogas já existentes, para o tratamento da doença de Alzheimer. Sua utilização como modelo é maior em relação a outros modelos, pois ela apresenta a característica de induzir déficits cognitivos e impedir a formação da memória (Cunha G.M.A *et al*., 2008; Deiana *et al*., 2009) com a possibilidade de reversão após a utilização de fármacos anticolinérgicos, como donezepil. Muitos estudos avaliaram

parâmetros comportamentais relacionados ao aprendizado e memória em ratos e primatas, como esquiva inibitória, labirinto em T, labirinto em Y e tarefa de esquiva passiva e todos demonstraram uma redução no desempenho cognitivo (Kunesova *et al*., 2008).

1.6. POLIFENÓIS

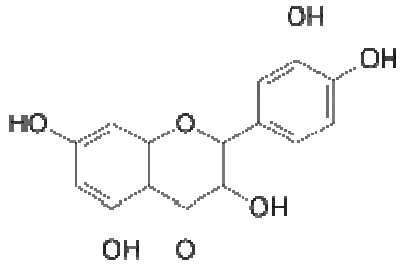
Atualmente, cerca de 10.000 metabólitos secundários de plantas são conhecidos e supõe-se que este número ultrapasse 100.000 (Halliwell e Gutteridge, 1999). Os três principais grupos de metabólitos secundários são os terpenos, os polifenóis e os alcalóides. Com algumas propriedades específicas, estes metabólitos secundários são essenciais para a fisiologia da planta (Rossi *et al*., 2008), contribuindo para a sua pigmentação, crescimento, reprodução e resistência à patógenos e predadores (Harvsteen, 2002). Os polifenóis são encontrados em diversas fontes alimentares, pois estão presentes em aparentemente todas as plantas, fazendo parte da nossa dieta diária (Rossi *et al*., 2008). Excluindo-se variações quanto às concentrações destes compostos em diferentes exemplares de plantas e variabilidade de consumo por humanos, é difícil o estabelecimento de uma dose diária de consumo de polifenol. Sabe-se que a ingesta humana diária de flavonóides corresponde a 13 mg/dia (Singh *et al*., 2008). Plantas da mesma espécie, cultivadas em diferentes localidades normalmente possuem os mesmos componentes, mas as concentrações de cada composto, como os antioxidantes, podem diferir. Sabe-se que uma vasta gama de compostos orgânicos naturais de origem vegetal é fisiologicamente ativa, isto é, apresenta ação tranqüilizante, analgésica, antiinflamatória,

citotóxica, anticoncepcional, antimicrobiana, antiviral e fungicida (Ross e Kasum, 2002). Dentre estas substâncias, esta a classe dos polifenóis (Cadenas e Packet, 2002).

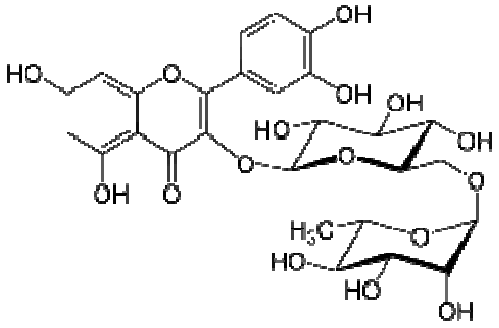
Os polifenóis podem ser divididos em duas grandes categorias de acordo com a sua estrutura molecular: flavonóides e não-flavonóides. Os flavonóides representam dois terços da totalidade dos polifenóis, e podem ser divididos em diferentes subclasses de acordo com o grau de oxidação do anel heterocíclico (Weinreb *et al*., 2004). São elas: flavonóis, flavanas, flavanóis, flavonas e isoflavonas. Os polifenóis não-flavonóides compreendem o grupo dos ácidos fenólicos, as lignanas e o grupo do resveratrol e participam com um terço da totalidade dos polifenóis encontrados na natureza (Singh *et al*., 2008).

A determinação de polifenóis em extratos de plantas tem sido realizada principalmente por métodos de separação (Zheleva-Dimitrova *et al*., 2007) como a cromatografia líquida de alta eficiência , a eletroforese capilar de zona e a cromatografia capilar electrocinética micelar. Dentre os compostos fenólicos mais comumente estudados por estes métodos estão a rutina e a quercetina (Figura 2).

Do ponto de vista fisiológico, toxicológico e farmacológico, o interesse por compostos fenólicos justifica-se pelo fato destes apresentarem uma série de vantagens em relação a outras drogas, principalmente em termos da ausência de efeitos colaterais. Neste sentido, o interesse pela descoberta de novos antioxidantes de fontes naturais tem aumentado, principalmente para prevenir a deterioração fisiológica de organelas e, consequentemente, minimizar o efeito oxidativo provocado pelos radicais livres.



Quercetina



Rutina

Figura 2: Estrutura química da quercetina e rutina.

Dentre os órgãos mais estudados nos últimos tempos, o cérebro destaca-se, devido à sua grande susceptibilidade em sofrer danos oxidativos e sua alta atividade metabólica, que induz a um alto consumo de oxigênio. Além disso, os radicais livres têm sido reconhecidos como moléculas que possuem um papel modulador na plasticidade sináptica, todavia, com um efeito deletério no processo de envelhecimento, aumentando o

número de mitocôndrias não-funcionais e diminuindo a plasticidade sináptica. Sabe-se, porém, que uma concentração moderada de radicais livres pode facilitar a memória através da ativação de cinases, enzimas que promovem a fosforilação protéica, o que favorece a memória de longo prazo. Desta forma, pode-se postular que a exposição de organismos a compostos como os polifenóis pode promover um aumento nas defesas antioxidantes, como também modular processos de memória nos organismos tratados com este tipo de compostos (Cadenas e Packet, 2002). Estudos têm demonstrado que a quercetina e outras substâncias polifenólicas podem inibir a ecto-5'-nucleotidase/ CD73 (Kavutcu e Melzig, 1999). Além disso, recentes estudos têm demonstrado que a quercetina tem um efeito antiproliferativo na linhagem de gliomas U138MG, provavelmente devido ao seu efeito inibitório sobre a atividade da 5'-nucleotidase (Braganhol *et al*., 2007). Muitas evidências sugerem o papel neuroprotetor dos flavonóides (Youdim *et al*., 2002), mas os mecanismos de ação destes compostos são complexos e envolvem muitos fatores (Zhang *et al*., 2008). Sabe-se que eles apresentam ação no sistema nervoso central, visto que possuem a capacidade de atravessar a barreira hemato-encefálica (Youdim *et al*., 2004). Vários estudos recentes mostram a relação da ingesta de polifenóis com o tratamento dos sintomas da doença de Alzheimer, já que vários polifenóis se mostram importantes agentes anticolinérgicos. Entre eles está o trabalho de Zhang *et al*., (2008), que demonstrou que a fração solúvel em água do própolis apresenta propriedades anticolinérgicas, antiinflamatórias e positivas em relação a prevenir a redução do déficit cognitivo e perda de memória associada a doenças neurodegenerativas.

2. OBJETIVOS

2.1. OBJETIVO GERAL

Avaliar o potencial neuroprotetor preventivo dos polifenóis quercetina e rutina seguido de indução de déficits cognitivos através da utilização de escopolamina. Esta avaliação se dará através da análise das enzimas envolvidas no controle da sinalização purinérgica e colinérgica em cérebro de peixe zebra em nível enzimático, além da avaliação de parâmetros comportamentais, como memória e locomoção.

2.2. OBJETIVOS ESPECÍFICOS

- Estabelecer o modelo de indução de déficit cognitivo associado ao tratamento com escopolamina em peixe zebra;

- Verificar o efeito *in vivo* do tratamento com escopolamina, do tratamento com os compostos polifenólicos (quercetina e rutina) e do tratamento com escopolamina associado ao tratamento com os compostos polifenólicos (quercetina e rutina) sobre as atividades de degradação de ATP, ADP e AMP e ACh em cérebro de peixe zebra.

- Avaliar parâmetros comportamentais de peixe zebra, como memória e atividade locomotora após tratamento com escopolamina e com os compostos polifenólicos (quercetina e rutina) isolados e em associação a tratamento com escopolamina.

CAPITULO 2 - ARTIGO CIENTÍFICO

RICHETTI, S.K., BLANK, M., CAPIOTTI K. M., PIATO A.L., BOGO, M.R., VIANNA., M.R., BONAN, C.D. Quercetin and rutin prevent scopolamine-induced memory impairment in zebrafish (*Danio rerio* Hamilton 1822).

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Quercetin and rutin prevent scopolamine-induced memory impairment in zebrafish (*Danio rerio* Hamilton 1822)

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Abstract

Demographic aging gives rise to a growing population with age-associated behavioral and cognitive deficits that may be associated at least partially to the increasing prevalence of neurodegenerative disorders, such as Alzheimer's disease. The aim of our study was to investigate the potential protective role of quercetin and rutin against scopolamine-induced inhibitory avoidance memory deficits in zebrafish. Scopolamine (200 uM dissolved in the tank water for one hour) given pre-training hindered memory formation while both quercetin and rutin pretreatments (50 mg/kg, single injection, i.p.) prevented the scopolamine induced amnesia. None of the compounds affected zebrafish general locomotor activity. Together, these results contribute to the increase of the knowledge about plant compounds applicability as medicines to prevent and treat neurodegenerative diseases, like Alzheimer´s disease.

Keywords: Quercetin; rutin; acetylcholinesterase; zebrafish; Alzheimer's disease; polyphenols; scopolamine.

1. Introduction

Aging in humans is accompanied by structural and neurophysiological changes in the brain and variable degrees of cognitive decline. A central issue arises when these behavioral and cognitive deficits are accompanied with severe dementia, impacting individuals and their caring personal life quality. Demographic aging, in combination with population increasing life expectancies [30], favors the manifestation of neurodegenerative diseases since the best known risk factor for age-related diseases, including Alzheimer's disease (AD), is aging itself [2].

The Alzheimer's disease is a multifactorial disorder with a complex combination of genetic and non-genetic components[43, 52], whereas the non-genetic or sporadic form represents the majority of the cases [52] and involves inflammation, glutamatergic toxicity, mitochondrial and proteassomal dysfunction, the activation of apoptosis pathways and oxidative stress [34, 37, 44]. Despite these components, AD patients experience disturbances in various neurotransmitter systems. Although deficits in glutamatergic, serotoninergic, and catecholaminergic systems are common in this pathology, functional deficits in the cholinergic system are strongly related to AD symptoms [7,18, 21, 27]. These changes include a reduction in acetylcholine (ACh) production, leading to a decreased availability of ACh at the neuronal synapse [5, 12, 28]. This reduction is believed to contribute to memory decline characteristic of AD. Furthermore, AD patients also present an augmented clearance of acetylcholine in the synaptic cleft, as a result of an increase in the enzymatic activity of the protein responsible for the hydrolysis of this neurotransmitter, acetylcholinesterase (AChE) [46].

These findings resulted in the establishment of the AD cholinergic hypothesis, in 1982 by Raymond T. Bartus [3].

This hypothesis was the basis for the development of drugs prescribed until today for AD treatment, called acetylcholinesterase inhibitors (ChEi), which prolong the action of acetylcholine in the synapse by preventing its breakdown. This strategy results in improvements of cognition, mood and behavior [36]. Even though there are modest benefits following the treatment with ChEi to the progression of AD, its use is limited because of the occurrence of severe side effects [9]. The identification of effective treatments will require a better understanding of the physiological mechanisms involved and the development of multi-targeted drugs as a result of innovative approaches [37].

There is a growing interest in the potential of phytochemicals to improve memory, learning and general cognitive ability [43, 48]. There is a large amount of excellent reviews highlighting the effects of phytochemicals as modulators of brain function [53-56, 59]. The main dietary sources of these phytochemicals are fruits, vegetables and plant-derived beverages such as tea and red wine [for a complete review see 52] This wide class of phytochemicals includes the polyphenols, the most abundant dietary antioxidants [29]. Some studies already reported a possible relationship between polyphenol ingestion and the prevention of AD [25, 52]. The polyphenols are a large group of compounds that can be divided into several subgroups, including the flavonoids. Two of the flavonoids most widely and abundantly present in herbs and plants consumed by men are quercetin and rutin [47, 52].

Quercetin has been reported to exert numerous pharmacological activities, such as free radical scavenging [24, 55, 56] and anticarcinogenic effects [8]. In addition,

quercetin has the potential to bind to the ATP-binding sites of a large number of proteins [10]. Studies have suggested that quercetin and other polyphenolic substances can inhibit ecto-5- NT/CD73 activity [26]. These actions can affect the cell function by altering the phosphorylation state of target molecules and/or by modulating gene expression [64]. In addition, rutin, studied since 1946, is a quercetin glycoside which has been reported to increase the scavenge of free radicals [24, 38, 39, 51], vascular resistance [50], decrease hepatic and blood cholesterol levels, and also shows antiplatelet activity [38, 51].

Therefore, considering that: (i) AD is a multifactorial disease which prevalence is expected to increase throughout the years (ii) available treatments present modest cognitive benefits but severe side effects, a fact that claims for the development of other strategies to treat this neurodegenerative disease (iii) recent studies suggest the multitargeted action of polyphenols and, finally (iv) polyphenols are natural compounds that do not present collateral effects, we sought to investigate the effects of acute quercetin and rutin treatment in a model of pharmacological cognitive impairment achieved with scopolamine, an antimuscarinic drug, in zebrafish.

2. Materials and methods

2.1 *Animals*

Wild-type adult (>8 months old) zebrafish of both sexes were obtained from specialized supplier (Redfish Agroloja, RS, Brazil). Animals were kept in 50L housing tanks with tap water previously treated with Tetra's AquaSafe® (to neutralize chlorine, chloramines, and heavy metals present in the water that could be harmful to fish) and continuously

aerated (7.20mgO2/l) at 25 ± 2ºC, under a 14-10 h light/dark photoperiod at a density of up to five animals per liter. Animals were acclimated for at least 2 weeks before the experiments. They were fed three times a day with TetraMin Tropical Flake fish®. The procedures were previously approved by the Animal Ethics Committee of Pontifical Catholic University of the Rio Grande do Sul (PUCRS) under the number 109/09-CEUA.

2.2 *Chemicals*

Quercetin ($C_{15}H_{10}O_7$, CAS number 117-39-5), Rutin hydrate ($C_{27}H_{30}O_{16} \cdot H_2O$, CAS number 207671-50-9), (−)-Scopolamine hydrobromide trihydrate ($C_{17}H_{21}NO_4 \cdot HBr \cdot 3H_2O$, CAS number 6533-68-2), Benzocaine ($C_9H_{11}NO_2$, CAS number 94-09-7), Tween 20 ($C_{58}H_{114}O_{26}$ CAS number 9005-64-5) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). All other reagents used were of analytical grade.

2.3. Animal procedures

Quercetin and Rutin were suspended in Tween 20 (1%) in saline. The doses of the polyphenols were chosen based on [42], which have shown that the i.p. treatment with quercetin and rutin at 50mg/kg reduced the spatial memory impairment and neuronal death induced by repeated cerebral ischemia in rats. The drugs were administered intraperitoneally (i.p.) in a volume of 20 mL/kg body weight (mean injection volume was 10 μL). I.p. injections were conducted using a 3/10-ml U-100 BD Ultra-Fine™ Short

Insulin Syringe 8mm (5/16”) x 31G Short Needle (Becton Dickinson and Company, New Jersey, USA) according to the protocol established by Phelps, Runft and Neely [40]. Briefly, anesthesia of the animals prior to the injection was obtained by its immersion in a Benzocaine solution (1 mM in MeOH 1%) until the animal shows lack of motor coordination and reduced respiration rate. The anesthetized animal was gently put in a water-soaked gauze-wrapped hemostat with the abdomen facing up and the head of the fish positioned at the hinge of the hemostat (the pectoral fins were used as a landmark on the abdomen). The needle was inserted parallel to the spine into the midline of the abdomen posterior to the pectoral fins. The injection procedure was conducted to guarantee that the animal do not spend more than 10 seconds out of the water. After the injection the animals were placed in a separate tank with highly aerated unchlorinated tap water (25 ± 2ºC) to facilitate the animals recovery from the anesthesia. Quercetin and rutin were injected two hours before the beginning of experiment. One hour before the beginning of the training session, the animals were transferred another tank to receive the second treatment, consisted of the scopolamine treatment (200 μM dissolved in the water for one hour as described by [29]). The animals that did not receive scopolamine were also transferred to another tank filled with water to ensure the homogeneity of stress presented to the fish. After the training the animals were transferred to another empty tank equal to the previous one. Tween 20 was used as control. Both drugs and vehicle were prepared freshly in the experimental day. All the animals have recovered after 2-3 minutes following the injection. Animals that did not recover during this period were discarded.

2.4. Behavioral analysis:

2.4.1 Inhibitory avoidance:

Zebrafish were individually trained and tested for long-term memory in the inhibitory avoidance paradigm as described in detail by [6]. Briefly, an 18 cm L x 9 cm W x 7 cm H glass tank divided in two equally sized compartments, designated hereon as dark and white, by a sliding guillotine-type partition (9 cm x 7 cm) was used. The tank water level was 3 cm and the partition raised 1 cm above the tank floor to allow zebrafish to swim freely from one side of the tank to the other. Two electrodes extending through the wall height and placed on each end side of the dark walls attached to an 8 V stimulator administered a final 3 ± 0.2 V AC shock when manually activated. On training session, animals were placed in the white side of the tank while the partition between compartments was closed. After 1 min of familiarization with the new environment the partition was raised, allowing fish to cross to the dark side of the tank. When animals entered the dark side with their entire body the sliding partition was closed and a pulsed electric shock administered for 5 seconds. Fish were then removed from the apparatus and placed in the dedicated temporary tank. Animals were tested 24 hr after training. The test session repeated the training protocol except that no shock was administered and animals immediately removed from the dark compartment. The latency to completely enter the dark compartment was measured on both sessions and the test latencies used as an index of retention. The saline and Tween 20 control groups did not differ on any measure; therefore, the saline treated-groups were excluded from the graphic representation of the results. Tween 20 will be referred to as the control group.

2.4.2 Exploratory assessment

Behavioral testing of drug effects took place during the light phase between 10:00 a.m. and 12:00 a.m. The behavioral screening test was performed thirty minutes after the injection (saline, Tween 20, quercetin or rutin) or after the beginning of the second drug treatment (water or scopolamine), on the inhibitory avoidance training day. The animals were individually transferred to a 2,7 L tank (24 cm L X 8 cm W X 20 cm H) with laterals and bottom white covered, except of the front to avoid any visual disturbances, and were first habituated to the tank for 30 s, as previously described [17]. There was no drug exposure during behavioral experiments. The locomotor activity of the animals was video recorded using Logitech Quickcam PRO 9000 for 5 min after the habituation period and further analyzed using the ANY-Maze recording software (Stoelting Co., Wood Dale, IL, USA). The tank was divided into two equal sections with one horizontal line, and the following behavioral patterns were measured: distance traveled, mean speed, number of line crossings (horizontal line), absolute turn angle, and time spent in upper and lower half.

2.5 *Statistical Analysis*

Inhibitory avoidance memory data are presented as median + interquartile range since a ceiling of 50s was used to each session. Training and test sessions for each group were compared by Wilcoxon matched pairs test. Training latencies were compared using by Mann-Whitney test. Data of the exploratory assessment were expressed as mean ± S.E.M. of 4 representative animals for each group and were analyzed by one-way

ANOVA test followed by Tukey test as post-test. *$p < 0.05$ denotes a significant difference from the control group.

3. Results

3.1 Scopolamine induces memory deficits in an inhibitory avoidance paradigm

Adult (>8 months old) male zebrafish long-term memory was tested in the inhibitory avoidance apparatus developed by [6]. To first establish the effectiveness of the scopolamine in the one-trial inhibitory avoidance task, we have treated the animals with scopolamine (200 μM dissolved in tank water) for one hour previous to training session (Figure 1). While water-exposed animals showed a robust retention of memory on the test session performed 24h after training when training and test latencies were compared (p = 0.0355), pre-training scopolamine hindered memory formation (p = 0.7792, comparison between training and test session of scopolamine treated animals). Importantly, no differences were found between training latencies for both groups (Mann-Whitney test). Scopolamine´s ability to induce memory deficits when given pre-training at the inhibitory avoidance has been shown in rodents and our data in zebrafish supports its effect to evaluate memory enhancing drugs.

3.2 Scopolamine deficits are prevented by quercetin and rutin treatment

The ability of quercetin and rutin to prevent scopolamine-induced inhibitory avoidance memory deficits was evaluated by combining an initial quercetin and rutin i.p. treatment with the pre-training scopolamine exposure (Figure 2). Tween 20 was used as the vehicle for quercetin and rutin and it did not differ from water being therefore considered the

standard control treatment of the experiment. Animals receiving Tween 20 (control group), quercetin or rutin only effectively learned, showing significant differences from their respective training and test sessions ($p<0.05$ for each group analyzed separately). Scopolamine pre-training again hindered memory formation, as observed in the lack of long-term memory retention when training and test sessions were compared in animals that received Tween 20 i.p. followed by scopolamine ($p = 0.2291$). Acute single pre-treatment with quercetin or rutin one hour before the beginning of scopolamine treatment prevented the memory impairment caused by scopolamine, as shown by a statistically different latency between training and test sessions for both rutin and quercetin- treated animals ($p <0.05$ for either group).

3.3 Scopolamine and polyphenols effects on exploratory assessment

Distinct parameters of zebrafish swimming activity were examined in the tank diving behavioral test. As indicated by the distance traveled, mean speed and line crossings, no differences were found in the locomotor activity of animals receiving any of the treatments when compared to the control group (Fig. 3 A, B, and C, respectively). None of the treatments have affected the swimming coordination neither the general swimming pattern of the animals, as shown by the absence of statistically different alterations in the absolute turn angle (Fig. 3 D). No differences in the time spent in the upper half or the lower half were found in treated animals in comparison with the control group (Fig. 3 E and F respectively) showing no anxiogenic properties of any treatment.

4. Discussion

In the present study, we have evaluated the potential preventive role of the flavonoids quercetin and rutin to prevent the scopolamine-induced memory deficits in zebrafish. Interestingly, we have found that one single intraperitoneal injection of 50 mg/kg of quercetin or rutin at one hour before the scopolamine treatment prevented scopolamine induced memory deficits. Scopolamine, the tropane alkaloid originally isolated from classical nightshade, such as *Atropa belladonna* L. [64] is a competitive antagonist of the muscarinic acetylcholine receptor (mAChR) [60]. Besides its use as a treatment for central nervous system dysfunctions such as motion sickness, shaking palsy and oppioid addiction [63], it became widely used as a standard/reference drug for inducing cognitive deficits in a wide range of animal models, especially after the postulation of the cholinergic hypothesis of geriatric memory dysfunction [3].

Despite the scopolamine classical use as an amnesic agent, there is a lot of discrepancy in relation to scopolamine effects in the locomotion. Some studies, in fact, challenge the viability of scopolamine use as a cognitive impairer, questioning if the alterations in behavior are related to peripheral locomotor effects, instead of memory disruption [for a review see 31]. To address this problem we have performed a general analysis on zebrafish locomotor behavior. As shown in the Fig. 3, there were no changes in none of the parameters analyzed. We have also shown that pre-exposure to scopolamine for one hour immediately before inhibitory avoidance training did not impact training performance, since control and scopolamine-treated animals training session latencies did not differ. In light of this evidence we believe that the scopolamine

induced memory deficits observed were solely due to the drug effect on the cholinergic system.

The cholinergic system is involved in many physiological processes, including synaptic plasticity and learning and memory [14, 41, 57, 62]. Cholinergic agonists can facilitate memory, whereas cholinergic antagonists can impair memory [34]. Studies of the effects on brain plasticity of cholinergic agents, particularly those engaging muscarinic receptors, have provided robust and clarifying information about learning and memory processes [23]. In addition, the cholinergic hypothesis of geriatric memory dysfunction and evidence of the involvement of this system in the etiology of AD have brought global attention to cholinergic interventions as a treatment for this disease.

In that sense, molecules that could modulate the cholinergic hipoactivity related cognitive effects have potential clinical use [13]. Cholinesterase inhibitors, such as Tacrine, Galantamine, Physostigmine and Donepezil were designed to ameliorate cholinergic deficits by slowing the rate of acetylcholine degradation after its synaptic release, but the use of these medicines is not always well accepted by the patients due to their severe side effects [9, 45], high cost and scarcity of robust benefits [7, 33].

Quercetin and rutin, natural compounds widely found in the diet, have been studied for a long time and shown to have wide physiological effects [52]. The effects of flavonoid-rich diet on cognitive function have been linked to the ability of flavonoids to interact with the cellular and molecular framework involved in learning and memory, including synaptic potentiation and plasticity [22]. Flavonoids also have known antioxidant abilities, effectively protecting neurons against neurotoxins, suppressing neuroinflammation, and enhancing neuronal function [reviewed in 61], stimulating

neuronal regeneration and revascularization [22, 52]. Flavonoids have also been reported to act as ChEi [1].

Here we have shown that these polyphenols could protect against scopolamine-induced cognitive deficits, using an inhibitory avoidance task. Aversive conditioning tasks, such as the inhibitory avoidance, have been shown useful to analyze cholinergic effects on memory, as muscarinic and nicotinic neurotransmission have been demonstrated to affect every aspect of aversive conditioning. Compelling evidence shows that cholinergic manipulations can affect memory acquisition, consolidation, and retrieval in inhibitory avoidance behavioral task, since cholinergic neurons are presented in areas engaged in learning and memory processes from this task in rodents [58].

Zebrafish (*Danio rerio*, Hamilton 1822), is a powerful animal model in many areas or biological research. Its use ranges from toxicology, developmental biology, biomedicine, neurophysiology, drug discovery [49] model for human diseases [4, 32] and behavioral analysis [6, 16, 17, 35]. In addition to the known advantages of its use, such as the small size and maintenance cost, the transparency of embryos and larvae, and the speed at which these develop *ex utero* [19, 20], zebrafish has been suited for large throughput screening for drug discovery, including those from natural sources [11, 15]. Zebrafish combines the ability to perform large scale screenings yet requiring a smaller infrastructure when compared to rodents, bringing new perspectives for the drug discovery process to yet untreatable diseases, such as Alzheimer's disease.

According to our results, quercetin and rutin administration prevented scopolamine induced memory deficits in zebrafish, suggesting that these flavonoids might be a preventive strategy against the development of AD. These findings, although

restrict to behavioral analysis, raise a new perspective to the prevention and treatment of AD. More experiments are already being conducted to investigate candidate biochemical targets of polyphenols in the scopolamine-induced memory deficits.

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Conflict of Interests

The authors declare no conflict of interests.

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Figure legends:

Figure 1: Scopolamine induces long-term memory deficits in inhibitory avoidance

Effect of scopolamine on latency to cross to dark compartment in training and long-term memory test sessions in the inhibitory avoidance task. Animals were pre-treated with scopolamine 1 hour prior to training session. Control animals were manipulated similarly but exposed to water only. Data are presented as median + interquartile range (N = 7 per group). * indicates $p < 0.05$ between training and test sessions compared by Wilcoxon matched pairs test. No differences were found between training latencies for both groups (Mann-Whitney test).

Figure 2: Scopolamine induced cognitive deficits are prevented by quercetin and rutin

Effects of scopolamine on latencies to enter the dark compartment for animals trained on the inhibitory avoidance task. T, tween treated animals, Q, quercetin treated animals, R, Rutin treated animals. Animals received single intraperitoneal injection of Tween 20 (1%), quercetin or rutin (50 mg/kg) two hours before the training session. The i.p. treatment was combined by water or scopolamine 1h exposure prior to test. Control animals were manipulated as equal as the treated animals, except that they received water instead scopolamine. Data are presented as median + interquartile range (N > 7 per group). * indicates $p < 0.05$ between training and test sessions for each group compared by Wilcoxon matched pairs test. No differences were found between training latencies for

both groups (Mann-Whitney test). # Comparison between test sessions in relation to the Tween 20 test session latency.

Figure 3: Neither scopolamine nor quercetin or rutin affects zebrafish locomotor activity. Effect of exposure to saline, Tween 20, quercetin and rutin, followed by water or scopolamine exposure on the distance traveled (A), mean speed (B), number of line crossings (C), absolute turn angle (D), time spent in the upper zone (E) and in the lower zone (F) determined during 5 min of video recording in the tank diving behavioral test. SW, saline + water, SS, saline + scopolamine, TW, tween + water, TS, tween + scopolamine, QW, quercetin + water, QS, quercetin + scopolamine, RW, rutin + water, RS, rutin + scopolamine. Animals received single intraperitoneal injection of Saline, Tween 20 (1%), quercetin and rutin (50 mg/kg) two hours before the beginning of the video recording, and were transferred to the second treatment (water or scopolamine 200uM dissolved in the tank water) one hour before the video recording. Data were expressed as mean ± S.E.M. of 4 representative animals for each group and were analyzed by one-way ANOVA test. *$p < 0.05$ denotes a significant difference from the control group.

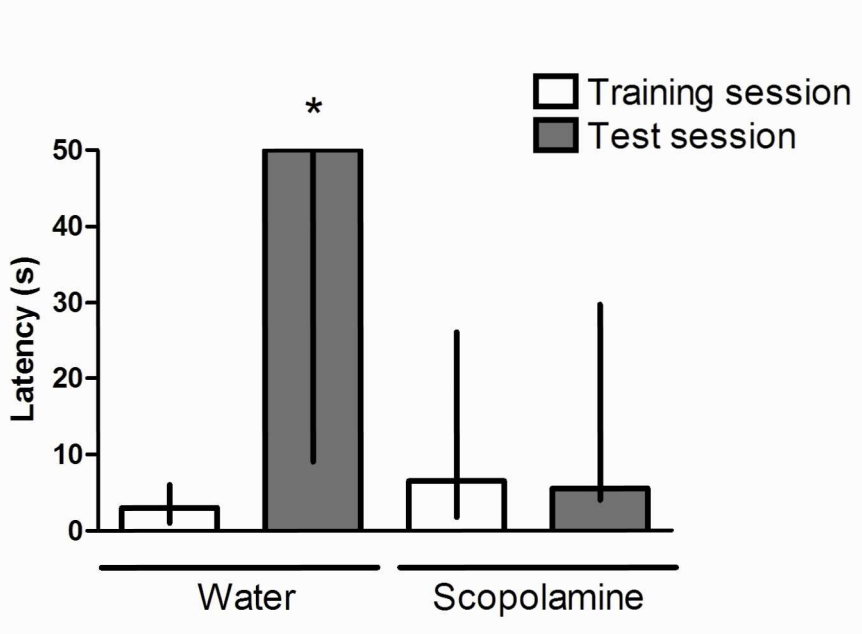

Figure 1

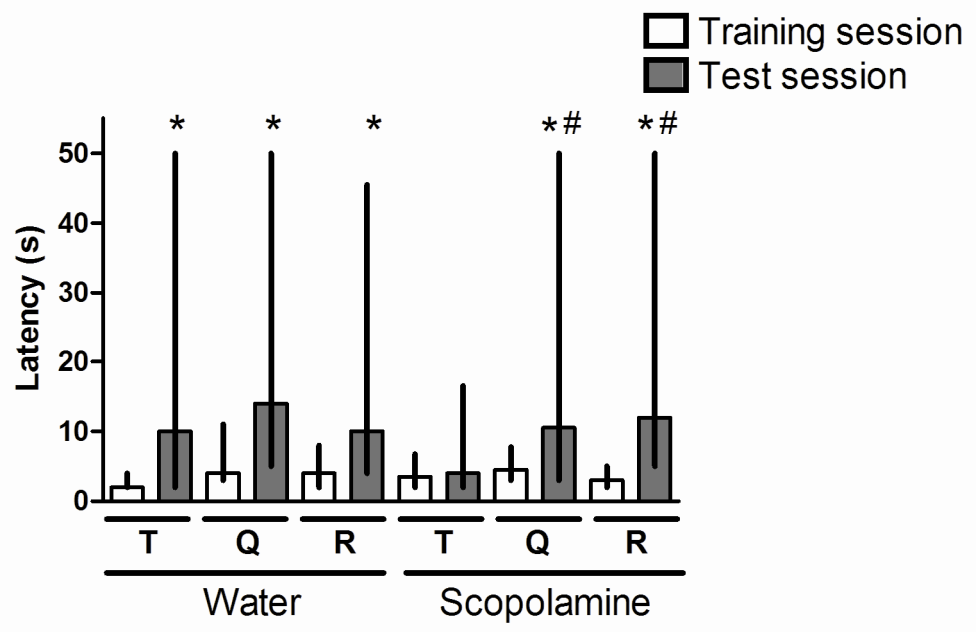

Figure 2

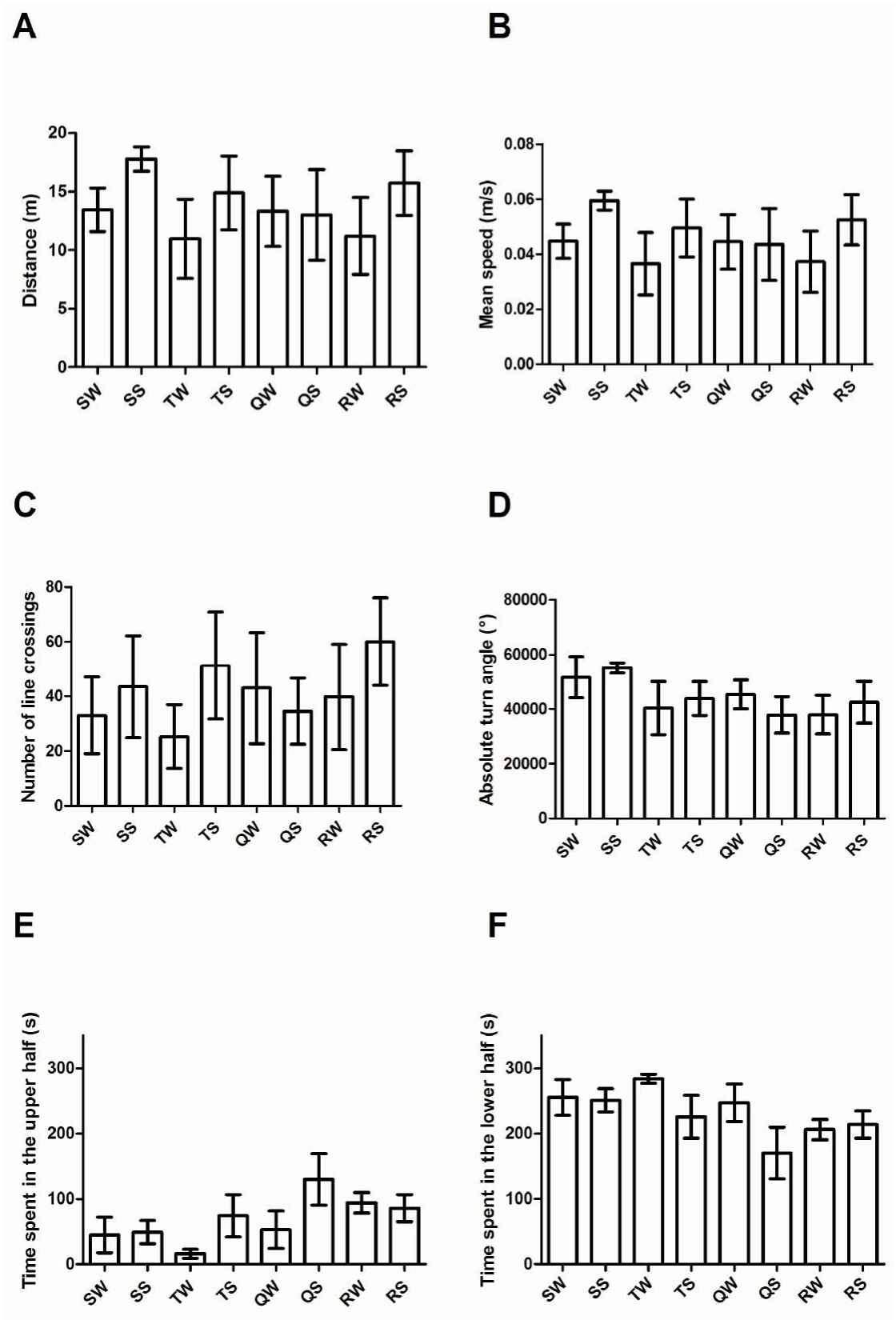
A
Distance (m)
20
15
10
5
0
SW
SS
TW
TS
QW
QS
RW
RS
B
Mean speed (m/s)
0.08
0.06
0.04
0.02
0.00
C
Number of line crossings
80
60
40
20
0
D
Absolute turn angle (°)
80000
60000
40000
20000
0
E
Time spent in the upper half (s)
300
200
100
0
F
Time spent in the lower half (s)

Figure 3

CAPITULO 3 - ARTIGO CIENTÍFICO

RICHETTI, S.K., CAPIOTTI K. M., BLANK, M., PIATO A.L., BOGO, M.R., VIANNA., M.R., BONAN, C.D. Quercetin and rutin affects acetylcholine and adenine nucleotide hydrolysis in zebrafish (*Danio rerio* Hamilton 1822)

Artigo em preparação que será submetido ao periódico

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Quercetin and rutin affects acetylcholine and adenine nucleotide hydrolysis in zebrafish (*Danio rerio* Hamilton 1822)

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Abstract

The purpose of this manuscript was to analyze the effects of the polyphenols quercetin and rutin on the ATP, ADP, AMP, and acetylcholine hydrolysis in a model of pharmacological cholinergic disruption achieved with scopolamine, an antimuscarinic drug, in zebrafish. To address this question, animals were treated with quercetin or rutin (50mg/kg, i.p. injection) followed by exposure in water or scopolamine (200 μM dissolved in the water) during one hour. Rutin treatment followed by exposure to water inhibited the acetylcholine hydrolysis (27% inhibition) whereas the treatment with rutin followed by exposure to scopolamine reduced ATP hydrolysis (48.2% inhibition). Regarding the effects of quercetin, it inhibited the AMP hydrolysis either when its administration was followed by exposure to water or scopolamine (54.4 and 50.7% inhibition respectively). Moreover, our results showed that rutin and quercetin *per se* are able to modulate the levels of acetylcholine, ATP, and adenosine in zebrafish brain. Considering the important role of adenine nucleotides and acetylcholine in learning and memory processes, the results demonstrated in this study give rise to new perspective in relation to preventive strategies to be conducted throughout the lifespan, seeking for the prevention of age-associated cognitive decline.

Keywords: Quercetin, Rutin, Acetylcholinesterase, Ectonucleotidases, Zebrafish, Alzheimer.

1. Introduction

The increase in the life expectancy, as a result of medicine and medical technologies advances, contributes to a phenomenon related to the augment in the elder population worldwide. As a consequence, many age-related diseases are expected to raise as serious public health problems. The best-known risk factor in ageing is dementia and dementia-related diseases, such as Alzheimer's disease (AD). AD is expected to commit millions of people with 65 years or more in the next decades (Wimo et al., 2003; Mount and Downton, 2006).

This multifatorial disease is a consequence of the combination of genetic and non genetic factors (Ramassamy, 2006; Singh et al., 2008). Between the genetic factors, mutations in genes related to the β-amyloid peptide synthesis pathway are involved (Ramassamy, 2006; Reddy, 2007), resulting in a disturbance of this route. The non genetic form of the disease counts for the majority of the cases. These non genetic factors include for example inflammation, glutamatergic toxicity, dysfunction of mitochondrial activity, activation of apoptotic pathways and alteration of the homeostasis of antioxidants/oxidation (Ramassamy, 2006; Reddy, 2007; Roberson and Mucke, 2006). In addition, alterations in many neurotransmission systems, such as the purinergic and cholinergic systems are common in this pathology (Cuello et al., 2010; Cunha, 2008; Stone et al., 2009; Thathiah and De Strooper, 2009).

ATP and adenosine are important endogenous signaling molecules of the purinergic system that exerts a wide range of actions. ATP, besides its energetic role, also acts as an excitatory or inhibitory neurotransmitter, as a trophic factor with effects on the cellular proliferation, as an excitotoxicity biomarker and as a component of pathologies

(Abbracchio and Burnstock, 1998). These actions are triggered by ATP binding to specific receptors called P2 receptors, subdivided in the P2X family, composed by ionotropic receptors, and the P2Y family, composed by metabotropic receptors (Burnstock, 2004, 2007, 2008; Burnstock and Knight, 2004). ATP is released in the synaptic cleft together with other neurotransmitters such as dopamine and acetylcholine (Burnstock 1999, 2004) and has its signal cleared by the action of a family of ecto-enzymes, the ectonucleotidases, which hydrolyze the neurotransmitter ATP to the neuromodulator adenosine. Adenosine is a neuromodulator that regulates neurotransmitter release in the synaptic cleft, protects organism against injury (Cunha, 2001, 2005; Fredholm, 2003) and is involved in the regulation of anxiety, sleep, memory, and cognition (El Yacoubi et al., 2000; Porkka-Heiskanen, 1999; Ribeiro et al., 2003). Adenosine acts through P1 metabotropic receptors divided in A_1, A_{2A}, A_{2B} and A_3. The A_1 and A_3 receptors are coupled to $G_{i/o}$ proteins, leading to an inhibition in cyclic AMP (cAMP) synthesis, in contrast to the A_{2A} e A_{2B} receptors, which are coupled to G_s proteins, resulting in stimulation of cAMP synthesis. Alterations in the purinergic system are common in neurodegenerative diseases, such as AD (For a review see Burnstock 2008; Jenner et al., 2009; Rosso et al., 2008).

In the cholinergic system, the signaling molecule acetylcholine acts in the synaptic cleft by its binding to nicotinic receptors (nAChr), which are ionotropic and the muscarinic receptors (mAChr), which are metabotropic coupled to different G proteins and are strongly related to learning and memory (Anagnostaras et al., 2003; Picciotto et al., 1998). Acetylcholine clearance and the control of its signal are performed by the enzyme acetylcholinesterase (AChE) in the synaptic cleft. Alzheimer's disease patients

present an augmented clearance of acetylcholine in the synaptic cleft, as a result of an increase in AChE activity (Roger et al., 2004). Besides that, other cholinergic alterations are common to AD patients. These alterations include a reduction in acetylcholine (ACh) production, leading to a decreased availability of ACh at the neuronal synapse (Birks, 2006; Kim et al., 2008). This reduction is believed to contribute to memory decline characteristic of AD.

Recently, there is an intense interest in the development of new strategies to treat neurodegenerative diseases. In that sense, there is a broad interest in analyzing the properties of phytochemicals, based on reportings of its ability to influence cognition and learning, enhancing existing neuronal function and stimulating neuronal regeneration (reviewed in Spencer 2008, 2009). The polyphenols, a group of phytochemicals are the most abundant dietary antioxidants (Kim et al., 2010). Some studies already reported a possible relationship between polyphenol ingestion and the prevention of AD (Weinreb et al., 2004). The polyphenols are large group that can be divided into several subgroups, including the flavonoids. Two of the flavonoids most widely and abundantly present in herbs and plant foods are quercetin and rutin (Singh et al., 2008).

Therefore, considering that AD is a multifactorial disease with alterations in purinergic and cholinergic neurotransmission systems and recent studies suggest the multitargeted actions of polyphenols, the aim of this study was to investigate the effects of acute quercetin and rutin treatment in the hydrolysis of ATP, ADP, AMP, and ACh in a model of pharmacological cholinergic disruption achieved with scopolamine, an antimuscarinic drug, in zebrafish.

2. Materials and methods

2.1 *Animals*

Adult (>8 months old) and healthy zebrafish of both sexes were obtained from specialized supplier (Redfish Agroloja, RS, Brazil) and were of genetically heterogeneous (randomly bred) stock. Animals were kept in standard conditions (tap water treated with Tetra's AquaSafe® to neutralize chlorine, chloramines, and heavy metals present in the water that could be harmful to fish) continuously aerated water (7.20mgO2/l , 25 ± 2ºC, under a 14-10 h light/dark cycle photoperiod) in 50L housing tank and a density of up to five animals per liter (Westerfield, 2007) for at least 2 weeks to acclimate before the experiments. The animals were maintained healthy and free of any signs of disease, according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996) and fed three times a day with TetraMin Tropical Flake fish. The Ethics Committee of Pontifical Catholic University of the Rio Grande do Sul (PUCRS) approved the protocol under the number 109/09- CEUA.

2.2 *Chemicals*

Quercetin ($C_{15}H_{10}O_7$, CAS number 117-39-5), Rutin hydrate ($C_{27}H_{30}O_{16} \cdot H_2O$, CAS number 207671-50-9), (−)-Scopolamine hydrobromide trihydrate ($C_{17}H_{21}NO_4 \cdot HBr \cdot 3H_2O$, CAS number 6533-68-2), Benzocaine ($C_9H_{11}NO_2$, CAS number 94-09-7), Tween 20 ($C_{58}H_{114}O_{26}$ CAS number 9005-64-5), Trizma Base, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin, acetylthiocholine, 5,5′ -dithiobis-2-nitrobenzoic acid (DNTB) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). KCl was purchased from Labsynth (Brazil). $MgCl_2$ was purchased from Isofar and Vetec (Brazil) respectively. All other reagents used were of analytical grade.

2.3. Animal procedures

Quercetin and Rutin were suspended in Tween 20 (1%). The doses of the polyphenols were chosen based on Pu et al., 2007, which has shown that the i.p. treatment with quercetin and rutin at 50mg/kg reduced the spatial memory impairment and neuronal death induced by repeated cerebral ischemia in rats. The drugs were administered intraperitoneally (i.p.) in a volume of 20 mL/kg (mean injection volume was 10 μL) body weight. I.p. injection was conducted using a 3/10-ml U-100 BD Ultra-Fine™ Short Insulin Syringe 8mm (5/16") x 31G Short Needle (Becton Dickinson and Company, New Jersey, USA) according to the protocol established previously (Phelps et al., 2009). Briefly, animal's anesthesia prior to the injection was obtained by its immersion in a benzocaine solution (1 mM in MeOH 1%) until the animal shows lack of motor coordination and reduced respiration rate. The anesthetized animal was gently put in a gauze-wrapped hemostat with the abdomen facing up and the head of the fish positioned

at the hinge of the hemostat (the pectoral fins were used as a landmark on the abdomen). The needle was inserted parallel to the spine into the midline of the abdomen posterior to the pectoral fins. The injection procedure was conducted to guarantee that the animal don't spend more than 10 seconds out of the water. After the injection the animals were placed in a separate tank with highly aerated unchlorinated tap water (25 ± 2ºC) to facilitate the animals recovery from the anesthesia. Quercetin and rutin were injected two hours before the euthanasia. One hour before the euthanasia, the animals were transferred another tank to receive the second treatment, consisted of the scopolamine treatment (200 μM dissolved in the water for one hour as described by Kim et al., 2010). The animals that did not receive scopolamine were also transferred to another tank filled with water to ensure the homogeneity of stress presented to the fish. Tween 20 was used as control. Both drugs and vehicle were prepared freshly in the experimental day. All the animals have recovered after 2-3 minutes following the injection. Animals that did not recover during this period were discarded.

2.4 *In vitro assays*

Scopolamine (50-500 μM) was added to the reaction medium before the pre-incubation with the enzyme and maintained throughout the enzymatic assay. Control group was performed with no addition of scopolamine in the enzyme assay.

2.5 *Determination of AChE activity*

Zebrafish brains were homogenized on ice in 60 vol (v/w) of 0.05 M Tris-HCL, pH 8.0, using a Teflon-glass homogenizer. AChE activity was determined according the method of Ellmann et al., (1961) with minor modifications. Briefly, the activity on the homogenate was measured by determining the rate of hydrolysis of acetilthiocholine iodide (ACSCh, 0.88 mM) in a final volume of 300 μL, with 33μL of 100 mM phosphate buffer, pH 7.5, and 2 mM DTNB. In this solution, 5 μg of protein of each sample were added and pre-incubated at 25ºC for 10 min. The reaction was started with the addition of the substrate acetylthiocholine, and as soon as the substrate was added the hydrolysis and the formation of the dianion of DTNB were analyzed in 412 nM for 2.5 min (in intervals of 30 s) using a microplate reader. AChE activity was expressed as micromole of thiocholine (SCh) released per hour per milligram of protein. To ensure the consistence of our results, the tests were performed at least in triplicates.

2.6 *Determination of ATP, ADP and AMP hydrolysis:*

2.6.1 *Membrane preparation*

The preparation of brain membranes was performed as described previously by Barnes et al. (1993). Zebrafish were euthanized by decapitation, their brains were removed from the cranial skull by the dissection technique. For each sample (membrane preparation), a pool of fifteen zebrafish brains was used for *in vitro* experiments, and five for *in vivo* experiments. Zebrafish brains were briefly homogenized in 60 volumes (v/w) of chilled Tris–citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) in a motor driven Teflon–glass homogenizer. The samples were centrifuged at 1000 x g for

10 min and the pellet was discarded. The supernatant was centrifuged for 25 min at 40000 x g. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris–citrate buffer, and centrifuged for 20 min at 40000 x g. This fresh-thaw-wash procedure was used to promote the lysis of the brain membranes. The final pellet was resuspended and used in the enzyme assays. All samples were maintained at 2–4 ºC.

2.6.2 *Enzyme assays*

The conditions of NTPDase and 5′-nucleotidase assays were performed as described previously (Rico et al., 2003, Senger et al., 2004). Zebrafish brain membranes (3–10 μg protein) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for the NTPDase activity) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for the 5′-nucleotidase activity) in a final volume of 200 μL. The samples were preincubated for 10 min at 37 °C and the reaction was initiated by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was stopped by the addition of trichloroacetic acid in a final concentration of 5% and the samples were chilled on ice for 10 min. Samples were then removed and it was added 1 ml of a mixture containing 2.3% polyvinyl alcohol, 5.7% ammonium molybdate and 0.08% Malachite Green in order to determine the inorganic phosphate released (Pi) (Chan et al., 1986). Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid were used to correct non-enzymatic hydrolysis of the substrates. Incubation times and protein concentrations were chosen in order to ensure the linearity

of the reactions. Specific activity was expressed as nanomol of Pi released per minute per milligram of protein. All enzyme assays were run at least in triplicate.

2.7 *Protein determination*

Protein was measured by the Coomassie Blue method considering bovine serum albumin as standard (Bradford, 1976).

2.8 *Statistical Analysis*

Data were expressed as means ± S.E.M. and analyzed by one-way analysis of variance (ANOVA), following the post-hoc test of Tukey, considering $P < 0.05$ as significant. Before ANOVA analysis, its assumptions (normality and variances homogeneity) were checked.

3. Results

In vitro effects of scopolamine treatment on AChE activity and on ATP, ADP and AMP hydrolysis.

To analyze possible effects of scopolamine on the hydrolysis of ACh, ATP, ADP, and AMP we have performed *in vitro* experiments, investigating a wide range of scopolamine concentrations (50-500 μM). As shown by Figure 1, scopolamine *in vitro* was not able to change neither in ACh (A), ATP (B), ADP (C) nor AMP (D) hydrolysis.

In vivo effects of scopolamine and polyphenols treatment on the AChE activity.

As shown by Figure 2, the effects of scopolamine and polyphenols treatment were investigated on AChE activity in zebrafish brain. Rutin treatment reduced the AChE activity in 27% ($P>0.05$) when compared to the Tween 20 + water group. This effect was absent when rutin treatment was followed by scopolamine treatment. Quercetin did not alter the enzyme activity neither when administered alone nor in combination with scopolamine treatment.

In vivo effects of scopolamine and polyphenols treatment on the ATP, ADP and AMP hydrolysis

In Figure 3 we have analyzed the effects of the treatment with polyphenols and scopolamine on the ATP, ADP, and AMP hydrolysis in zebrafish brain. According to the Figure 3A, rutin was able to strongly inhibit the ATPase activity (48.2% inhibition, p

<0.05). In Figure 3B, we can see that Tween 20 + scopolamine and quercetin + scopolamine have shown an inhibition of the ADPase activity (38.5 and 45% inhibition respectively, $p < 0.05$) in comparison with the saline + scopolamine-treated group. Since Tween 20, used as the vehicle, altered the activity of the enzyme per se, we cannot exclude the possibility that the effect attributed to quercetin is in fact a consequence of Tween 20 actions. Finally, in Figure 3C, we observe the effect of the treatment with polyphenols and scopolamine on the AMP hydrolysis. As shown, quercetin inhibited the AMPase activity when administered in combination with water or scopolamine (54.4 and 50.7% inhibition respectively, $p < 0.05$) in comparison with saline + water and saline + scopolamine groups, respectively.

4. Discussion

In the present study, we have evaluated the effects of scopolamine, quercetin, and rutin on ACh, ATP, ADP, and AMP hydrolysis in zebrafish brain. To first understand if scopolamine could influence directly to the enzymes analyzed, we have performed *in vitro* experiments, and according to our results scopolamine does not affect any of the enzymes evaluated, suggesting that the scopolamine effects on memory are due to alterations in signaling cascades that involve other targets than the enzymes investigated here.

In addition, we have found that the polyphenols have different effects in the enzymes investigated. Many studies describe the relation between plant extracts ingestion and the treatment of Alzheimer's disease symptoms, since a great portion of these extracts presents beneficial characteristics, including anticholinergic properties (Adams et al., 2007; Houghton and Howes, 2005; Howes et al., 2003; Loizzo et al., 2008; Marchalant et al., 2008; Mukherjee et al., 2007; Perry et al., 1998; Zhang et al., 2006). Considering the reports of the plant extracts anticholinergic properties, as well as the great importance to an AD drug to have anticholinergic properties, we have investigated the effects of rutin and quercetin on the AChE activity. As shown, rutin reduced this enzymatic activity when administered alone, but this effect was not visible when rutin was administered followed by scopolamine treatment. Quercetin does not show any effects on AChE activity.

Purine derived nucleotides and nucleosides are widely recognized as signaling molecules (Burnstock e Knight, 2004; Dunwiddie e Masino, 2001; Ralevic e Burnstock,

1998) in peripheral and central nervous system, affecting several physiological and pathological processes, including cognition and memory (Ribeiro et al., 2003). Adenosine is considered a neuromodulator in the central nervous system (Williams, 1989) and acts by depressing cholinergic, noradrenergic and GABAergic transmission via adenosine A_1 receptors (Phillis and Kostopoulos, 1975; Hollins and Stone, 1980). Considering that the cholinergic system is involved in learning and memory processes (Bartus et al., 1982), a reduction in adenosine levels could lead to a decrease in A_1 activation, resulting in an attenuation of the depression of cholinergic transmission, and thereby promoting an enhancement in cognition. In addition, there is evidence on the ability of the nonselective adenosine receptor antagonist, caffeine, to protect against cognitive impairment in different animal models, an effect that mainly seems to involve adenosine A_{2A} receptors (for review, see Cunha, 2008b; Takahashi et al., 2008). Also, caffeine consumption inversely correlates with the incidence of AD (Maia and de Mendonca, 2002) and prevents memory impairment in animal models of AD (Arendash et al., 2006; Dall'Igna et al., 2007). Additionally, Canas et al., (2009) have shown that A_{2A} blockade prevents Aβ1-42-induced synaptotoxicity and subsequent memory dysfunction by a mechanism involving the control of the p38 mitogen-activated protein kinase (MAPK) pathway. This evidence suggests that a reduction in the adenosinergic signaling would have beneficial effects on cognition. In this study we show that rutin treatment has reduced the ATP hydrolysis and that quercetin treatment has induced a decrease in AMP hydrolysis. Considering that the extracellular nucleotide hydrolysis is an important pathway t for the production of adenosine, the results presented here can suggest that the reduction of ATP

and AMP hydrolysis rate could be a protective strategy to avoid effects of adenosine by reducing the activation of A_1 and A_{2A} receptors.

In summary, we have shown that the polyphenols quercetin and rutin are capable to modulate per se the levels of the signaling molecules, such as ACh, ATP, and adenosine in zebrafish brain. Considering the important role of adenine nucleotides and acetylcholine in learning and memory processes, the results demonstrated in this study give rise to a new perspective in relation to preventive strategies to be conducted throughout the lifespan, seeking for the prevention of cognitive decline age-associated.

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Conflict of Interests

The authors declare no conflict of interests.

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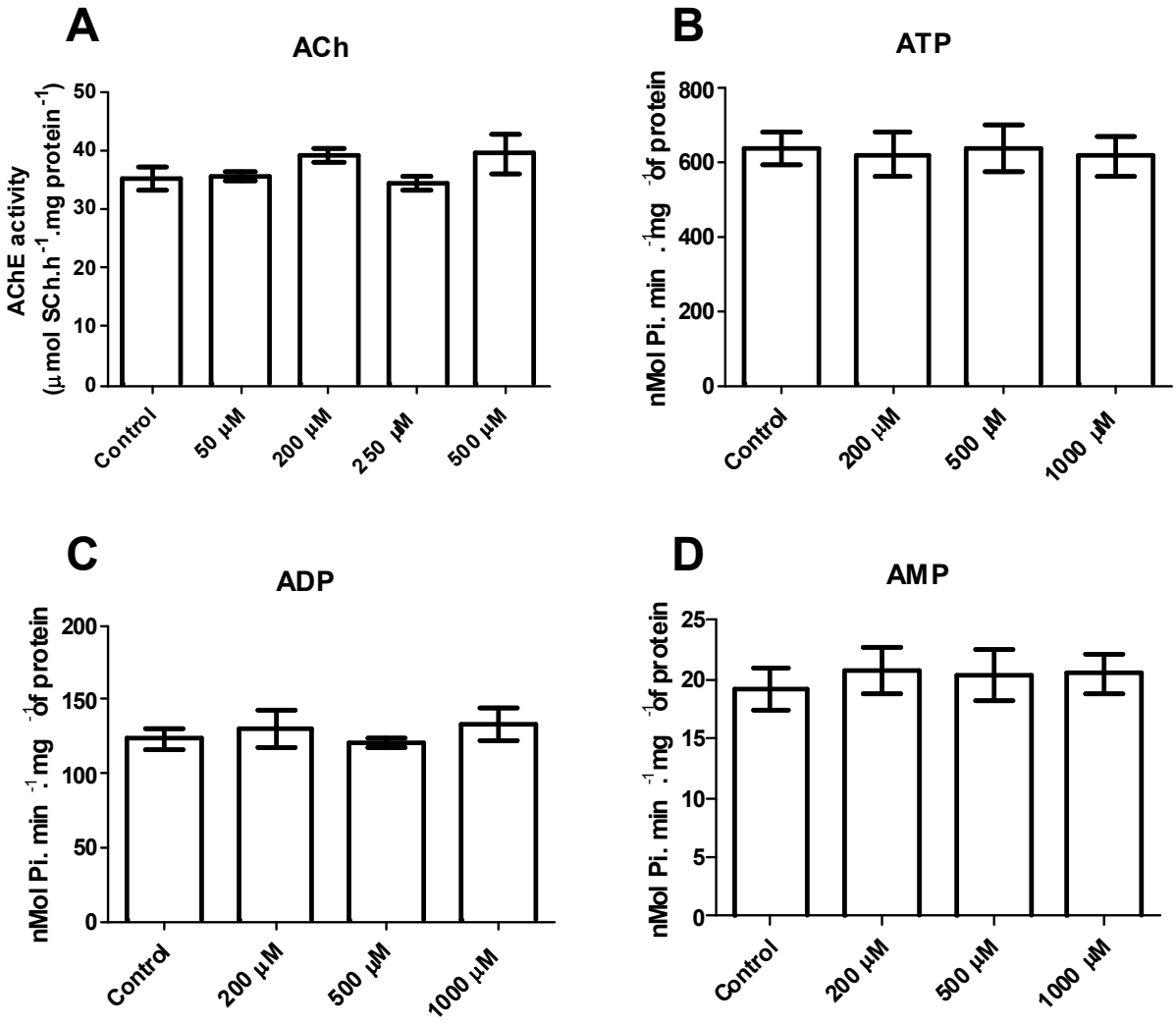
Figure Legends

Figure 1: *In vitro* effect of scopolamine on ACh (A), ATP (B), ADP (C) and AMP hydrolysis evaluated in different concentrations (50–1000 μM). Bars represent mean ± S.E. of at least four independent experiments. *Significantly different from control group (without scopolamine added). (ANOVA, followed by Dunnett's Multiple Comparison Test as post-hoc, $P \leq 0.05$).

Figure 2. *In vivo* effect of different treatments (SW, saline + water; SS, saline + scopolamine; TW, tween + water; TS, tween + scopolamine; QW, quercetin + water; QS, quercetin + scopolamine; RW, rutin + water; RS, rutin + scopolamine) in ACh hydrolysis in zebrafish brain. Animals received single intraperitoneal injection of saline, tween 20 (1%), quercetin and rutin (50 mg/kg) two hours before the euthanasia, and were transferred to the second treatment (water or scopolamine 200μM dissolved in the tank water) one hour before the euthanasia. Data were expressed as mean ± S.E.M. of at least 4 independent experiments. Data were analyzed by one-way ANOVA test followed by Tukey´s test as post hoc. *$p < 0.05$ denotes a significant difference from the tween + water group.

Figure 3. *In vivo* effect of different treatments (SW, saline + water; SS, saline + scopolamine; TW, tween + water; TS, tween + scopolamine; QW, quercetin + water; QS, quercetin + scopolamine; RW, rutin + water; RS, rutin + scopolamine) in ATP, ADP, and AMP hydrolysis in zebrafish brain. Animals received single intraperitoneal injection of

saline, tween 20 (1%), quercetin and rutin (50 mg/kg) two hours before the euthanasia, and were transferred to the second treatment (water or scopolamine 200 μM dissolved in the tank water) one hour before the euthanasia. Data were expressed as mean ± S.E.M. of at least 4 independent experiments. Data were analyzed by one-way ANOVA test followed by Tukey´s test as post hoc. * denotes a significant difference ($p < 0.05$) from the saline + scopolamine group and # denotes a significant difference ($p < 0.05$) from the saline + water group.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AChE activity
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50 µM
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ATP
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600
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200
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Control
200 µM
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C
ADP
nMol Pi. min -1.mg -1of protein
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Figure 1

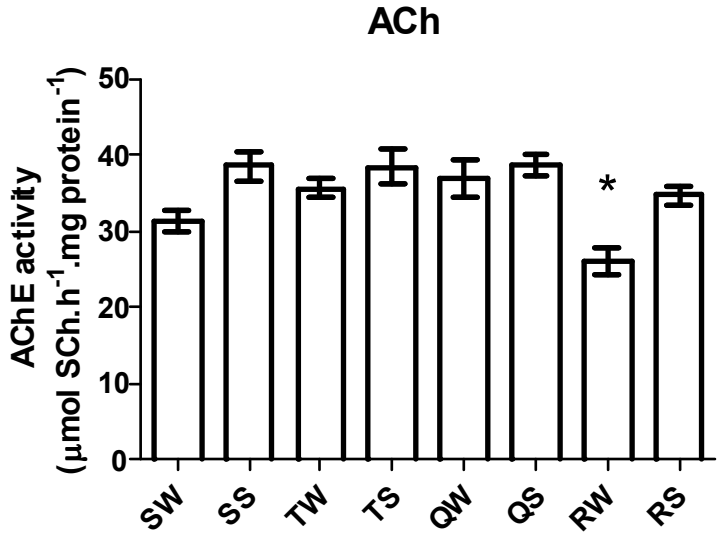
ACh
AChE activity
(µmol SCh.h-1.mg protein-1)
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Figure 2

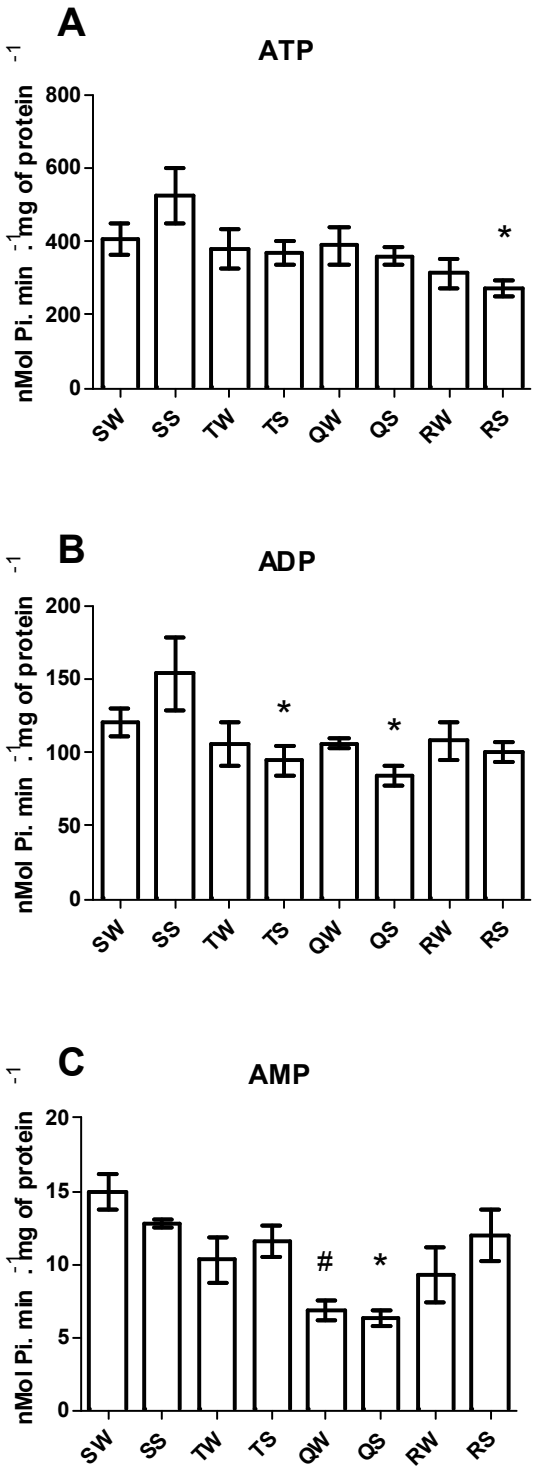
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Figure 3

CAPÍTULO 4 - CONSIDERAÇÕES FINAIS

O envelhecimento populacional observado nas estatísticas ao redor do mundo gera sérias preocupações governamentais em relação à possibilidade de concomitante aumento da incidência de déficits cognitivos e comportamentais que podem estar associados, pelo menos parcialmente com o aumento da prevalência de doenças neurodegenerativas, como a doença de Alzheimer (Kinsella e Wan, 2009). Hoje, os tratamentos eficazes estão disponíveis para muitas condições neurológicas. Entretanto, para milhões de novos pacientes que serão afetados pela Doença de Azheimer em todo o mundo (Alzheimer´s Association, 2010), a realidade parece desanimadora. Apesar de vários medicamentos já terem sido aprovados, os benefícios do seu uso são poucos. Mais de cem anos após a descoberta da doença de Alzheimer, ainda não foram estabelecidas abordagens farmacológicas com melhorias robustas. Os inibidores da colinesterase, como a tacrina, galantamina, donepezil e fisostigmina, são a principal abordagem utilizada atualmente, apesar de apresentarem modestos benefícios associados aos severos efeitos colaterais, o que contribui para reduzir a, muitas vezes já diminuta, qualidade de vida do paciente (Blennow *et al*., 2006; Casey *et al*., 2010; Maggini *et al*. 2006; Roberson e Mucke, 2006). Os polifenóis, metabólitos secundários de plantas amplamente distribuídos na natureza e na ingesta diária da população humana, apresentam diversas características que os tornam moléculas interessantes com potencial farmacológico. Entre elas, destaca-se a ausência de efeitos colaterais. Ainda, diversos estudos relacionam os efeitos dos flavonóides na função cognitiva, evidenciado pela capacidade destes compostos em interagir com os processos moleculares e celulares envolvidos na aprendizagem e memória, incluindo a potenciação sináptica e plasticidade (Harvsteen, 2002).

Considerando a necessidade do desenvolvimento de novas abordagens para o tratamento da doença de Alzheimer, o amplo repertório de alvos bioquímicos dos polifenóis e ainda, a ausência de efeitos colaterais severos relacionados ao seu uso, foi objetivo do nosso trabalho entender os mecanismos de ação dos polifenóis quercetina e rutina, bem como seus alvos bioquímicos, para avaliar a possibilidade de utilização destes compostos na profilaxia ou terapia desta doença. Quercetina e rutina, dois dos polifenóis mais prevalentes na natureza (Ross and Kasum, 2002; Singh *et al*., 2008), foram abordados nesta dissertação.

No capítulo dois, nós avaliamos o potencial papel dos polifenóis quercetina e rutina na prevenção dos deficits cognitivos induzidos por escopolamina em peixe zebra. O emprego de escopolamina tornou-se uma estratégia amplamente utilizada para induzir déficits cognitivos em uma grande variedade de modelos animais, especialmente após a postulação da hipótese colinérgica da disfunção da memória associada ao envelhecimento (Bartus *et al*., 1982). A escopolamina, o alcalóide originalmente isolado a partir de plantas como *Atropa belladonna L*. (Zhang *et al*., 2008) é um antagonista competitivo do receptor colinérgico muscarínico (mAChR) (Wang *et al*., 2003). Considerando que o sistema colinérgico está envolvido em muitos processos fisiológicos, incluindo processos cognitivos (Flood *et al*., 1981; Power, *et al*., 2003; Stratton e Petrinovich, 1963; Weinberger, 2006), agonistas colinérgicos podem facilitar a memória, enquanto os antagonistas colinérgicos pode prejudicar a memória (Mattson, 2004). Estudos dos efeitos sinápticos de agentes colinérgicos, principalmente aqueles relacionados à receptors muscarínicos contribuíram para o entendimento de processos relacionados ao aprendizado e memória (Hasselmo, 2006).

Neste estudo, surpreendentemente, apenas uma injeção intraperitoneal de quercetina ou rutina (50 mg/kg) administrada uma hora antes do tratamento com escopolamina foi suficiente para prevenir os déficits cognitivos induzidos por este fármaco. Esta rápida ação dos polifenóis pode acontecer, uma vez que a quercetina e rutina apresentam uma rápida elevação da sua concentração no plasma sanguíneo (Yang *et al*. 2005), que demonstraram que em ratos a quercetina e a rutina apresentam um pico de concentração plasmática 60 minutos após a sua administração. Tais resultados levam ao surgimento de diversas abordagens científicas, a partir de subseqüentes estudos visando confirmar a sugestão de que estes flavonóides podem ser uma estratégia preventiva contra o desenvolvimento da AD. Estes resultados, apesar de se restringirem a análise comportamental, trazem uma nova perspectiva para a prevenção e tratamento da doença de Alzheimer.

No capítulo três, representado por um artigo científico em processo de preparação, investigamos possíveis alvos bioquímicos dos polifenóis nos sistemas purinérgico e colinérgico. Tal estudo foi desenvolvido visando entender os mecanismos de ação que poderiam estar desencadeando a prevenção da perda de memória observada com o tratamento prévio com os polifenóis quercetina e rutina seguido de tratamento com escopolamina, demonstrado no capítulo 2 desta dissertação. Neste trabalho foi demonstrado que o tratamento com rutina reduziu a atividade enzimática da acetilcolinesterase, porém este efeito não foi mantido quando o tratamento com rutina foi seguido de tratamento com escopolamina. Em relação à quercetina, esta não apresentou efeito sobre a atividade enzimática da acetilcolinesterase. Vários estudos recentes mostram a relação da ingesta de polifenóis com o tratamento dos sintomas da doença de

Alzheimer, já que vários polifenóis se mostram importantes agentes anticolinérgicos. Zhang *et al*. (2008) demonstraram que a fração solúvel em água do própolis possui propriedades anticolinérgicas, apresentando resultados positivos em relação a prevenção e redução do déficit cognitivo e perda de memória associado a doenças neurodegenerativas. Ainda, neste trabalho foram avaliados os efeitos dos polifenóis sobre a hidrólise de nucleotídeos da adenina, no qual foi observado que tanto quercetina quanto rutina podem afetar a hidrólise de nucleotídeos da adenina. Os nucleosídeos e nucleotídeos derivados de purinas são amplamente reconhecidos como moléculas sinalizadoras em vários tecidos, entre eles o sistema nervoso central e periférico (Burnstock e Knight, 2004; Dunwiddie e Masino, 2001; Ralevic e Burnstock, 1998). No sistema nervoso central, estas moléculas estão envolvidas em diversos processos fisiológicos e patológicos, inclusive desempenhando papéis na cognição e memória (Ribeiro *et al*., 2003). A adenosina é considerada um neuromodulador do sistema nervoso central (Burnstock, 2009; Sebastião e Ribeiro, 2009) e age deprimindo a neurotransmissão colinérgica, noradrenergica e GABAergica via os receptores adenosinérgicos do tipo A_1 (Hollins e Stone, 1980; Phillis e Kostopoulos, 1975). Considerando que o sistema colinérgico é fortemente relacionado a processos de aprendizado e memória (Bartus *et al*., 1982), uma redução nos níveis de adenosina poderia resultar em uma redução da ativação dos receptores A_1, o que levaria a uma atenuação da depressão da transmissão colinérgica, com conseqüente aumento na cognição (Pitsikas e Borsini, 1997). Ainda, evidências demonstram a habilidade da cafeína, um antagonista não-seletivo dos receptors adenosinérgicos, de proteger o organismo contra deficits cognitivos em diversos modelos animais (Arendash *et al*.,

2006; Dall'Igna *et al*., 2007). Tal efeito é mediado principalmente pelos receptores A_{2A} (para revisão veja Cunha, 2008 e Takahashi *et al*., 2008). Ainda, já foi relatada a relação inversa entre o consumo de cafeína e a incidência de AD (Maia e De Mendonça, 2002). Canas *et al*, (2009) também demonstraram que o bloqueio de receptores A_{2A} previne a sinaptotoxicidade induzida por Aβ1-42 e a consequente perturbação na memória através de um mecanismo envolvendo o controle da via de sinalização dependente da proteína p38 tirosina quinase ativada por mitógenos (p38MAPK). Portanto, estas evidências sugerem que uma redução na sinalização adenosinérgica poderia resultar em benefícios para a cognição.

Como demonstrado no nosso estudo, o tratamento com rutina reduziu a hidrólise de ATP, enquanto o tratamento com quercetina reduziu a hidrólise de AMP. Considerando que a hidrólise destes nucleotídeos é necessária para ocorrer a formação de adenosina, os resultados demonstrados aqui podem indicar que a redução na atividade ATPásica e AMPásica pode ser uma estratégia para proteger o organismo das ações adenosina, pela redução da ativação dos receptores A_1 e A_{2A}.

Considerando dados estatísticos que demonstram o provável aumento da prevalência de doenças neurodegenerativas nas próximas décadas, a ocorrência de benefícios insatisfatórios do tratamento atualmente disponível para a AD, associado a efeitos colaterais severos, surge a necessidade de desenvolvimento de novas abordagens terapêuticas para o tratamento da doença de Alzheimer. Os resultados apresentados nesta dissertação demonstram pela primeira vez o potencial protetor dos polifenóis quercetina e rutina com relação ao prejuízo cognitivo induzido pela escopolamina em zebrafish. Além disso, nossos resultados demonstram que rutina e quercetina *per se* são capazes de

modular os níveis de acetilcolina, ATP e adenosina, moléculas sinalizadoras fortemente relacionadas a processos de cognição e memória, em encéfalo de peixe zebra. Estes resultados são promissores em relação à possibilidade de terapia preventiva a ser realizada ao longo da vida, visando a não ocorrência de declínio cognitivo associado ao envelhecimento.

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ANEXOS

ANEXO I - ARTIGO CIENTÍFICO

PROJETO PARALELO EXECUTADO PELA MESTRANDA

RICHETTI, S.K., ROSEMBERG, D.B., VENTURA-LIMA, J., MONSERRAT, J.M., BOGO, M.R., BONAN, C.D. Acetylcholinesterase activity and antioxidant capacity of zebrafish brain is altered by heavy metal exposure.

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Abstract: Pollution is a world problem with immeasurable consequences. Heavy metal compounds are frequently found as components of anthropogenic pollution. Here we evaluated the effects of the treatment with cadmium acetate, lead acetate, mercury chloride, and zinc chloride in acetylcholinesterase activity and gene expression pattern, as well as the effects of these treatments in antioxidant competence in the brain of an aquatic and well-established organism for toxicological analysis, zebrafish (Danio rerio, Cyprinidae). Mercury chloride and lead acetate promoted a significant decrease in acetylcholinesterase activity whereas they did not alter the gene expression pattern. In addition, the antioxidant competence was decreased after exposure to mercury chloride. The data presented here, together with bioinformatic analysis, allowed us to hypothesize a signal transmission impairment, through posttranslational modifications, alterations in cholinergic transmission, and also in the antioxidant competence of zebrafish brain tissue as some of the several effects elicited by these pollutants.

Cover Letter

August 6, 2010

Joan Marie Cranmer,
Editor-in-chief
Neurotoxicology,
Department of Pediatrics - Mail #512-19C, University of Arkansas for Medical Sciences, & Arkansas Children's Hospital, 11 Children's Way, Little Rock, AR 72202, USA

Dear Dr. Cranmer

We are sending for submission to Neurotoxicology the manuscript entitled **"Acetylcholinesterase activity and antioxidant capacity of zebrafish brain is altered by heavy metal exposure".** Authors: Richetti, S.K., Rosemberg, D.B., Ventura-Lima, J., Monserrat, J.M., Bogo, M.R., Bonan, C.D.

The paper contains original data and it is not been submitted for publication elsewhere. All authors approve the submission of the manuscript.

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Acetylcholinesterase activity and antioxidant capacity of zebrafish brain is altered by heavy metal exposure.

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25

Abstract

Pollution is a world problem with immeasurable consequences. Heavy metal compounds are frequently found as components of anthropogenic pollution. Here we evaluated the effects of the treatment with cadmium acetate, lead acetate, mercury chloride, and zinc chloride in acetylcholinesterase activity and gene expression pattern, as well as the effects of these treatments in antioxidant competence in the brain of an aquatic and well-established organism for toxicological analysis, zebrafish (*Danio rerio*, Cyprinidae). Mercury chloride and lead acetate promoted a significant decrease in acetylcholinesterase activity whereas they did not alter the gene expression pattern. In addition, the antioxidant competence was decreased after exposure to mercury chloride. The data presented here, together with bioinformatic analysis, allowed us to hypothesize a signal transmission impairment, through posttranslational modifications, alterations in cholinergic transmission, and also in the antioxidant competence of zebrafish brain tissue as some of the several effects elicited by these pollutants.

Keywords: Heavy metal, Pollution, Acetylcholinesterase, Zebrafish, Oxidative stress.

1. Introduction

The pollutant emissions are increasing worldwide and bringing huge health and environmental problems, particularly in aquatic milieu. The pollution is frequently composed of a mixture of heavy metals, including the three most expressive: cadmium (Cd^{2+}), lead (Pb^{2+}) and mercury (Hg^{2+}); and the second most important trace metal in the body, zinc (Zn^{2+}) (Coleman, 1992; Monnet-Tschudi et al., 2006; Vallee and Falchuk, 1981).

Cadmium is incorrectly disposed in the environment as a result of anthropogenic activities, as mining exploration, as a constituent of color pigments and re-chargeable nickel-cadmium batteries (Jarup, 2003). The cadmium effects on health are countless, and vary from kidney damage, bone effects and also many types of cancer, being classified by the IARC (International Agency for Research on Cancer) as carcinogenic to humans (Group 1) (IARC, 1993).

Another metal which the general population is exposed is the lead, being the mines, industries of glass, food bowls, and wine considered important sources of its emission. In relation to its health effects, they are well established, including its action in various neurotransmitter systems (Cory-Slechta, 1995). There is evidence of the carcinogenic potential of lead compounds, which ranks it as a compound probably carcinogenic to humans (Group 2A) (IARC, 2006).

Mercury is used since the pre-history as a pigment, passing through a cure for syphilis in the 1800´s and arriving in the present as diuretics, dental amalgam fillings, thermometers and several uses that despise its toxicity. A high intake of mercury by

humans comes together with an elevated consumption of fish. When in contact with animals, mercury can cause lung damage, neurological and psychological disturbances and also, but in a lesser extent, development of cancer, being classified by the IARC as possibly carcinogenic to humans (Group 2B) (IARC, 1993)

Although the transition metal zinc plays a neuromodulatory role in the CNS, it may be a neurotoxic agent following Zn^{2+} influx, carried out in large part through voltage-gated Ca^{2+} channels (Sheline et al., 2002). The increase of cytosolic Zn^{2+} concentrations triggers several downstream mechanisms which culminate in neuronal cell death (Cai et al., 2006). In contrast to the very low levels of free intracellular Zn^{2+}, toxic exposures to this metal strongly rises $[Zn^{2+}]i$ to 400-600 nM (Sensi et al., 1999). At this concentration, Zn^{2+} can decrease the activity of key glycolytic enzymes, such as GAPDH (Krotkiewska and Banas, 1992) and phosphofructokinase in purified forms (Ikeda et al., 1980). Studies also reported that Zn^{2+} induces mitochondrial dysfunction by inhibiting the activities of enzymes involved in electron transport, leading to an increase in neuronal reactive oxygen species and, consequently, oxidative stress (Kim et al., 1999; Manev et al., 1997; Noh et al., 1999). Furthermore, a recent data showed that Zn^{2+}-mediated neurotoxicity is dependent of intracellular NAD^{+} levels and the sirtuin activity, indicating that alterations in energy metabolic pathways could be regulated at transcriptional level (Cai et al., 2006).

The cholinergic system, with acetylcholine (ACh) as the neurotransmitter, is involved in cognitive processes, through the activation of metabotropic muscarinic and ionotropic nicotinic cholinergic receptors. The reaction responsible for the maintenance of levels of ACh is catalyzed by two cholinesterases (ChE): Acetylcholinesterase (AChE)

(E.C. 3.1.1.7) and Butirylcholinesterase (BuChE) (E.C. 3.1.1.8). Zebrafish (*Danio rerio*) is an emergent vertebrate model for studying several biological events, such as neurochemical alterations promoted by heavy metal toxicity (Senger et al., 2006). This teleost possesses only the gene for AChE, which is responsible for the whole ACh degradation, being the BuChE absent. The AChE gene was already identified, cloned and functionally detected in the zebrafish brain (Bertrand et al., 2001). Acetylcholinesterase is an important biomarker for several environmental contaminants in zebrafish (Rico et al., 2006; Senger et al., 2006). In addition, it is also known the important role of this enzyme in diseases with an increasing incidence in the elderly population, such as Alzheimer disease (Han et al., 2007, Kim et al., 2008).

There is evidence of the interaction between heavy metals as mercury and lead and the etiology of neurodegenerative diseases, since many of these metals can cross the blood brain barrier and accumulate in the brain, promoting the generation of oxidative stress and alterations in the metabolism of some proteins associated with the development of neurodegenerative diseases, such as Alzheimer disease, Parkinson disease, and Amyotrophic Lateral Sclerosis (reviewed in Monnet-Tschudi et al., 2006).

Therefore, considering the increase of the pollution, incorrect disposal of heavy metals as industrial effluents, the immersion of organisms in this impaired environment and the possible consequences of this exposure, the aim of the present study was to investigate the effects of four heavy metal compounds in AChE activity and its gene expression pattern. Furthermore, we have analyzed the effects of some of these heavy metal treatments in parameters related to antioxidant defenses and lipid peroxidation in the brain of an aquatic and well-stabilished organism for toxicological analysis, zebrafish.

2. Materials and methods

2.1 *Chemicals*

Zinc chloride ($ZnCl_2$; CAS number 7648-85-7) was purchased from Nuclear (Brazil) and cadmium acetate [$Cd(CH_3COO)_2$; CAS number 543-90-8], mercury chloride ($HgCl_2$, CAS Number 7487-94-7) and lead acetate [$Pb(CH_3COO)_2$ CAS Number 301-04-2] were purchased from QM (Brazil). Trizma Base, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin, acetylthiocholine, 5,5´ -dithiobis-2- nitrobenzoic acid (DNTB), HEPES, BHT (99%), 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (ABAP) and 1,1,3,3-tetramethoxypropane were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). KCl and SDS (90%) were purchased from Labsynth (Brazil). Tetramethoxypropane (TMP) and 2',7'-dichlorodihydrofluorescein diacetate were purchased from Acros Organics (Morris Plains, NJ, USA) and Molecular Probes Inc. (Eugene, OR, USA) respectively. $MgCl_2$ and Acetic acid 99,7% was purchased from Isofar and Vetec (Brazil) respectively. TRIzol, GelRed and Taq DNA polymerase were purchased from Invitrogen Corp. (Carlsbad, CA, USA). All other reagents used were of analytical grade.

2.2 *Animals*

Adult and healthy Zebrafish of both sexes were obtained from specialized supplier (Redfish Agroloja, RS, Brazil) and kept in standard conditions (tap water treated with Tetra's AquaSafe® to neutralize chlorine, chloramines, and heavy metals present in tap

water that can be harmful to fish) continuously aerated water, 25 ± 2ºC, under a 14-10 h light/dark cycle photoperiod) in 50L housing tank for at least 2 weeks to acclimate before the experiments. The animals were maintained healthy and free of any signs of disease and fed three times a day with TetraMin Tropical Flake Fish. The Ethics Committee of Pontifical Catholic University of the Rio Grande do Sul (PUCRS) approved the protocol under the number 0703854- CEUA.

2.3 *Determination of Acetylcholinesterase activity*

Zebrafish were cryoanesthetized and immediately euthanized by decapitation, and whole brains were homogenized on ice in 60 vol (v/w) of 0.05 M Tris-HCL, pH 8.0, using a Teflon-glass homogenizer. For the AChE activity analysis, a pool of 3 brains was considered as an sample. To ensure the consistence of our results, the tests were performed at least in quadruplicates, using a total of 12 animals per group. AChE activity was determined according the method of Ellmann et al, (1961) with minor modifications. Briefly, the activity on the homogenate was measured by determining the rate of hydrolysis of acetilthiocholine iodide (ACSCh, 0.88 mM) in a final volume of 300 μL, with 33μL of 100 mM phosphate buffer, pH 7.5, and 2 mM DTNB. In this solution, 5 μg of protein of each sample were added and pre-incubated at 25ºC for 10 min. The reaction was started with the addition of the substrate acetilthiocholine, and as soon as the substrate was added the hydrolysis and the formation of the dianion of DTNB were analyzed in 412 nM for 2.5 min (in intervals of 30 s) using a microplate reader. AChE

activity was expressed as micromole of thiocholine (SCh) released per hour per milligram of protein.

2.4 *In vivo treatments*

The animals were separated in groups of 12 animals and housed in 3L tanks with the respective treatment. The animals were transferred to the test aquarium filled with reverse osmosis water to avoid the presence of any metal in the tap water and also to avoid the use of the metal chelant Tetra's AquaSafe®. The animals were kept in continuously aerated water, 25 ± 2ºC, under a 14-10 h light/dark cycle photoperiod, fed three times a day with TetraMin Tropical Flake fish. The treatments were as follows: mercury chloride or lead acetate at a final concentration of 20 μg/L, which has been chosen in previous studies from our laboratory (Senger et al., 2006) based on reportings about the aquatic environment (Berzas Nevado et al., 2003, Jha et al., 2003 and). The concentrations of zinc chloride (5 mg/L) or cadmium acetate (0.1 mg/L) were chosen according the National Council for the Environment (Brazil) (Resolution 357/2005), that allow the disposal of these heavy metal concentrations as industrial effluents in the environment. Control group was kept in the same conditions as the other groups, but without the addition of any metals in the reverse osmosis water. The animals were maintained in the test aquarium for 24, 96 h and 30 days for acute, subchronic and chronic exposures, respectively. The water of the tanks was changed every two days to guarantee the concentration desired of the heavy metal treatment.

183 2.5 *In vitro assays*

184

185 Mercury chloride and Lead acetate (1–250 μM) were added to the reaction medium
186 before preincubation with the enzyme and maintained throughout the enzyme assays
187 described in the section 2.3. Zinc Chloride and Lead acetate were not analyzed *in vitro*
188 because they were already analyzed by Senger et al., 2006. Each metal was added to the
189 reaction medium at 75nm, 150nm, 500nm, 1000nm , 25μM and 250 μM; these
190 concentrations were chosen in order to analyze the effect of a wide spectrum of
191 concentrations, ranging from the concentration of heavy metal found in the test tank
192 water, which was already shown in the environment (Berzas Nevado et al., 2003 and Jha
193 et al., 2003) to higher concentrations chosen based on previous studies showing the effect
194 of these metals on other enzymes involved in nucleoside/nucleotide metabolism (Aikawa
195 et al., 1980, Senger et al., 2006a), Control group was performed with no addition of metal
196 in the enzyme assay.

197

198 2.6 *Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)*

199

200 Total RNA was isolated from a pool of 5 zebrafish brains constituting each group using
201 TRIzol reagent according manufacturer instructions. The RNA was quantified
202 spectrophotometrically and all samples were adjusted to 160 ng/μl. cDNA species were
203 synthesized with SuperScript^{TM} First-Strand (Synthesis System for RT-PCR, Invitrogen),
204 in accordance with the suppliers. PCR reactions were performed as previously described
205 in Rico et al., (2006). The *AChE* and *β-actin* gene amplifications were conducted in a

final volume of 20 µL, with 0.1 µM of each primer, 0.2 µM of dNTP, 2 mM of $MgCl_2$ and 0.5 U of Taq DNA polymerase. The PCR reaction were performed following the conditions below: 2 min at 94°C , 1 min at 60ºC or 58.5°C for *AChE* and *β-actin* gene respectively, 1 min at 72 °C for 35 cycles. A post-extension period of 10 min at 72ºC was performed. A negative control for the PCR product was conducted. The PCR products were analyzed in a 1% agarose gel in an UV transiluminator using GelRed 10X. The band intensities were analyzed in a semi-quantitative way using Image J software. The primers used for the gene amplification were CCAAAAGAATAGAGATGCCATGGACG (forward) and TGTGATGTTAAGCAGA CGAGGCAGG (reverse) for *AChE* (Rico et al., 2006) and GTCCCTGTACGCCTCTGGTCG (forward) and GCCGGACTCATCGTACTCCTG (reverse) for *β-actin* (Chen et al., 2004). To ensure the consistence of our results, the tests were performed at least in quadruplicates, using a total of 20 animals per group.

2.7 *Antioxidant capacity against peroxyl radicals*

Total antioxidant competence against peroxyl radicals was evaluated through reactive oxygen species (ROS) determination in tissues samples treated or not with a peroxyl radical generator (Amado et al., 2009). Briefly, on a white 96-well microplate, 10 µL of brain homogenates were disposed into the wells, six wells per sample. The reaction buffer (127.5 µl) containing 30 mM HEPES (pH 7.2), 200 mM KCl and 1 mM $MgCl_2$ were added to the wells containing the samples. In three of the six wells of each sample, 7.5 µL of 2,2´-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM) were added. In

229 the other three wells the same volume of ultrapure water was pipetted. After this, the
230 microplate was put into a fluorescence microplate reader (Victor 2, Perkin Elmer),
231 programmed to keep temperature at 35 °C. At this temperature, peroxyl radicals are
232 produced by thermal decomposition of ABAP (Winston et al., 1998). Immediately before
233 microplate reading, it was added in all wells 10 µL of the fluorescent probe 2′,7′
234 dichlorofluorescein diacetate (H_2DCF-DA) in a final concentration of 40 µM, according
235 to the methodology employed by Ferreira-Cravo et al. (2007). H_2DCF-DA is deacetylated
236 and the product H_2DCF is oxidized by ROS to the fluorescent compound DCF, which is
237 detected at wavelengths of 488 and 525 nm, for excitation and emission, respectively.
238 Fluorescence readings (fluorescence units or FU) were performed every 5 min during 30
239 min. Total fluorescence production was calculated by integrating the fluorescence units
240 (FU) along the time of the measurement, after adjusting FU data to a second order
241 polynomial function. The results were expressed as area difference of FU x min in the
242 same sample with and without ABAP addition and standardized to the ROS area without
243 ABAP (background area). The relative difference between ROS area with and without
244 ABAP was considered a measure of antioxidant capacity, with high area difference
245 meaning low antioxidant capacity, since high fluorescence levels were obtaining after
246 adding ABAP, meaning low competence to neutralize peroxyl radicals (Amado et al.,
247 2009).
248
249
250 2.8 *Measurement of lipid peroxidation*
251

Lipid peroxidation was measured through determination of thiobarbituric acid reactive substances (TBARS), following the methodology of Oakes and Van der Kraak (2003). Brain homogenates (10 µl) were added to a reaction mixture made with 150 µl of 20% acetic acid, 150 µl of thiobarbituric acid (0.8%), 50 µl of Milli Q water and 20 µl of sodium dodecyl sulfate (SDS, 8.1 %). Samples were heated at 95°C during 30 min and after cooling by 10 min, 100 µl of Milli Q water and 500 µl of n-buthanol was added. After centrifugation (3,000 x g during 10 min at 15 °C), the organic phase (150 µl) was placed in a microplate reader and the fluorescence registered after excitation at 515 nm and emission of 553 nm. The concentration of TBARS (nmols/mg of wet tissue) was calculated employing tetramethoxypropane (TMP) as standard.

2.9 *Protein determination*

Protein was measured with two different methods in accordance with the sensitivity required for the analysis. For the AChE activity analysis, protein was measured by the Coomassie Blue method considering bovine serum albumin as standard (Bradford, 1976). For the antioxidant procedures, total protein content was measured by the Biuret method using a commercial Total Protein Kit (Doles Inc. Brazil) in accordance with the supplier instructions.

2.10 *Statistical Analysis*

Data for the enzymatic and antioxidant analyses were expressed as means ± S.E.M. and analyzed by one-way analysis of variance (ANOVA), following the post-hoc test of Tukey, considering $P < 0.05$ as significant. Before ANOVA analysis, its assumptions (normality and variances homogeneity) were checked.

279

280 **3. Results**

281

282 ***Acetylcholinesterase enzymatic activity and gene expression***

283

284 As shown by Figure 1, the effect of zinc chloride, cadmium acetate, mercury chloride and
285 lead acetate were investigated on AChE activity in zebrafish brain. Zinc chloride did not
286 affect the enzyme activity (P>0.05), even when in the graph is observed a slight augment
287 of the activity after 24 h exposure (Fig. 1A). As with zinc chloride, cadmium acetate did
288 not alter the enzyme activity in the concentration tested (Fig. 1B; P>0,05). The two
289 compounds that altered AChE activity were lead acetate and mercury chloride. In relation
290 of the effects of the treatment with mercury chloride, we can observe a reduction (-25%,
291 P <0.05) of the AChE activity in the 24h-treated animals, following an elevation (+16%,
292 P <0.05) of the AChE activity in the 96h-treated animals in relation to control. This
293 alteration is stabilized after 30 days of treatment (Fig. 1C). Lead acetate induced a
294 reduction (-18%, P <0.05) of the AChE activity in the 24h-treated animals, following a
295 progressive restoration of the normal activity after 96 h and 30 d of treatment (Fig. 1D).
296 We also evaluate the gene expression of this enzyme to investigate in what molecular
297 level these compounds were acting. analyzed the effects of the 24 h treatment with lead
298 acetate and mercury chloride and after 96 h of mercury chloride treatment. As shown by
299 Figure 2, there were no significant changes (P>0.05) in the *Ache* mRNA transcript levels
300 after lead acetate and mercury chloride exposure.

In vitro effects of heavy metal treatments on acetylcholinesterase activity

According to the Figure 3, neither mercury chloride nor lead acetate were able to interfere directly in the enzyme at the concentrations reached with the in vivo treatment. Only mercury chloride at higher concentrations tested in vitro (25 and 250 μM) was capable to inhibit AChE activity (77% and 55%, respectively) (Fig. 3A). Although we have observed a significant effect on AChE activity, these concentrations are much higher than the mercury levels detected in the aquatic environment.

Antioxidant analysis

Samples of animals treated for 24 hours with mercury chloride showed a decrease in total antioxidant competence (<0.05) against peroxyl radicals (Fig. 4A) as a result of an augmented relative area (162%) when compared to the control group ($p<0.05$). The treatment with lead acetate for 24 hours was not able to interfere in the total antioxidant competence, as shown by a relative area statistically similar to the control group ($P>0.05$). No differences in the TBARS content (Fig. 4B) were observed under the experimental conditions.

4. Discussion

In present study, we have evaluated the effect of different treatments with four heavy metal compounds (zinc chloride, cadmium acetate, lead acetate, and mercury chloride) on the AChE activity and gene expression in zebrafish brain. In the concentrations tested, only the animals treated for 24h with lead acetate and mercury chloride and after 96h with mercury chloride have shown alterations in the AChE activity. In addition, we have shown the effects of the treatments above in the antioxidant competence and lipid peroxidation in zebrafish brain. The 24 h-treatment with mercury chloride had the ability to reduce the antioxidant competence against peroxyl radicals.

Environmental pollutants, such as insecticides, herbicides and heavy metal compounds, have been associated with the development of PD and AD (Allam, 2005; Gorell, 1999; Mutter, 2004; Sherer, 2003). The slow accumulation of heavy metals in the brain, due to their widespread and permanent presence in the environment (Bahn, 2005; Björkman, 1997; Bloom, 1994; Ely, 2001; Halbach 1998; Lopez, 2005; Swarup, 2005) can be a potential predictor for the development of a neurodegenerative disease. Yet, a short-term exposure but during early or critical and vulnerable developmental periods (Carpenter, 2001; Finkelstein, 1998) also contributes to increase the susceptibility for developing a neurodegenerative disease later in life (Bolin et al., 2006; Finkelstein, 1998; Landrigan, 2005; Monnet-Tschudi, 2006; Mutter, 2004; Pabello, 2005).

Acute and chronic toxicoses caused by cadmium, mercury, and lead are known for all forms of life (Hu, 2005, Kosnett, 2007). A general consensus holds that the

harmful effects of heavy metals as cadmium, mercury, and lead mainly result from their interactions with protein and/or DNA (Sharma et al., 2008). Sharma et al., (2008) have shown that heavy metal ions are potent inhibitors of protein folding, suggesting that the interference of metal ions with non native forms of proteins might result in quantitative deficiencies of the affected proteins and in the formation of proteotoxic aggregates, which can contribute to explain the pleiotropic symptomatology of heavy metal poisoning (Hu, 2005; Kosnett, 2007; Waisberg, 2003). There is also evidence that heavy metals can affect diverse post-translational modifications of proteins, which are a decisive step for some proteins to achieve its correct folding and enzyme activity. Other action attributed to heavy metals is the alteration of ion-dependent events at the synapses, such as the impairment of calcium channels and NMDA receptors-mediated events, and also the disruption of zinc-fingers motif containing proteins. Zinc finger proteins are the largest class of transcription factors, which, in the presence of this ion, shows the correct folding and stability allowing the binding to nucleic acids to regulate transcription (Zawia et al., 2000; Zeng and Kagi, 1995). This motif is present in a number of critical brain specific proteins and the substitution of zinc by environmental heavy metal ions, mainly as a consequence of lead exposure can induce structural and functional changes in these proteins, contributing to the cellular degeneration, disturbed gene expression, signal transduction, and DNA repair (Zawia et al., 2000). Considering this hypothesis, we have evaluated the possible influence of the heavy metals tested in AChE gene expression. AChE transcript levels were not affected by the treatment with heavy metals, suggesting other metabolic target for the treatments.

As multi-targeted disturbing agents, heavy metals can induce or exacerbate pathogenic cascades by acting in signal transduction elements, such as enzymes, ion channels or receptors. Considering that these networks of signal transducers determine the conversion of environmental cues into cellular actions (Rosse et al., 2010), an alteration in this balanced pathway may trigger a variety of altered signals, including the activation of messengers that can modulate the actions of other cell components. The main players in these networks are protein kinases, such as PKC and PKA.

There is data that demonstrate that Pb^{+2} can activate PKC in immature rat brain microvessels and in brain tissues, by mimicking calcium (Markovac and Goldstein 1988a, 1988b). The prevalent theory is that lead may affect calcium metabolism, and, in several systems, it can mimic calcium actions at cellular level or disrupt calcium homeostasis (Lu, Guizzetti and Costa, 2002). Pb can substitute calcium action in activating PKC (reviewed in Costa, 1998). It also have been reported the inhibitory effects of lead in purified PKC enzymes from rat brains and hepatoma cultured cells (Murakami, Feng and Chen, 1993, Tonner and Heiman, 1997). Despite these data, there is also evidence that lead plays a dual role, activating or inhibiting PKC according to the lead dose tested (Sun et al., 1999; Tomsig and Suszkiw, 1995). This discrepancy of lead effects can be a result of a partial agonist role of lead at low concentrations, activating the enzyme with its interaction with high affinity sites that would normally interact with calcium at the regulatory domain of the enzyme. At higher concentrations, lead may also interact with a low affinity site, and this interaction results in inhibition of calcium-stimulated PKC activity (Tomsig and Suszkiw, 1995). PKA is other kinase that is a target to heavy metals.

391 According with the literature data, heavy metal can inhibit adenylate cyclase (Rodrigues
392 et al., 1999), altering cAMP levels, which in turn can restrict PKA activity.
393 Considering that post-translational modification are targets for heavy metals, and
394 also may be responsible for regulating AChE activity (reviewed in Nalivaeva and Turner,
395 2001), we have analyzed AChE zebrafish protein sequence (using NetPhosK) to search
396 for possible phosphorylation sites. Interestingly, we found possible sites of
397 phosphorylation involving different enzymes, including PKA (7 sites, maximum score of
398 0.75 at positions Ser612 and Ser613), PKC (9 sites, highest score of 0.76 at position
399 Thr271) and p38MAPK (2 sites, score of 0.61 in Thr128 and Ser237). Existing data
400 shows that PKA phosphorylation of AChE increased up to 10 times the rate of
401 acetylthiocholine hydrolysis by the recombinant human enzyme (Grifman et al., 1997).
402 Therefore, we propose that the pattern of activity restoration shown here after 96h and
403 30d of treatments could be due to an influence of possible post-translational
404 modifications. Previous studies demonstrated that *in vivo* phosphorylation of AChE by
405 PKA may play a protective feedback role against long lasting impairments of cholinergic
406 neurotransmission (Grisaru et al., 1999; Soreq and Zakut, 1993). Further studies are
407 required to elucidate the susceptibility of signal transduction pathways of zebrafish brain
408 to heavy metal toxicity.
409 Oxygen, a vital fuel for all eukaryotic organisms is also a reason of concern. This
410 occurs by its ability to continuously generate reactive oxygen species (ROS) that can be
411 extremely harmful to cell constituents when in high cellular levels. However, organisms
412 have a protective machinery composed of enzymatic and non-enzymatic defenses
413 (Halliwell and Gutteridge, 2007), that are responsible to counteract the actions of ROS

414 and prevent oxidative stress. These actions range from lipid peroxidation, protein
415 oxidation, enzyme inactivation and DNA breakage up to carcinogenesis, ageing and
416 neurodegenerative diseases, which occur when ROS formation exceeds antioxidant
417 defense capability or disrupt redox signaling, affecting cell functionality (Jones, 2006 and
418 reviewed in Monnet-Tschudi, 2006). There is evidence that environmental contaminants
419 can alter antioxidant status of the cell, being the antioxidant and oxidative stress
420 parameters used as biomarkers of pollution (Halliwell and Gutteridge, 2007; Regoli et al.,
421 2002). Because of their multitargeted actions, heavy metals can increase ROS levels by
422 the perturbation of diverse pathways, including enzymatic processes, mitochondrial
423 functions, and endogenous antioxidant defense mechanisms. ROS-induced protein
424 oxidation promotes the aggregation of protein as synuclein and Aβ peptides, which are
425 characteristics of PD and AD, respectively (Liu and Yang , 2005; Moreira et al., 2005;
426 Tabner et al., 2005).

427 The stress oxidative analysis were perfomed only in groups where a kinetic
428 alteration has occurred. Here we have analyzed the effects of 24h treatment with mercury
429 chloride and lead acetate in the total ROS production to look for general alterations rather
430 than be restricted to one unique component of this pathway (Amado et al., 2009).
431 Mercury chloride has reduced the antioxidant competence, as shown by a higher relative
432 area than the control group in the analysis of competence against peroxyl radicals.

433 The data presented herein contributes to increase the knowledge about heavy
434 metal brain contamination. In accordance with the literature we have shown that the
435 heavy metal targets occur at different levels, such as alteration in cholinergic transmission

436 and also in the antioxidant competence of the tissue, which are a small portion of the
437 wide spectrum of actions promoted by these pollutants.
438
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446
447 **Conflict of Interests**
448 The authors declare no conflict of interests.
449

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Figure legends

Figure 1: In vivo effect of different treatments (24 h, 96 h, and 30 days) with $ZnCl_2$ (A), $Cd(CH_3COO)_2$ (B), $HgCl_2$ (C), and $Pb(CH_3COO)_2$ (D) in AChE activity in zebrafish brain. Data represent means ± S.E.M of at least three independent experiments. * Significantly differences from control group (ANOVA followed by Tukey's test as post-hoc, $P < 0.05$).

Figure 2: Relative gene expression pattern of *AChE* after $Pb(CH_3COO)_2$ (24 h) and $HgCl_2$ (24 and 96 h) treatments in zebrafish brain. Data represent means ± S.E.M of the *AChE* versus □-*actin* mRNA ratio of at least four independent experiments. The results were analyzed by densitometry using Image J 1.37 for Windows.

Figure 3: In vitro effect of varying concentrations of $HgCl_2$ (A) and $Pb(CH_3COO)_2$ (B) in AChE activity in zebrafish brain. Data represent means ± S.E.M of four independent experiments. * Significantly differences from control group (ANOVA followed by Tukey's test as post-hoc, $P < 0.05$).

Figure 4: Total antioxidant capacity against peroxyl radicals (A) and lipid peroxides content expressed as thiobarbituric acid reactive substances (TBARS) (B) in zebrafish brain after 24 h treatments with $Pb(CH_3COO)_2$ and $HgCl_2$. Data represent means ± S.E.M of at least three independent experiments. * Significantly differences from control group (ANOVA followed by Tukey's test as post-hoc, $P < 0.05$).

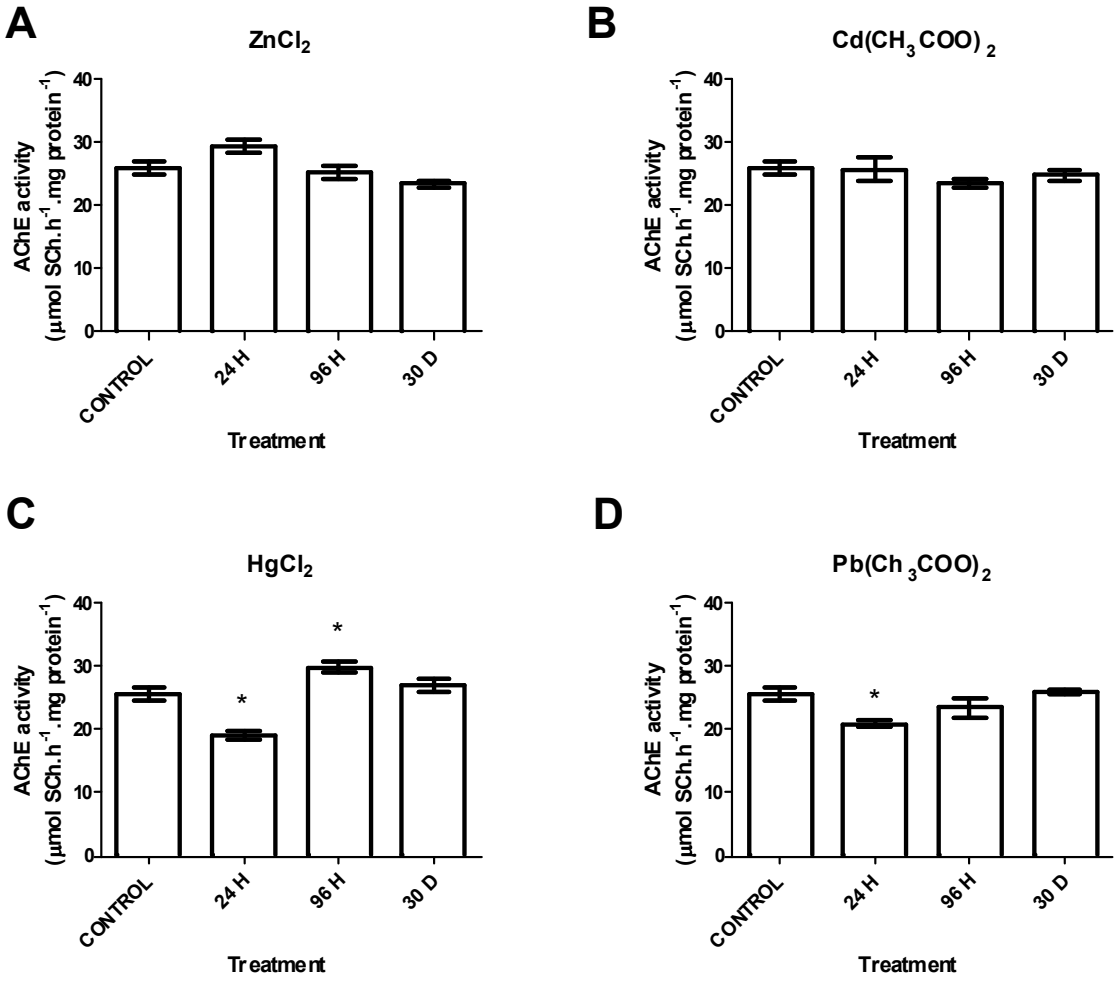
A
ZnCl2
AChE activity
(µmol SCh.h-1.mg protein-1)
0
10
20
30
40
CONTROL
24 H
96 H
30 D
Treatment
B
Cd(CH3COO)2
AChE activity
(µmol SCh.h-1.mg protein-1)
0
10
20
30
40
CONTROL
24 H
96 H
30 D
Treatment
C
HgCl2
AChE activity
(µmol SCh.h-1.mg protein-1)
0
10
20
30
40
*
*
CONTROL
24 H
96 H
30 D
Treatment
D
Pb(Ch3COO)2
AChE activity
(µmol SCh.h-1.mg protein-1)
0
10
20
30
40
*
CONTROL
24 H
96 H
30 D
Treatment

Fig. 1

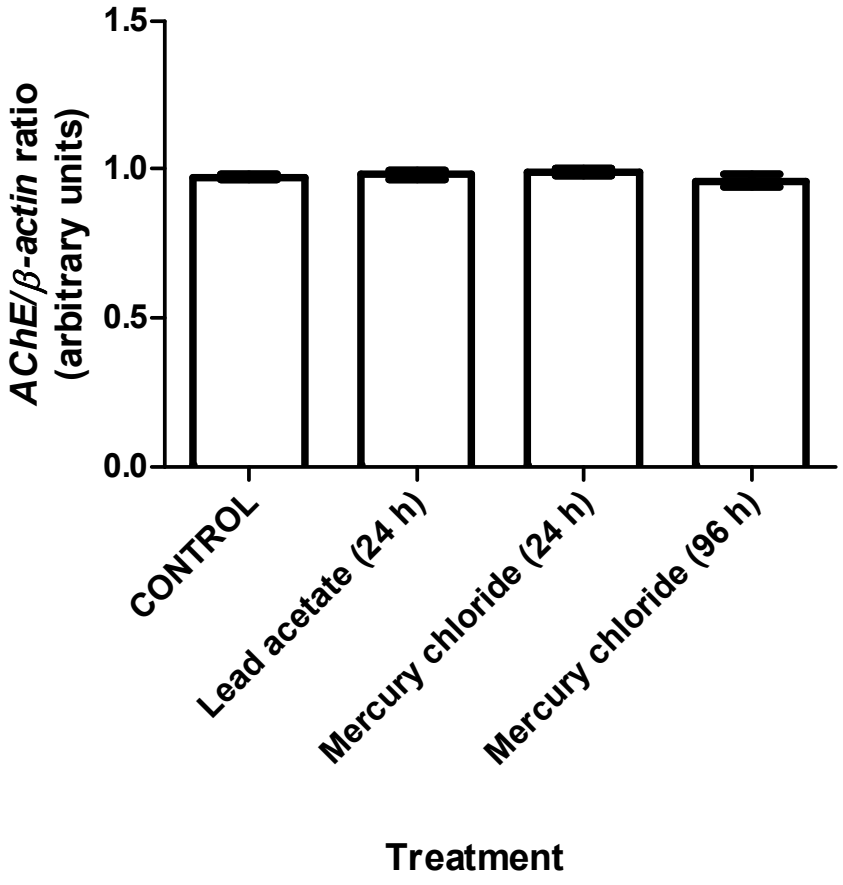
1.5
1.0
0.5
0.0
AChE/β-actin ratio
(arbitrary units)
CONTROL
Lead acetate (24 h)
Mercury chloride (24 h)
Mercury chloride (96 h)
Treatment

Fig 2

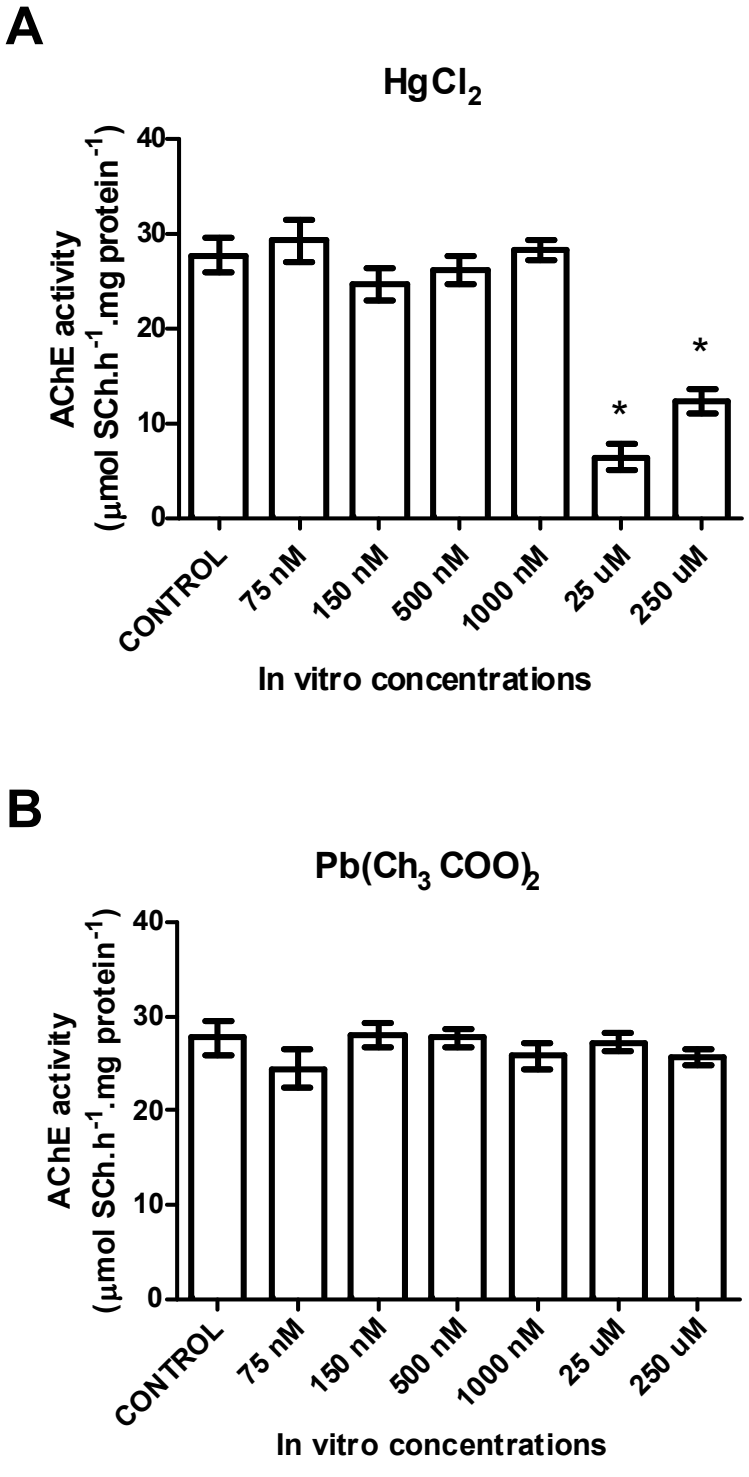
A
HgCl2
AChE activity
(µmol SCh.h-1.mg protein-1)
40
30
20
10
0
*
*
CONTROL
75 nM
150 nM
500 nM
1000 nM
25 uM
250 uM
In vitro concentrations
B
Pb(Ch3 COO)2
AChE activity
(µmol SCh.h-1.mg protein-1)
40
30
20
10
0
CONTROL
75 nM
150 nM
500 nM
1000 nM
25 uM
250 uM
In vitro concentrations

Fig 3

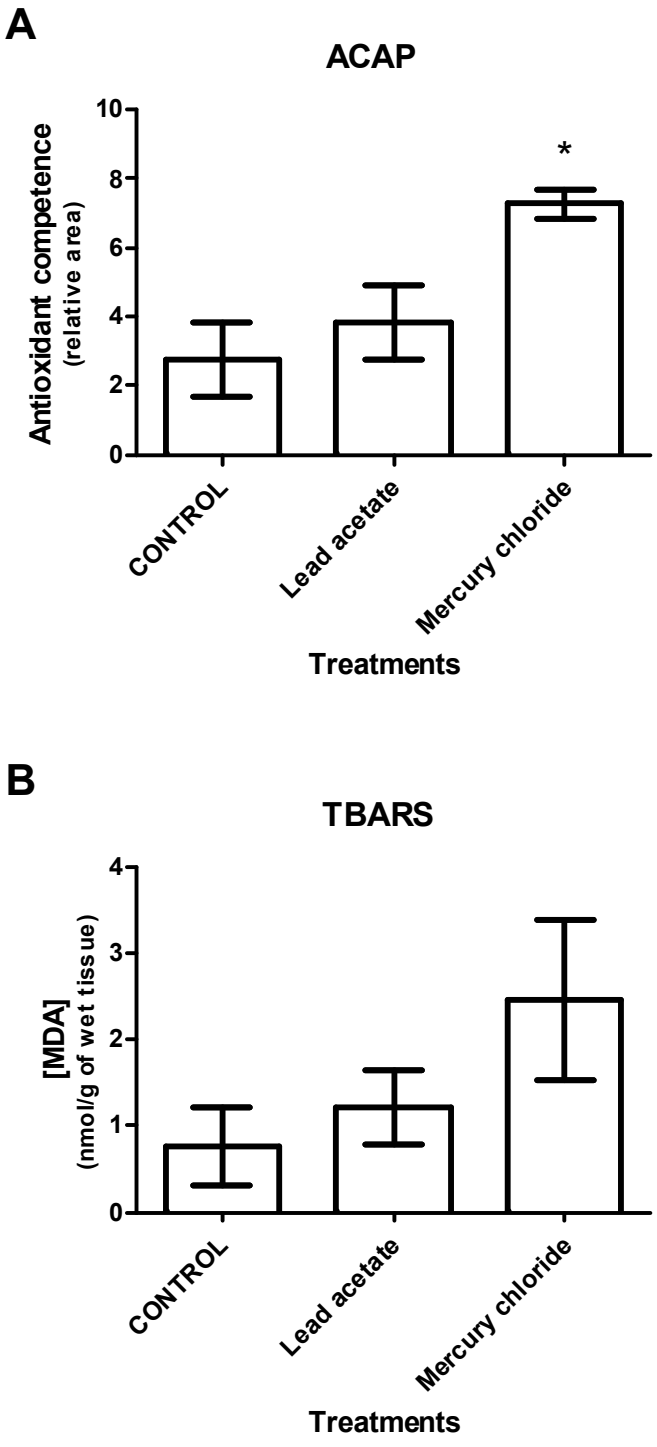
A
ACAP
Antioxidant competence
(relative area)
10
8
6
4
2
0
*
CONTROL
Lead acetate
Mercury chloride
Treatments
B
TBARS
[MDA]
(nmol/g of wet tissue)
4
3
2
1
0
CONTROL
Lead acetate
Mercury chloride
Treatments

Fig 4

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