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# INFLUÊNCIA DO METANOL E DO ETANOL SOBRE A ATIVIDADE E A EXPRESSÃO GÊNICA DAS ECTONUCLEOTIDASES E ACETILCOLINESTRASE EM CÉREBRO DE ZEBRAFISH (*Danio rerio*)

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# Parte I

## I.1. Resumo

O zebrafish (*Danio rerio*) é um modelo experimental consolidado em diversas áreas da ciência, como a neurociências e toxicologia. O genoma desta espécie já está quase todo seqüenciado e estudos demonstraram que muitos genes deste peixe são similares ao de mamíferos, incluindo a espécie humana. Evidências demonstram que nucleotídeos e nucleosídeos, principalmente o ATP e a adenosina exercem diversos efeitos sinalizadores no espaço extracelular. No sistema nervoso, o neurotransmissor ATP é armazenado de forma vesicular e liberado na fenda sináptica, onde pode agir em receptores específicos localizados na membrana celular denominados receptores purinérgicos do tipo P2. Estes receptores são subdivididos em receptores ionotrópicos P2X e receptores metabotrópicos P2Y. A inativação do sinal mediado pelo ATP extracelular é realizada por uma família de enzimas denominadas ecto-nucleotidases. Dentre este grupo de enzimas, destacam-se as NTPDases (nucleosídeo trifosfato difosfoidrolases) e a ecto-5'-nucleotidase. Após sofrer catabolismo pelas ectonucleotidases, o neurotransmissor ATP é hidrolisado ao neuromodulador adenosina. Este nucleosídeo exerce seus efeitos através dos receptores metabotrópicos denominados purinoceptores P1. Estudos do nosso laboratório demonstraram a presença de ectonucleotidases como as NTPDases e a ecto-5'-nucleotidase no SNC de zebrafish. Além disso, já foi descrito na literatura que esta espécie apresenta purinoceptores do tipo P2X e P2Y. A acetilcolina é um neurotransmissor secretado pelos nervos colinérgicos terminais, juntamente com o ATP. Após exercido seu sinal nos receptores nicotínicos e muscarínicos, a acetilcolina é inativada pela ação de uma enzima denominada acetilcolinesterase (AChE), que hidrolisa a acetilcolina até colina e acetato. O gene da AChE já foi clonado e seqüenciado e esta atividade enzimática foi detectada em cérebro de zebrafish. O metanol é um composto neurotóxico responsável por sérios danos ao SNC. Além de ser encontrado como um contaminante ambiental, este álcool é também empregado como componente de soluções crioprotetoras para embriões de zebrafish. Os efeitos do consumo agudo de etanol exercem uma variedade de modificações como coordenação motora, percepção sensorial e cognição. O etanol promove diversas alterações bioquímicas e fisiológicas em células do sistema nervoso central. Portanto, nós investigamos o efeito *in vitro* e *in vivo* dos álcoois metanol e etanol sobre as ectonucleotidases e acetilcolinesterase em SNC de zebrafish. Após o tratamento agudo com metanol a 0,5 e 1,0% houve uma redução significativa na hidrólise de ATP e ADP. Entretanto, não foram verificadas alterações na atividade da ecto-5´-nucleotidase em cérebro de zebrafish. Uma inibição significativa na atividade da AChE foi observada na faixa de 0,25 a 1,0% de exposição ao metanol. Quatro seqüências de NTPDase foram identificadas através de uma análise filogenética, na qual uma é similar a NTPDase1 e as outras a NTPDase2. O metanol foi capaz de inibir os transcritos para a NTPDase1, duas isoformas da NTPDase2 e AChE. Metanol a 1,5 e 3,0% inibiu *in vitro* a hidrólise de ATP, e a hidrólise de ADP somente a 3,0%. No entanto, a hidrólise de AMP e a atividade da AChE não foram alteradas. A exposição ao etanol a 0,5 e 1,0% diminuiu a hidrólise de ATP e ADP, enquanto que a atividade da AChE apresentou um aumento significativo a 1,0%. Nenhuma alteração foi observada na atividade da ecto-5'-nucleotidase. Etanol *in vitro* a 0,5 e 1,0% não alterou a hidrólise de ATP, ADP e a atividade da AChE, mas a hidrólise de AMP foi inibida. Acetaldeído *in vitro*, na faixa de 0,5-1,0%, inibiu a hidrólise de ATP e ADP, mas a hidrólise de AMP e acetiltiocolina foi reduzida a 0,25, 0,5 e 1,0%. Acetato *in vitro* não alterou a atividade destas enzimas. O tratamento com etanol reduziu os níveis de mRNA para AChE, NTPDase1 as três isoformas de NTPDase2. Estes resultados demonstram que a intoxicação aguda por metanol e etanol pode influenciar as enzimas envolvidas na degradação dos neurotransmissores ATP e acetilcolina, sugerindo que os sistemas purinérgico e colinérgico podem ser alvos para os efeitos neurotóxicos destes álcoois.

## I.2. Abstract

Zebrafish (*Danio rerio*) is a consolidated model system in many research areas, including neuroscience and toxicology. The genome of this specie is almost sequenced and studies have shown that many genes of this specie are similar to mammals, including the human specie. Evidence has shown that nucleotides and nucleosides, mainly ATP and adenosine, exert extracellular signaling effects. In the nervous system, the neurotransmitter ATP is stored in vesicles and released in the synaptic cleft, where can act on specific cellular membrane receptors named purinergic P2 receptors. These receptors are subdivided on ionotropic P2X receptors and metabotropic P2Y receptors. The inactivation of ATP extracellular signaling is mediated by a family of enzymes named ectonucleotidases. This group of enzymes includes NTPDases (nucleoside triphosphate diphosphohydrolases) and ecto-5'-nucleotidase. After the catabolism promoted by ectonucleotidases, the neurotransmitter ATP is hydrolyzed to the neuromodulator adenosine. This nucleoside exerts its effects by the activation of P1 metabotropic receptors. Studies from our laboratory have demonstrated the presence of NTPDases and ecto-5'-nucleotidase in CNS of zebrafish. Furthermore, P2X and P2Y purinoceptors already are described in this specie. Acetylcholine (ACh) is a neurotransmitter secreted by the cholinergic nerve endings and ATP is co-released with ACh. After exerts its signaling on nicotinic e muscarinic receptors, ACh is inactivated through an enzyme named acetylcholinesterase (AChE), that can hydrolyze ACh into choline and acetate. The AChE gene is already cloned and sequenced and this enzyme activity was detected in zebrafish brain. Methanol is a neurotoxic compound responsible for serious damage on CNS. Besides it can be found as an environmental contaminant, this alcohol is also employed as a component of cryoprotector solutions for zebrafish embryos. The effects of acute ethanol consumption promote several changes related to motor coordination, sensory perception and cognition. Ethanol promotes many biochemical and physiological alterations on nervous cells. Therefore, we investigated the *in vitro* and *in vivo* effect of alcohols methanol and ethanol on ectonucleotidases and acetilcholinesterase in CNS of zebrafish. After acute treatment, there was a significant decrease of ATP and ADP hydrolysis at 0.5 and 1.0%. However, no significant alteration on ecto-5′-nucleotidase activity was verified in zebrafish brain. A significant inhibition on AChE activity was observed in the range of 0.25 to 1.0% methanol exposure. Four NTPDase sequences were identified from phylogenetic analyses, which one is similar to NTPDase1 and the others to NTPDase2. Methanol was able to inhibit NTPDase1, two isoforms of NTPDase2 and AChE transcripts. Methanol inhibited *in vitro* ATP hydrolysis at 1.5 and 3.0% and ADP hydrolysis only at 3.0%. Nevertheless, AMP hydrolysis and AChE activity were not changed. Ethanol exposure decreased ATP and ADP hydrolysis at 0.5 and 1.0%, while the AChE activity presented a significant increased at 1.0%. No changes on ecto-5'-nucleotidase activity were observed in zebrafish brain membranes. Ethanol *in vitro* did not alter ATP, ADP hydrolysis and AChE activity, but AMP hydrolysis was inhibited at 0.5 and 1.0%. Acetaldehyde *in vitro*, in the range 0.5-1.0%, inhibited ATP and ADP hydrolysis, but AMP and acetylthiocholine hydrolysis were reduced at 0.25, 0.5 and 1.0%. Acetate *in vitro* did not alter these enzyme activities. Semi-quantitative expression analysis of NTPDase and ecto-5′-nucleotidase was performed. Ethanol treatment reduced AChE, NTPDase1 and three isoforms of NTPDase2 mRNA levels. These results demonstrate that acute methanol and ethanol intoxication may influence the enzymes involved in the degradation of neurotransmitters ATP and ACh, suggesting that the purinergic and colinergic system can be a target to the neurotoxic effects of these alcohols.

## I.3. Lista de Abreviaturas

Acetil CoA – acetil coenzima A

ACh – acetilcolina

AChE – acetilcolinesterase

ADH – álcool desidrogenase

ADP – adenosina 5'-difosfato

ALDH – aldeído desidrogenase

AMP – adenosina 5'-monofosfato

AMPc – adenosina 3',5'- monofosfato cíclico

ASCh – acetiltiocolina

ATP – adenosina 5'-trifosfato

BuChe – butirilcolinesterase

CD73 – proteína de superficie de linfócitos

CHAT – acetilcolina transferase

CYP2E1 – citocromo P450

E-NPP – ecto-nucleotídeo pirofosfatase

ERK – cinases reguladas por sinalização extracelular

FSH – hormônio folículo estimulante

G6PD – glicose-6-fosfato desidrogenase

GABA – ácido γ-aminobutírico

GPI – glicosil-fosfatidilinositol

GTP – guanosina 5'-trifosfato

$K_M$ – constante de Michaelis

$LC_{50}$ – concentração letal 50

LDH – lactato desidrogenase

$NAD^+$ – nicotinamida adenina dinucleotídeo

NTPDase – nucleosídeo trifosfato difosfoidrolase

MAP – proteína cinase ativada por mitógeno

mRNA – RNA mansageiro

PKC – proteína cinase C, proteína cinase dependente de AMP cíclico

SNC – sistema nervoso central

SNP – sistema nervoso periférico

# I.4. Introdução

## I.4.1. Zebrafish

O Zebrafish ou peixe-zebra (*Danio rerio*) é um pequeno teleósteo (3-4 cm) da família Cyprinidae, sendo uma espécie bastante conhecida pelo seu uso como peixe ornamental (Figura 1). O pioneiro a estudar esta espécie foi George Streisinger que, no final da década de 60, aplicou as técnicas de análise mutacional para estudar o desenvolvimento embrionário do zebrafish (GRUNWALD & EISEN, 2002). Atualmente, este peixe é um modelo experimental consolidado em diversas áreas da ciência, tais como: genética e genômica, desenvolvimento, teratologia, comportamento, toxicologia e neurociências (VASCOTTO et al., 1997). Este peixe possui diversas características favoráveis, tais como: pequeno custo e espaço requerido para manutenção, rápido desenvolvimento e ciclo biológico, grande prole e embriões translúcidos e suscetíveis à manipulação e microinjeção (LELE & KRONE, 1996). O interesse pela espécie pode ser observado pelo número crescente de laboratórios que tem utilizado este teleósteo como um modelo experimental em suas pesquisas (SPRAGUE et al., 2001). Foi criada uma rede de informações na web sobre o zebrafish (http://zfin.org), na qual laboratórios do mundo inteiro podem depositar informações sobre esta espécie (SPRAGUE et al., 2003). Além disso, existe um excelente, compreensivo e freqüentemente atualizado manual de manutenção e controle das condições em laboratórios para este teleósteo (WESTERFIELD, 2000).

Figura 1: Zebrafish (*Danio rerio*)

Nos últimos anos, houve um progresso considerável na genética e genômica do zebrafish. Em 2001, o Instituto Sanger iniciou o seqüenciamento do genoma total (VOGEL, 1992; STERN & ZON 2003), e o genoma mitocondrial já está seqüenciado, servindo de base para estudos filogenéticos (BROUGHTON et al., 2001). O estudo do genoma do zebrafish pode servir como um complemento funcional para o projeto genoma humano, o qual produz enormes quantidades de seqüências, mas carece de informações funcionais para a maioria dos genes identificados (DOOLEY & ZON, 2000). Além disso, grandes segmentos dos cromossomos do zebrafish estão em sintonia com os cromossomos humanos e de camundongo, e muitos genes apresentam um alto grau de similaridade, quando comparados em sua seqüência (BARBAZUK et al., 2000).

O zebrafish se tornou o principal modelo experimental para o estudo do desenvolvimento de vertebrados (ANDERSON & INGHAM, 2003). As características básicas de sua embriogênese são bem conhecidas, assim como o destino celular durante o seu desenvolvimento (KIMMEL & WARGA, 1988; KIMMEL, 1989). Atualmente, um amplo espectro de estudos sobre o desenvolvimento de diversos sistemas, órgãos e patologias relacionadas são realizados nesta espécie (DODD et al., 2000; ACKERMANN & PAW, 2003). Comparando-se as seqüências do genoma humano e de zebrafish, muitos genes como os do ciclo celular, supressores tumorais e oncogenes se mostram conservados

(AMATRUDA et al., 2002). Já foram observados muitos tipos de neoplasias no zebrafish, as quais são semelhantes histologicamente e geneticamente com as de humanos, o que mostra que a biologia do câncer é muito similar nestes organismos (AMATRUDA et al., 2002; STERN & ZON, 2003). Além disso, animais transgênicos podem ser gerados com alterações em genes específicos envolvidos no câncer (LONG et al., 1997).

Recentemente, estudos avaliando características comportamentais do zebrafish foram desenvolvidos (GUO, 2004; GERLAI et al, 2000). A maioria dos trabalhos avaliou o efeito de pesticidas, drogas e xenobióticos na atividade comportamental desta espécie (LEVIN & CHEN, 2004; SWAIN et al., 2004). Alguns estudos também observaram a importância do comportamento inato e adquirido em modelos de agressividade, sociabilidade e sua preferência por ambientes claros ou escuros (SERRA et al., 1999).

Devido às vantagens de se usar o zebrafish como modelo experimental, o efeito agudo e crônico de diversas substâncias tóxicas pode ser avaliado facilmente. Devido ao pequeno espaço requerido por estes animais, uma quantidade menor de toxinas é empregada nos testes toxicológicos (YAMAZAKI et al., 2002).

Atualmente, muitos estudos são realizados nesta espécie para estudar as bases moleculares da neurobiologia, identificando genes envolvidos na formação de circuitos neuronais, no comportamento e nos mecanismos envolvidos na neuropatogênese (VASCOTTO et al., 1997; GUO, 2004). Muitos sistemas de neurotransmissão já foram identificados no zebrafish tais como: glutamatérgico (EDWARDS & MICHEL, 2002), colinérgico (BEHRA et al., 2002; CLEMENTE et al., 2004), dopaminérgico (BOEHMLER et al., 2004), serotoninérgico (RINK & GUO, 2004), histaminérgico (KASLIN & PANULA, 2001), gabaérgico (KIM et al., 2004) e purinérgico (KUCENAS et al., 2003; RICO et al., 2003; SENGER et al., 2004).

I.4.2. Sinalização colinérgica

A acetilcolina (ACh) é um neurotransmissor clássico que desempenha diversas funções no SNC e SNP, sendo sintetizada pela reação catalisada pela colina acetiltransferase (ChAT; EC 2.3.1.6).

Sua síntese ocorre a partir de Acetil CoA, formada durante o metabolismo celular mitocondrial, e da colina, um importante produto do metabolismo dos lipídios. A etapa final da síntese da ACh ocorre no citoplasma, sendo o neurotransmissor transportado para o interior de vesículas sinápticas (KAPCZINSKI et al., 2000). A colina usada na síntese de ACh pode vir diretamente da reciclagem da ACh, que é hidrolisada pela AChE na fenda sináptica ou a partir da fosfatidilcolina. Essas duas fontes de colina são particularmente importantes para o SNC, porque a colina presente no plasma não ultrapassa a barreira hemato-encefálica (TAYLOR & BROWN, 1994).

A liberação de ACh depende das variações no potencial elétrico das membranas dos terminais nervosos e este processo é dependente da concentração de cálcio intracelular (BRADFORD, 1986). A ACh é um neurotransmissor de fundamental importância nas funções desempenhadas pelo córtex cerebral. Esse transmissor tem sido associado com as funções cognitivas, o processamento das funções sensoriais, com a organização cortical de movimento e com o controle do fluxo sanguíneo cerebral (SCREMIM et al., 1997). A ação excitatória ou inibitória da ACh depende da via nervosa que é estimulada (BRADFORD, 1986). Além de sua ação neurotransmissora, a ACh possui função neuromoduladora, pois os níveis da mesma podem regular a concentração de outros neurotransmissores no cérebro (COOPER et al., 1991).

Os efeitos intracelulares da acetilcolina são mediados pela ativação de receptores colinérgicos nicotínicos e muscarínicos (BURGEN, 1995). No SNC, existem evidências de que estes receptores estejam estão envolvidos no controle motor cardiovascular, na regulação da temperatura e na memória (CAULFIELD & BIRDSALL, 1998). Quando ativado, o receptor poderá abrir ou fechar canais de $K^+$, $Ca^{2+}$ ou $Cl^-$, dependendo do tipo de célula. A estimulação dos receptores muscarínicos conduzirá à despolarização ou hiperpolarização da membrana. A estimulação dos receptores muscarínicos também é capaz de inibir a enzima adenilato ciclase e ativar a enzima fosfolipase C (COOPERR et al., 1991).

Os receptores nicotínicos para a acetilcolina consistem de cinco subunidades designadas $\alpha$, $\beta$, $\gamma$ e $\delta$, sendo que a subunidade $\alpha$ é espressa em duas formas. Estudos estruturais mostram que as subunidades estão arranjadas ao redor de uma cavidade central, com uma grande porção de proteína voltada para a superfície extracelular. A ACh se liga normalmente a subunidade $\alpha$, produzindo mudanças conformacionais, que permitem a passagem, principalmente de cátions. A desensibilização do receptor aumenta quando o mesmo é fosforilado por proteína quinase dependente de AMPc, PKC ou tirosina quinase (COOPER et al, 1991).

### I.4.3. Acetilcolinesterase (AChE; E.C.3.1.1.7)

As colinesterases hidrolisam a ACh na fenda sináptica e são enzimas que desempenham um papel muito importante na neurotransmissão colinérgica, além de outras funções fisiológicas. Elas são classificadas de acordo com suas propriedades catalíticas,

especificidade de inibidores e distribuição nos tecidos: a AChE é encontrada, principalmente nas sinapses do SNC, SNP parassimpático e junção neuromuscular; e a butirilcolinesterase (E.C. 3.1.1.8, BuChe) encontrada no plasma, no intestino e em outros tecidos (MASSOULIÉ & BOM, 1982). A AChE é uma importante enzima regulatória que controla a transmissão de impulsos nervosos através da sinapse colinérgica pela hidrólise do neurotransmissor excitatório ACh (MILATOVIC & DETTBARN, 1996). A AChE é uma serina hidrolase que desempenha um papel essencial no mecanismo colinérgico, catalisando a hidrólise natural do substrato acetilcolina em acetato e colina (QUINN, 1987). A seqüência de aminoácidos da AChE mostrou que serina, histidina e glutamato são resíduos importantes para a atividade catalítica (SUSSMAN et al, 1991). Os níveis de AChE parecem ser controlados pela interação da ACh com seus receptores; quando a interação é acentuada, aumentam os níveis de AChE. No entanto, a AChE pode ser usada como um marcador da função colinérgica, e mudanças na atividade da enzima podem indicar alterações na disponibilidade de ACh e do nível de seus receptores (FERNANDES & HODGES-SAVOLA, 1992).

ARENZANA et al., 2005 estudaram o desenvolvimento do sistema colinérgico em cérebro e retina de zebrafish, sendo também mostrado através de análise histoquímica e imunohistoquímica em SNC desta espécie (CLEMENTE et al., 2004). O gene da AChE já foi clonado e sequenciado, e sua atividade enzimática já foi detectada no cérebro (BERTRAND, et al., 2001). Este peixe apresenta uma única situação entre os vertebrados, pois a BuChE, responsável pela hidrólise de butirilcolina, não está presente no seu genoma. Além disso, subunidades de receptores muscarínicos e nicotínicos também são expressos nesta espécie (ZIRGER, et al., 2003).

I.4.4. Sinalização purinérgica

O ATP foi descrito inicialmente como neurotransmissor através dos estudos que mostravam a sua liberação a partir de nervos sensoriais (HOLTON & HOLTON, 1954 e HOLTON, 1959). Entretanto, sua ação como neurotransmissor só foi reconhecida pelos estudos realizados pelo grupo de Geoffrey Burnstock e colaboradores, que desenvolveu a hipótese purinérgica (BURNSTOCK et al., 1970; BURNSTOCK, 1972). O ATP pode ser armazenado e liberado para o meio extracelular juntamente com outros neurotransmissores, tais como: acetilcolina, glutamato, norarenalina, serotonina e GABA (BURNSTOCK, 1999; BURNOSTOCK, 2004) através de vesículas pré-sinápticas dependentes de cálcio, (PHILLIS & WU, 1982). DOWDALL et al. (1974) demonstraram que o ATP pode ser estocado, junto com acetilcolina, nas vesículas sinápticas dos terminais nervosos colinérgicos de órgão elétrico de *Torpedo marmorata*.

No sistema nervoso central e periférico, o ATP age como neurotransmissor excitatório e possivelmente como neuromodulador (CUNHA & RIBEIRO, 2000; SALGADO et al., 2000). Os nucleotídeos e o nucleosídeo da adenina podem exercer seus efeitos através da ativação de receptores purinérgicos subdivididos em dois grandes grupos: P1 e P2. Os purinoreceptores do tipo P1 são mais eficientemente ativados por adenosina, enquanto que os purinoreceptores P2 são ativados por ATP (RALEVIC & BURNSTOCK, 1998). Estes receptores são denominados purinoceptores P2 e são divididos em duas subclasses, os receptores ionotrópicos P2X, que são canais iônicos dependentes de ligantes, e os receptores metabotrópicos P2Y, que são acoplados à proteína G. Membros de ambos tipos de receptores são distribuídos no SNC e SNP e estão envolvidos em uma miríade de funções (BARNARD et al., 1997, BURNSTOCK & KNIGTH, 2004).

A clonagem e caracterização molecular dos subtipos dos receptores P2X do zebrafish já foram realizadas (DIAZ-HERNANDEZ et al., 2002; BOUÉ-GRABOT et al., 2000; EGAN et al., 2000; NORTON et al., 2000). A análise da seqüência de nove genes sugere que cinco deles são ortólogos a genes dos receptores P2X de mamíferos, dois são parálogos e um ainda precisa ser devidamente classificado (KUCENAS et al., 2003). Todos os subtipos de receptores P2X do zebrafish contêm resíduos altamente conservados, os quais são encontrados nas subunidades de mamíferos. Até o momento, na família de receptores P2Y foram identificadas oito proteínas que possuem respostas funcionais (RALEVIC & BURNSTOCK, 1998; LAZAROWSKI et al., 2003; ILLES & RIBEIRO, 2004). Também existem evidências que os receptores P2Y estão envolvidos na transdução de sinal mediada por proteínas ligantes de GTP e proteínas quinases como a PKC, MAP, ERK1 e 2 (COMMUNI et al., 2000; BOEYNAEMS et al., 2000). Entretanto, até o momento, somente foram identificados receptores P2Y1 em trombócitos de zebrafish (GREGORY & JAGADEESWARAN, 2002).

A ação sinalizadora dos nucleotídeos é terminada por uma cascata de enzimas localizadas na superfície celular. No caso da degradação extracelular do ATP, o produto final é o neuromodulador adenosina. Esta degradação pode inativar a sinalização mediada pelo ATP através dos receptores P2 e aumentar a sinalização mediada pela adenosina através dos receptores P1 (KATO et al., 2004).

A adenosina é uma substância que pode ser formada nos espaços intracelular e extracelular. No meio extracelular esta purina se comporta como uma molécula sinalizadora, influenciando a transmissão sináptica e a atividade do sistema nervoso central (RIBEIRO et al., 2003). Sua formação intracelular é devido a ação, principalmente, da enzima 5'-nucleotidase que hidrolisa AMP à adenosina e da hidrólise do substrato S-

adenosil-homocisteína pela S-adenosil-homocisteína hidrolase. A adenosina gerada intracelularmente pode ser transportada ao espaço extracelular através de transportadores bidirecionais, por um mecanismo de difusão facilitada que regula os níveis intracelulares e extracelulares deste nucleosídeo (FREDHOLM et al., 2001). Entretanto, a adenosina não é considerada um neurotransmissor, pois não há indícios de que é armazenada em vesículas sinápticas (BRUNDEGE & DUNWIDDIE, 1997; RIBEIRO et al., 2003).

A adenosina atua como um importante modulador sináptico no sistema nervoso central (DUNWIDDIE E MASINO, 2001). Suas ações são exercidas através de um grupo de receptores, que estão divididos em quatro subtipos: $A_1$, $A_{2A}$, $A_{2B}$ e $A_3$. Grande parte do conhecimento sobre a distribuição e funcionalidade destes receptores corresponde aos receptores de baixa afinidade $A_1$ e $A_{2A}$. Os receptores $A_1$ e $A_2$ são acoplados à proteína G inibitória e estimulatória, respectivamente, porém estudos evidenciam que estes receptores podem ser acoplados a outras proteínas G (FREDHOLM et al., 2001). Estudos têm demonstrado que a adenosina formada a partir dos nucleotídeos da adenina age preferencialmente nos receptores $A_{2A}$ e a adenosina liberada como tal preferencialmente age sobre os receptores $A_1$ (CUNHA, 2001).

A adenosina está envolvida nos efeitos comportamentais e neuronais induzidos pelo etanol (DOHRMAN et al., 1997). Diversos estudos demonstraram que o etanol altera a função neuronal por modificar rotas de transdução de sinais mediadas por hormônios e neurotransmissores. Estudos em cultura de células neurais demonstraram que o etanol, através da ativação dos receptores $A_{2A}$, estimula a sinalização mediada pela via da adenosina monofosfato cíclico/protein kinase A (AMPc/PKA) e a expressão gênica mediada pelo elemento regulator de AMPc e que este efeito é bloqueado pela subunidades beta e gama da proteína G inibitória (AROLFO et al., 2004).

I.4.5. Ectonucleotidases

A sinalização mediada por nucleotídeos extracelulares necessita de mecanismos eficientes para a inativação de seu sinal. Muitos estudos evidenciaram a presença de uma variedade de enzimas localizadas na superfície celular denominadas ectonucleotidases, que são capazes de hidrolisar, e assim, controlar os níveis de nucleotídeos para os receptores P2 quanto de nucleosídeos para os receptores P1 (BONAN et al., 2001; SEVIGNY et al., 2002).

Este conjunto de enzimas inclui a família das E-NPP (ectonucleotídeo pirofosfatase/fosfodiesterase), a família das NTPDases (nucleosídeo trifosfato difosfoidrolase), as fosfatases alcalinas e a ecto-5'-nucleotidase (EC 3.1.3.5) (ROBSON et al., 2006).

A família das E-NPPs consistem em sete membros localizados tanto na superfície celular quanto secretadas. Estas proteínas hidrolisam ligações fosfato ou fosfodiéster de moléculas como nucleotídeos e dinucleotídeos (NPP1-3), (liso)fosfolipídios (NPP2) e ésteres de colina fosfato (NPP6 e NPP7) (STEFAN et al., 2005).

Na família das NTPDases, já foram descritas até o momento em mamíferos oito membros, que foram clonados e caracterizados (ZIMMERMANN, 2001; BIGONNESSE et al., 2004). As NTPDases1, 2, 3 e 8 possuem seus sítios catalíticos voltados para a superfície celular. Já os outros membros (NTPDase 4-7) possuem seu sítio catalítico voltados para o lúmen de organelas intracelulares, como o sistema de Golgi, o retículo endoplasmático e/ou vacúolos lisossomais/autofágicos. As NTPDases 5 e 6 também podem ser encontradas na membrana plasmática e possivelmente secretadas por clivagem proteolítica (ZIMMERMANN, 2001). Esta família de enzimas possui uma topologia de

membrana comum com dois domínios transmembrana e uma alça extracelular, contendo cinco domínios denominadas ACRs (regiões conservadas da apirase) (ZIMMERMANN, 2001). Estas enzimas possuem uma ampla especificidade de substrato, hidrolisando nucleotídeos púricos e pirimídicos. Para a sua atividade catalítica máxima, estas enzimas necessitam de cátions divalentes, como cálcio e magnésio e um pH alcalino. Na maioria dos casos, os valores de $K_M$ para ATP e ADP estão geralmente na ordem micromolar (PLESNER, 1995, ZIMMERMANN, 2001).

A ecto-5'-nucleotidase, também conhecida como a proteína linfocitária CD73 hidrolisa nucleotídeos 5'-monofosfatatos púricos e pirimídicos aos respectivos nucleosídeos. Esta atividade enzimática é dependente de cátions divalentes, como cálcio e magnésio. A ecto-5'-nucleotidase é uma enzima ancorada à membrana plasmática por GPI, sendo que formas solúveis da enzima podem ser originadas mediante a ação de uma fosfolipase específica (ZIMMERMANN, 1992). Esta enzima encontra-se presente na maioria dos tecidos e sua principal função é a hidrólise de nucleosídeos monofosfatados extracelulares, tais como AMP, GMP ou UMP, a seus respectivos nucleosídeos, sendo o AMP o nucleotídeo hidrolisado com maior eficiência com valores de $K_M$ na faixa de micromolar (ZIMMERMANN, 1996). O ATP e o ADP são inibidores competitivos da 5'-nucleotidase com valores de $K_i$ também na faixa de micromolar (ZIMMERMANN, 1996). Em SNC, a ecto-5'-nucleotidase está predominantemente associada à glia, mas várias evidências têm demonstrado que esta atividade também está associada a neurônios (ZIMMERMANN, 1996; ZIMMERMANN et al., 1998; ZIMMERMANN, 2001).

Outra família de enzimas pertencentes ao grupo das ecto-nucleotidases são as fosfatases alcalinas (EC 3.1.3.1). Estas enzimas catalisam a hidrólise de monoésteres de ácido fosfórico e também catalisam a transfosforilação na presença de altas concentrações

de aceptores de fosfato. As fosfatases alcalinas são enzimas homodiméricas e cada sítio catalítico contém três íons metálicos, dois Zn e um Mg, necessários para a atividade enzimática (Millán, 2006).

A hidrólise extracelular de ATP por essa via resulta na formação de ADP, AMP e do nucleosídeo adenosina, que pode agir sobre seus próprios receptores ou ser captado pela célula e participar na rota de salvação do metabolismo das purinas (ROBSON et al., 2006).

Muitos estudos demonstraram a presença de ectonucleotidases como a ATP difosfoidrolase (SARKIS & SALTÓ, 1991; SCHETINGER et al., 2001) e 5´-nucleotidase (VOGEL et al., 1992; VOLKNANDT, 1991) em teleósteos. Em zebrafish, estudos do nosso laboratório demonstraram a presença de uma NTPDase e uma ecto-5'-nucleotidase em membranas cerebrais, apresentando um pH ótimo entre 7.2 e 8.0, um $K_M$ na faixa do micromolar e uma ampla especificidade por outros nucleotídeos (RICO et al., 2003; SENGER et al., 2004). Além disso, as atividades destas enzimas já foram avaliadas quanto às ações promovidas por contaminantes ambientais, tais como pesticidas e metais pesados (SENGER, et al., 2005; 2006a; 2006b).

### I.4.6 Metanol

O metanol é um álcool bastante utilizado industrialmente como matéria prima para diversos produtos, incluindo pesticidas, sabões, solventes e removedores (BUDVARI, 1989). Devido ao seu uso em grande quantidade, este componente pode ser encontrado em efluentes de indústrias, sendo descrito como um importante contaminante ambiental afetando a biota aquática (KAVIRAJ et al., 2004). Estudos têm mostrado que a exposição ao metanol pode causar danos ao SNC de ratos no estágio de gastrulação (DEGITZ et al.,

2004) e é também reconhecido como uma neurotoxina capaz de causar cegueira, afetando o nervo óptico e retina (MURRAY et al., 1991; EELLS, 1991).

A investigação dos efeitos causados pela exposição de compostos solventes ao ecossistema tem despertado o interesse em pesquisas envolvendo a utilização de organismos aquáticos, como espécies de peixes. Na literatura, o metanol tem sido testado na busca de parâmetros bioquímicos para esclarecer os mecanismos da sua toxicidade. Exposições de carpas (*Cyprinus carpio*) ao metanol em concentrações subletais são capazes de induzir aumento nos níveis de cortisol, além de alterações nos níveis de proteína e colesterol em soro (GLUTH & HANKE, 1985). Além disso, seu efeito também foi evidenciado ao alterar o sistema endócrino de salmonídeos, inibindo a síntese e secreção de hormônios FSH e esteróides, (DICKEY & SHANSON, 1998; GIESY et al., 2000), mostrando sua possível capacidade de afetar o potencial reprodutivo de diversas espécies. A perturbação de um sistema por concentrações letais de metanol pode provocar séria toxicidade ao meio aquático, devido ao tempo que este agente tóxico leva para ser removido através de fotoxidação e processos de biodegradação (KAVIRAJ et al., 2004). Em 96 horas de exposição, o valor de $LC_{50}$ do metanol para peixes variou entre 15,4 e 29,4 mg/L (POIRIER et al., 1986), mostrando seu poder de toxicidade ao ambiente.

O metanol é catabolizado por uma enzima álcool desidrogenase formando formaldeído, que pode ser oxidado até formato ou ácido fórmico. O metabólito formaldeído pode facilmente reagir, causando destruição da função de membranas, proteínas e ácidos nucléicos (HECK & CASANOVA, 1990). A toxicidade aguda do metanol varia amplamente entre diferentes espécies, sendo bastante alta em grupos com relativa dificuldade em remover formato (JOLIN et al., 1987). A toxicidade ocular é caracterizada por um seletivo acúmulo de formato na retina e humor vítreo comparado com outras

regiões do SNC (WALLACE et al., 1997), devido a uma possível capacidade limitada de oxidar o formato (EELLS et al., 1994). A toxicidade promovida por este metabólito é devido a sua capacidade de inibir a enzima citocromo oxidase, componente da cadeia transportadora de elétrons envolvida na síntese de ATP (WALLACE et al., 1997).

O metanol tem sido testado em protocolos de vitrificação, atuando como substância crioprotetora essencial na conservação de embriões. Seu uso vem sendo freqüente nesse tipo de técnica, evitando a formação de cristais de gelo nos meios celulares. Apesar de o metanol proporcionar benefícios às técnicas de criopreservação, sua adição também pode causar injúrias celulares, podendo promover efeitos tóxicos ou teratogênicos em sistemas biológicos (ROBLES et al., 2004). Pelo fato do zebrafish ser amplamente estudado também na área da biologia do desenvolvimento, seu uso vem aumentando quanto à exposição dos ovos a compostos crioprotetores no sentido de melhorar a conservação de diferentes linhagens (ZHANG et al., 2005). O efeito deste álcool sobre diferentes atividades enzimáticas, tais como a LDH e G6PD foi testado em embriões de zebrafish (ROBLES et al., 2004).

### I.4.7. Etanol

O consumo excessivo de álcool é um problema de saúde pública que afeta milhares de pessoas em todo o mundo. Seu consumo abundante está associado com a ocorrência de muitas condições patológicas, como câncer, doenças hepáticas, danos cerebrais, entre outros. Muitos órgãos são capazes de metabolizar o etanol, mas a maior parte (mais de 90%) é metabolizada no fígado (QUERTEMONT et al., 2005). Sua eliminação é através de uma cascata metabólica de degradação envolvendo múltiplas reações enzimáticas.

A principal via do metabolismo do álcool é através de duas reações enzimáticas que requerem nicotinamida adenina dinucleotídeo ($NAD^+$) como cofator. No primeiro passo, a enzima álcool desidrogenase (ADH) converte o etanol em acetaldeído, reduzindo o $NAD^+$ nesse processo. No segundo passo, o acetaldeído é metabolizado a ácido acético (acetato) pela enzima aldeído desidrogenase (ALDH), reduzindo também $NAD^+$ (SWIFT, 2003). Em mamíferos, cinco classes distintas de ADH têm sido caracterizadas, apresentando diversas características moleculares e cinéticas (REIMERS et al., 2004a). Além disso, duas classes de ADH foram caracterizadas em zebrafish e compartilham similaridade estrutural com as de mamíferos (REIMERS et al., 2004b), mas apresentam características funcionais distintas.

Além da principal rota de degradação envolvendo ADH e ALDH, há duas menores vias oxidativas para a degradação do etanol em acetaldeído: citocromo P450, (CYP2E1) que é responsável por uma pequena parte do total metabolizado, e a catalase, capaz de transformar etanol em acetaldeído a partir de um radical peróxido (SWIFT, 2003). Neste contexto, acetaldeído e acetato vêm sendo investigados no sentido de esclarecer o envolvimento dos metabólitos da degradação do etanol em respostas comportamentais e farmacológicas (ISRAEL et al., 1994; QUERTEMONT et al., 2005). Estudos têm demonstrado o papel do acetaldeído em diversos efeitos neuroquímicos e farmacológicos promovidos pelo etanol. O acetaldeído, produto intermediário da ADH, é uma molécula altamente reativa que pode formar complexos com proteínas e outros componentes biológicos formando aductos (NIEMELA, 2001).

A utilização do zebrafish em pesquisas envolvendo drogas de abuso vem aumentando consideravelmente, principalmente no que se refere à intoxicação por álcool. Estudos demonstram que o etanol causa alterações neste teleósteo, tais como anormalidades

craniofaciais, malformações cardíacas e prejuízos no seu desenvolvimento (CARVAN 3$^{RD}$ et al., 2004; REIMERS et al., 2004b; BILOTTA et al., 2004). Isso faz com que esta espécie sirva como um importante modelo para biologia do câncer devido às diversas ações teratogências promovidas por este composto (SCALZO & LEVIN, 2004). Recentemente, o zebrafish tem sido utilizado com sucesso em pesquisas que envolvem as mais diversas respostas comportamentais aos efeitos de drogas, dentre elas, o etanol. O etanol é capaz de alterar a atividade locomotora, aprendizado, agressividade e interação social (GERLAI et al., 2000), servindo de base para estudos genéticos, na qual linhagens de diferentes genótipos podem ser expostas ao etanol (DLUGOS & RABIN, 2003).

Então, devido ao fato do ATP ser um co-transmissor liberado junto com a ACh (BURNSTOCK, 2004) e estes dois neurotransmissores serem hidrolisados na fenda sináptica pelas NTPDases e AChE, respectivamente, o estudo dos efeitos do metanol e etanol sobre a atividade dessas enzimas em cérebro de zebrafish é de peculiar interesse.

## I.5. Objetivos

Considerando que: (1) o zebrafish é um importante e consolidado modelo experimental em estudos toxicológicos, (2) os sistemas purinérgico e colinérgico exercem um importante papel na sinalização no sistema nervoso central e (3) receptores e enzimas, envolvidos nestes importantes sistemas de neurotransmissão, já foram descritos nesta espécie, o objetivo geral deste estudo foi avaliar o efeito dos compostos metanol e etanol na atividade e padrão de expressão de enzimas envolvidas na hidrólise dos neurotransmissores ATP e acetilcolina em cérebro de zebrafish.

Objetivos específicos:

- Verificar o efeito *in vivo* da exposição aguda (1 hora) do metanol sobre a atividade e (expressão gênica) padrão de expressão das ecto-nucleotidades e AChE em cérebro de zebrafish.
- Avaliar o efeito *in vitro* do metanol na hidrólise de ATP, ADP, AMP e ASCh em cérebro de zebrafish.
- Estudar a influência da exposição aguda do etanol na atividade e expressão gênica padrão de expressão das ecto-nucleotidases e acetilcolinesterase em cérebro de zebrafish.
- Investigar possíveis alterações induzidas *in vitro* pelo etanol, acetaldeído e acetato sobre a hidrólise de ATP, ADP, AMP e ASCh em cérebro de zebrafish.

# Parte II

II.1. Capítulo 1 – RICO, E.P., ROSEMBERG, D.B., SENGER, M.R., ARIZI M.DE B., BERNARDI, G.F., DIAS, R.D., BOGO, M.R., BONAN, C.D. Methanol alters ecto-nucleotidases and acetylcholinesterase in zebrafish brain (2006). *Neurotoxicology and Teratology*, 28(4), 489-496.

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# Methanol alters ecto-nucleotidases and acetylcholinesterase in zebrafish brain

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**Abstract**

Methanol is a neurotoxic compound that is responsible for serious damage on CNS. Besides being found as an environmental contaminant, this alcohol is also employed as a component of cryoprotector solutions for zebrafish embryos. Here we tested the acute effect of methanol on ecto-nucleotidase (NTPDase, ecto-5′-nucleotidase) and acetylcholinesterase (AChE) activities in zebrafish brain. After acute treatment, there were significant decreases on ATP (26% and 45%) and ADP hydrolysis (26% and 30%) at 0.5% and 1.0%, respectively. However, no significant alteration on ecto-5′-nucleotidase activity was verified in zebrafish brain. A significant inhibition on AChE activity (39%, 33% and 30%) was observed at the range of 0.25% to 1.0% methanol exposure. Four NTPDase sequences were identified from phylogenetic analyses, which one is similar to NTPDase1 and the others to NTPDase2. Methanol was able to inhibit NTPDase1, two isoforms of NTPDase2 and AChE transcripts. To evaluate if methanol affects directly these enzymes activities, we have performed in vitro assays. ATP hydrolysis presented a significant inhibition (19% and 34%) at 1.5% and 3.0%, respectively, and ADP hydrolysis decreased only at 3.0% (29.2%). Nevertheless, AMP hydrolysis and AChE were not altered after in vitro exposure. The inhibitory effect observed on these enzymes could contribute to the neurodegenerative events promoted by methanol in zebrafish brain.


*Keywords:* Methanol; Ecto-nucleotidase; Acetylcholinesterase; NTPDase; Ecto-5′-nucleotidase; Zebrafish

## 1. Introduction

Methanol is an alcohol very useful in different industries as a raw material for many products, including pesticides, soap, solvents and removers [9]. Due to the large use of this compound, it can be found in the effluent of industries, being described as an environment contaminant that affects the aquatic biota [29].

Studies have shown that methanol exposure can cause several damages to mice CNS at gastrulation-stage [16]. In addition, methanol is also recognized as a neurotoxin capable of producing visual impairment or blindness, affecting optic nerve and retina [33,20].

The adenine nucleotide, ATP, is a well-known molecule released at synaptic cleft after nerve terminals depolarization, acting as a neurotransmitter or as a co-transmiter [14,51,11]. The effects of this nucleotide can be elicited by activation of ionotropic P2X or metabotropic P2Y receptors. Ecto-nucleotidase pathway constitutes an important route of extracellular ATP degradation [23]. It controls the level of nucleotides at the cell surface metabolizing ATP to adenosine, an important neuromodulator. NTPDase (nucleoside triphosphate diphosphohydrolase) family hydrolyzes both tri- and diphosphonucleosides and an ecto-5′-nucleotidase cleaves monophosphonucleosides to the respective nucleoside, controlling the purinergic neurotransmission. Adenosine can mediate different cellular functions by

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operating G-protein-coupled receptors ($A_1$, $A_{2A}$, $A_{2B}$, $A_3$), which can inhibit ($A_1$) or facilitate ($A_2$) neuronal communication [22].

Evidence suggests that ATP and acetylcholine are co-released together in a $Ca^{2+}$-dependent manner [11]. After being released, the neurotransmitter acetylcholine is cleaved into choline and acetate by acetylcholinesterase (AChE, EC 3.1.1.7). This enzyme is a serine hydrolase related to type B carboxylesterases family that exerts an important regulation of acetylcholine levels at synaptic cleft and at neuromuscular junctions [45]. Furthermore, ATP can facilitate [40] or inhibit [15] acetylcholine release in different preparations. It is also possible that the acetylcholine release might be under dual opposite regulation by acting on facilitatory P2X or inhibitory P2Y receptors [40] already identified in zebrafish genome [30,41].

Zebrafish (*Danio rerio*) is a teleost of only 3–5 cm in length, which belongs to Cyprinidae family. Many properties made this animal an important emerging vertebrate model in many biological areas [24]. Zebrafish has shown genetic and anatomic conservation with both mice and humans and a high degree of genetic similarities [3,19]. Recently, this fish has being useful in biochemistry and toxicological studies, due to the presence of similar physiological response to apomorphic animals when exposed to different compounds, including organophosphate and carbamate pesticides and metals, such as $Zn^{2+}$ and $Cd^{2+}$ [43,44]. This fish presents a unique situation among vertebrates, as the AChE is the only ACh-hydrolyzing enzyme present [6]. Butyrylcholinesterase, another enzyme that can also hydrolyze ACh, is not encoded by zebrafish genome. The AChE gene is already cloned, sequenced and this enzyme activity was detected in zebrafish brain [7]. Furthermore, muscarinic and nicotinic receptor subunits are also expressed in neurons of this specie [52,49] and the use of zebrafish to test methanol as cryoprotectant solution in vitrification protocols has been demonstrated in the literature [50].

The presence of NTPDase and ecto-5′-nucleotidase activities has been already described in zebrafish brain membranes [36,42]. Considering (i) the co-release of ATP and acetylcholine at nerve endings; (ii) the use of methanol in zebrafish embryo cryoconservation protocols; (iii) the fact that this compound is an environmental contaminant, the aim of this study was to test the in vivo (acute) and in vitro effects of methanol on ecto-nucleotidase and acetylcholinesterase activities in zebrafish brain, followed by an expression pattern analysis after short-term methanol treatment.

## 2. Methods

### 2.1. Animals

Adult zebrafish of both sexes were obtained from commercial supplier and acclimated for at least 2 weeks in a 50-l aquarium. The fish were kept between 25±2 °C under a natural light–dark photoperiod. The use of animals was according to the National Institute of Health Guide for Care and Use of Laboratory Animals.

### 2.2. In vivo and in vitro treatments

For acute treatment, fish were kept in 20-l aquariums and exposed to water contaminated with methanol for 1 h in three different concentrations (0.25%, 0.5% and 1.0%). For in vitro assay, methanol was added directly to reaction medium before the pre-incubation and maintained throughout the enzyme assays. The final concentration of methanol was in the range of 0.25% to 3%.

### 2.3. Determination of ecto-nucleotidase activities

The preparation of brain membranes was according described previously [4]. After being sacrificed, zebrafish brains were removed of cranial skull by dissection technique and 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 Teflon-glass homogenizer. The samples were centrifuged at 1000×*g* during 10 min and the pellet was discarded. The supernatant was centrifuged for 25 min at 40,000×*g*. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris-citrate buffer and recentrifuged for 20 min at 40,000×*g*. This fresh–thaw–wash procedure was used to ensure the lysis of the brain membranes. The final pellet was resuspended and used in the enzyme assays. All samples were maintained at 2–4 °C throughout preparation.

The conditions of enzyme assay were performed as described previously [36,42]. 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 ecto-5′-nucleotidase activity) in a final volume of 200 μl. The samples were pre-incubated 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 200 μl 10% trichloroacetic acid and chilled on ice for 10 min. Samples (0.4 ml) 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) [12]. 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 nanomole of Pi released per minute per milligram of protein. Four different experiments were performed and all enzyme assays were run at least in triplicate.

### 2.4. Determination of AChE activity

Zebrafish brains were homogenized on ice in 60 volumes (v/w) of 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 rate of acetylthiocholine hydrolysis (ACSCh, 0.8 mM) in 2 ml assay solutions with 100 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB was determined using a method

Table 1
PCR primer design

| Enzymes | Sequences (5′–3′) | Annealing temperature (°C) | PCR product (bp) | GenBank accession number |
|---|---|---|---|---|
| NTPDase1 | CCCATGGCACAGGCCGGTTG (forward) | | | |
| | GCAGTCTCATGCCAGCCGTG (reverse) | 54 | 380 | AAH78240 |
| NTPDase2_mg [a] | GGAAGTGTTTGACTCGCCTTGCACG (forward) | | | |
| | CAGGACACAAGCCCTTCCGGATC (reverse) | 64 | 554 | XP_697600 |
| NTPDase2_mq [a] | CCAGCGGATTTAGAGCACGCTG (forward) | | | |
| | GAAGAACGGCGGCACGCCAC (reverse) | 64 | 313 | XP_687722 |
| NTPDase2_mv [a] | GCTCATTTAGAGGACGCTGCTCGTG (forward) | | | |
| | GCAACGTTTTCGGCAGGCAGC (reverse) | 64 | 263 | AAH78419 |
| AChE | CCAAAAGAATAGAGATGCCATGGACG (forward) | | | |
| | TGTGATGTTAAGCAGACGAGGCAGG (reverse) | 60 | 556 | NP_571921 |
| β-Actin | GTCCCTGTACGCCTCTGGTCG (forward) | | | |
| | GCCGGACTCATCGTACTCCTG (reverse) | 54 | 678 | AAC13314 |

[a] Corresponds to the two first amino acid residues of the protein sequence.

previously described [21]. Before the addition of substrate, samples containing protein (10 μg) and the reaction medium described previously were pre-incubated for 10 min at 25 °C. The substrate hydrolysis was monitored by the formation of thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s). Controls without the homogenate preparation were performed in order to determine the non-enzymatic hydrolysis of ACSCh. The linearity of absorbance towards time and protein concentration was previously determined. AChE activity was expressed as micromole of thiocholine (SCh) released per hour per milligram of protein. We have performed four different experiments, all run in duplicate.

### 2.5. Protein determination

Protein was measured using Coomassie Blue as color reagent [8] and bovine serum albumin as a standard.

### 2.6. Phylogenetic analysis

Sequence alignment was performed using ClustalX program [47] and a phylogenetic tree was constructed according neighbor-joining method [39] using proportional ($p$) distance with MEGA 2.1 program [31].

### 2.7. Reverse transcription–polymerase chain reaction (RT-PCR)

In order to obtain NTPDase1 and NTPDase2 zebrafish orthologous genes, the mouse proteins sequences (AAH11278 and NP_033979) were used as query. NCBI Blast searches of GenBank yielded one zebrafish sequence similar to NTPDase1 (AAH78240) and three different isoforms of NTPDase2 (XP_697600, XP687722 and AAH78419). PCR NTPDase1, NTPDase2_mg, NTPDase2_mq, NTPDase2_mv and AChE primers were designed based on sequences obtained throughout GenBank and the optimal conditions for primers annealing were determined (Table 1). The β-actin primers were designed as described previously [13].

Total RNA was isolated from zebrafish brain using Trizol reagent (Invitrogen) in accordance with manufacturer instructions. RNA was quantified by spectrophotometry and all samples were adjusted to 160 ng/μl. cDNA species were synthesized with SuperScript™ First-Strand (Synthesis System for RT-PCR) Invitrogen Kit following the suppliers. PCR reactions for different NTPDase2 and β-actin genes were performed in a total volume of 20 μl, 0.1 μM primers (Table 1), 0.2 μM dNTP, 2 mM $MgCl_2$ and 0.5 U Taq DNA polymerase (Invitrogen). The PCR conditions for NTPDase1 were similar as described above, except that 1.5 mM $MgCl_2$ was employed. PCR reaction for AChE was performed in a total volume of 25 μl, 0.08 μM primer (Table 1), 0.2 μM dNTP, 2 mM $MgCl_2$ and 1 U Taq DNA polymerase (Invitrogen). The following

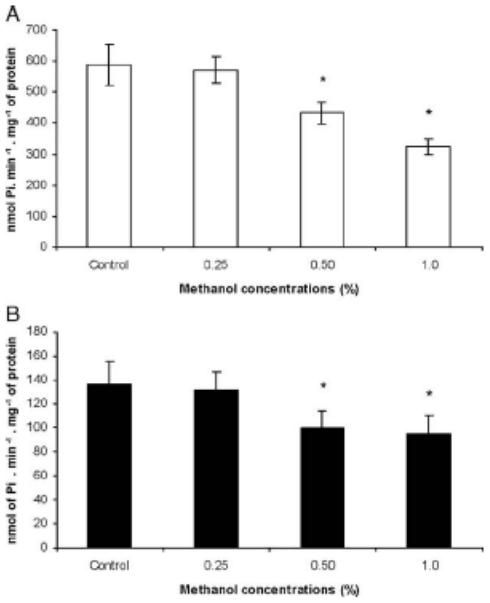


Fig. 1. Effect of acute methanol treatment on NTPDase activities in zebrafish brain membranes. ATP (A) and ADP (B) hydrolysis were evaluated in three different concentrations (0.25%, 0.5% and 1.0%). Bars represent the mean ± S.D. of at least three different experiments. Data were analyzed by ANOVA followed by a Duncan multiple range test, considering $P \leq 0.05$ as significant. *Significantly different from control group.

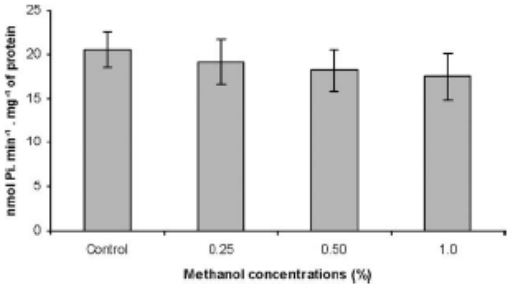


Fig. 2. Ecto-5′-nucleotidase activity from zebrafish brain membranes after acute methanol treatment. AMP hydrolysis was evaluated in three different concentrations (0.25%, 0.5% and 1.0%). Bars represent the mean±S.D. of at least three different experiments. Data were analyzed by ANOVA followed by a Duncan multiple range test, considering $P \leq 0.05$ as significant.

conditions were used for the PCR reactions: 1 min at 94 °C, 1 min for annealing temperature (see Table 1) and 1 min at 72 °C for 35 cycles. Post-extension at 72 °C was performed for 10 min. For each set of PCR reactions, negative control was included. PCR products were analyzed on 1.5% agarose gel, containing ethydium bromide and visualized with ultraviolet light. The Low DNA Mass Ladder (Invitrogen) was used as molecular marker and normalization was performed employing β-actin as a constitutive gene.

### 2.8. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), being expressed as means+ S.D. A Duncan multiple test range as post-hoc was performed, considering a level of significance of 5%.

## 3. Results

The acute methanol exposure was evaluated on ecto-nucleotidase and AChE activities in zebrafish brain. After 1 h in vivo methanol exposure at varying concentrations at the range of 0.25% to 1.0%, NTPDase activity was significantly inhibited at 0.5% and 1.0% methanol. After acute treatment, there were significant decreases of ATP (Fig. 1A) (26% and 45%) and ADP hydrolysis (Fig. 1B) (26% and 30%) at 0.5% and 1.0%, respectively. There were no significant changes on ecto-5′-nucleotidase activity in zebrafish brain membranes in all methanol concentrations tested (Fig. 2). This same treatment promoted a significant inhibition of zebrafish brain AChE activity in all concentrations tested (39%, 33% and 30.5%) at 0.25%, 0.5% and 1.0%, respectively (Fig. 3).

The inhibitory effect promoted by methanol could be consequence of transcriptional control. From the eight-well characterized enzymes of mammals NTPDase family, four members, NTPDase 1-3 and 8, are tightly bound to plasma membrane with active site facing the extracellular milieu. A phylogenetic analysis was performed in order to try to find orthologous genes in zebrafish. Protein sequences of *Homo sapiens* and *Mus musculus* NTPDase 1 (NP_001767 and AAH11278), NTPDase 2 (NP_982293 and NP_033979), NTPDase 3 (NP_001239 and AAQ86585) and NTPDase 8 (AAR04374 and AAQ84519), respectively, were retrieved from GenBank and used on phylogenetic analyses. When both human and mouse NTPDases were used as query, NCBI Blast searches of GenBank yielded in four complete zebrafish NTPDase sequences from which AAH78240 is similar to NTPDase1 and the others (XP_697600, XP_687722, AAH78419) to NTPDase2. At present, zebrafish NTPDases 3 and 8 similar sequences were not found on the GenBank database with the strategy adopted. The phylogenetic tree constructed used Neighbor-Joining method and proportional (*p*) distance (Fig. 4). The AAH78240 sequence grouped consistently with *H. sapiens* and *M. musculus* NTPDase1 sequences, suggesting homologous function in zebrafish. The other sequences must be paralogous since they grouped together and formed the NTPDase2 clade with *H. sapiens* and *M. musculus* sequences. The sequences obtained were used to construct specific primers (Table 1).

RT-PCR analyses were performed when kinetic alteration has occurred. For this reason: (i) ecto-5′-nucleotidase expression was not analyzed and (ii) the concentration of 0.25% was only tested to AChE. The expression patterns after acute methanol exposure were represented (Fig. 5) and have shown that NTPDase1 transcription was inhibited at 1% methanol. Interestingly, all NTPDase2 isoforms (mg, mq and mv) were expressed on zebrafish brain with different intensities. NTPDase2_mv transcription apparently was not affected by methanol, whereas the others were inhibited at 0.5% and 1.0%. All concentrations tested (0.25%, 0.5% and 1.0%) were able to decrease the AChE transcripts.

In order to verify if methanol could modify the ecto-nucleotidase and AChE activities by direct mechanisms, we have performed in vitro assays, with concentrations varying from 0.25% to 3.0%. When added directly to reaction medium, methanol promoted a significant inhibition of ATP hydrolysis (Fig. 6A) (19% and 34%) at 1.5% and 3.0%, respectively, and ADP hydrolysis (Fig. 6B) decreased only at 3.0% (29%). However, the ecto-5′-nucleotidase activity was not affected (Fig. 7). Methanol did not promote any significant effect on

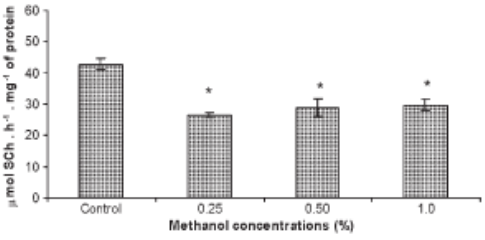


Fig. 3. Inhibition of zebrafish brain AChE activity after acute methanol treatment. ASCh hydrolysis was evaluated in three different concentrations (0.25%, 0.5% and 1.0%). Bars represent the mean±S.D. of at least three different experiments. Data were analyzed by ANOVA followed by a Duncan multiple range test, considering $P \leq 0.05$ as significant. *Significantly different from control group.

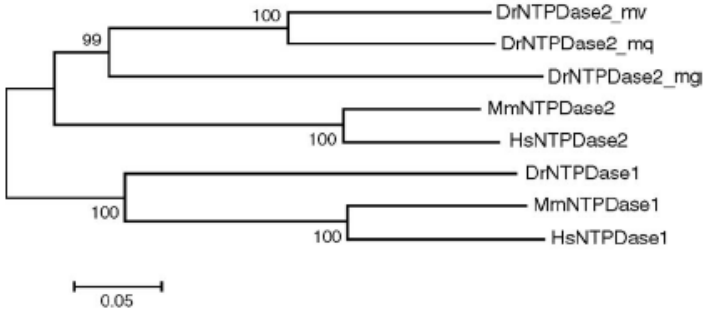


Fig. 4. Phylogenetic analysis of E-NTPDase family, demonstrating the existence of three different isoforms of NTPDase2 in zebrafish genome. The sequence was alignment by ClustalX program and the phylogenetic tree was constructed using neighbor-joining method, using proportional (*p*) distance with MEGA 2.1 program. The phylogenetic tree grouped consistently (Dr) *Danio rerio*, (Mm) *Mus musculus* and (Hs) *Homo sapiens* NTPDase1 and NTPDase2.

AChE activity in zebrafish brain when compared to control group (Fig. 8).

## 4. Discussion

The results presented herein demonstrate the influence of methanol treatment on ecto-nucleotidase and acetylcholinesterase activities and expression patterns in zebrafish brain. Acute exposure significantly inhibited NTPDase activity at higher concentrations of methanol tested. However, AMP hydrolysis did not present any significant modifications after in vivo treatment. Contrasting with the ecto-nucleotidases, zebrafish brain AChE activity was significantly inhibited in all methanol concentrations tested.

A probable direct effect of methanol on these enzymes activities was also evaluated. In vitro experiments demonstrated that methanol was able to promote a significant inhibition on

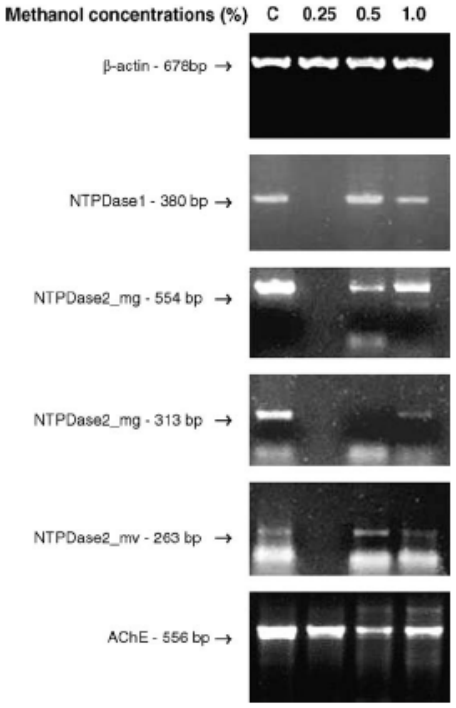


Fig. 5. Gene expression patterns after acute methanol exposure. The figure shows β-actin, NTPDase1, NTPDase2_mg, NTPDase2_mq, NTPDase2_mv and AChE mRNA expression in the brain of adult zebrafish. Fish were exposed to methanol concentrations (0.25%, 0.5% and 1.0%), the brains were excised and total RNA was isolated being subjected to RT-PCR for the indicated targets. RT-PCR products were subjected to eletrophoresis on a 1.5% agarose gel. Three independent experiments were performed, with entirely consistent results.

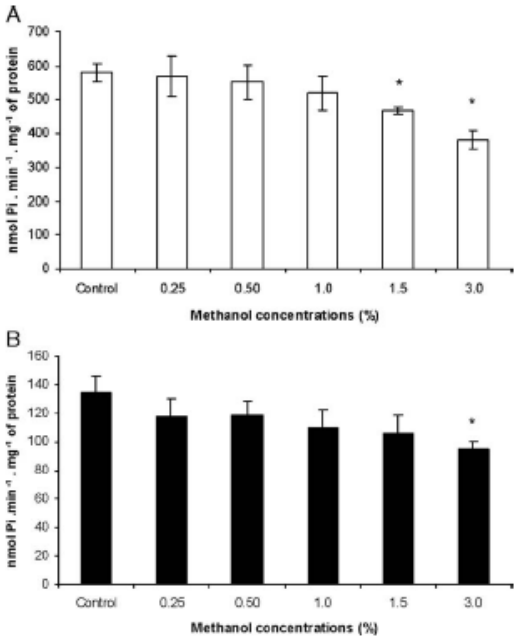


Fig. 6. In vitro effect of methanol on NTPDase activities in zebrafish brain membranes. ATP (A) and ADP (B) hydrolysis were evaluated in different concentrations (0.25–3.0%). Bars represent the mean±S.D. of at least three different experiments. Data were analyzed by ANOVA followed by a Duncan multiple range test, considering $P \leq 0.05$ as significant. *Significantly different from control group.

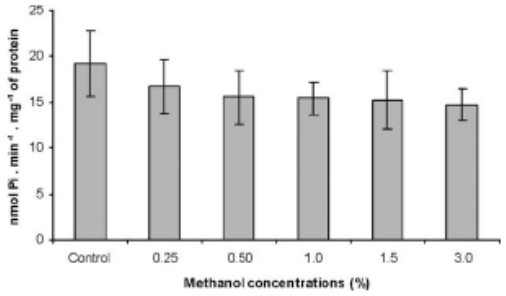


Fig. 7. In vitro effect of methanol on AMP hydrolysis in zebrafish brain membranes. The ecto-5′-nucleotidase activity was evaluated in different concentrations (0.25–3.0%). Bars represent the mean±S.D. of at least three different experiments Data were analyzed by ANOVA followed by a Duncan multiple range test, considering $P \leq 0.05$ as significant.

ATP and ADP hydrolysis, but no significant changes were observed on ecto-5′-nucleotidase activity. The zebrafish brain AChE activity was also not altered.

The inhibitory effect promoted by in vivo methanol treatment on NTPDase activity could be attributed to a possible indirect effect of this compound, since there were no significant changes on ATP and ADP hydrolysis when methanol (0.5% and 1%) was added directly to the reaction medium. Methanol in vitro did not cause any significant change on zebrafish brain AChE, but after 1 h exposure, ACh hydrolysis significantly decreased when compared to untreated fish. Based on these findings, it is possible to suggest that methanol did not act directly on zebrafish brain NTPDases and AChE at lower concentrations tested, leading us to investigate a possible indirect mechanism able to affect ecto-nucleotidases and AChE after exposure to this compound.

These changes in the enzyme activities may occur by modulations via transcriptional or post-translational mechanisms. A phylogenetic analysis was performed, suggesting the presence of orthologous NTPDase1 and three distinct isoforms of NTPDase2 activities on zebrafish. To verify if the inhibition observed in ATP, ADP and ACh hydrolysis from zebrafish brain could be due to alteration on gene expression, NTPDase and AChE primers were synthesized and RT-PCR experiments were conducted. The results have shown a decrease on NTPDase1, NTPDase2_mg, NTPDase2_mq and AChE transcript levels after methanol treatment. Interestingly, NTPDase2_mv, which is the isoform less expressed in zebrafish brain, apparently was not affected by methanol exposure in the concentrations tested. Hence, the lower NTPDase1, NTPDase2_mg, NTPDase2_mq and AChE genes expression could be involved in the significant decrease observed in these enzymes activities after methanol treatment.

Neurotoxins, such as methylazoxy-methanol are able to influence transduction signal pathways, modifying PKC activity [17,18]. Zebrafish AChE, NTPDase1 and all NTPDase2 isoforms protein sequences present possible PKC phosphorylation sites, according to analysis performed in NetPhosk, a kinase-specific prediction of protein phosphorylation sites tool. The inhibition on ecto-nucleotidase and AChE activities could also be attributed to a methanol effect on signaling pathways involved in the possible post-translational modulation of these enzymes.

Methanol is initially metabolized by alcohol dehydrogenase in liver to formaldehyde, which is further oxidized to formic acid or formate. The acute and short-term toxicity of methanol varies significantly between different species, being relatively higher in organisms with poor ability to metabolize formate [28]. Although the metabolism of this alcohol in fish is poorly understood, studies have shown that formaldehyde and formate are cytotoxic compounds. The metabolite formaldehyde can easily react with amino and sulfhydryl groups of biological molecules, causing alkylation, mutations and cross-links that destroy the function of membranes, proteins and nucleic acids [25]. Formaldehyde is able to regulate the expression of glutathione-dependent formaldehyde dehydrogenase [26]. Fish exposed during a short period to lethal concentrations of methanol exhibited hyperactivity and convulsion, demonstrating a decrease of opercular movements with signs of suffocation [29]. The toxicity of formic acid is able to promote morphologic changes in optic nerve cell cultures after this methanol metabolite exposure [48]. A possible influence of these metabolites on nucleotide and on acetylcholine hydrolysis in zebrafish brain should not be discarded.

Vitrification technique requires the use of cryoprotectants concentrations that avoid intracellular and extracellular ice crystal formation [50]. Studies have shown the use of methanol as an important cryoprotector [50,37]. This alcohol was tested in zebrafish and turbot embryos vitrification protocols and the influence on different enzymes activities, such as lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (G6PD) was described [37]. The methanol concentration used as cryoprotectant in these protocols was the same tested in our in vitro experiments (3% or 2 M) and demonstrated a significant dropped on LDH and G6PD zebrafish embryos activities. It is also already known that lipophilic (hydrophobic) interactions are mainly responsible for maintenance of the tertiary conformation of native proteins [46]. Alcohols, for example, can strongly bind to lipophilic moieties of proteins, alter the lipophilic core region, decrease the binding of lipophilic

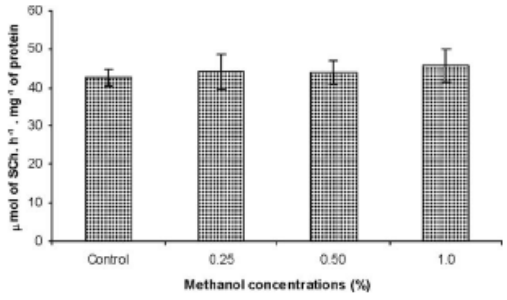


Fig. 8. Effect of methanol on AChE activity in zebrafish brain after in vitro assays. The AChE activity was evaluated in three different concentrations (0.25%, 0.5% and 1.0%). Bars represent the mean±S.D. of at least three different experiments. Data were analyzed by ANOVA followed by a Duncan multiple range test, considering $P \leq 0.05$ as significant.

moieties to each other and thus disrupt the tertiary structure [1,34]. While solvent concentrations in the in vitro studies are typically much higher those currently recommended, solvents exhibited significant inhibition of some isoenzymes at very low concentrations [27]. Based on these considerations, we tested in vitro several methanol concentrations in order to observe ecto-nucleotidases and acetylcholinesterase in situations that this solvent can act as potential contaminant, such as in environmental poisoning, occupational exposure and vitrification protocols.

Studies in rat demonstrated that methanol intoxication increased lipid peroxidation and depleted the free radical scavenging enzyme systems [35]. Methanol is a compound that can induce lipid peroxidation and the metabolism of this compound is accompanied by formation of superoxide anion and hydrogen peroxide that could modify the function of proteins, antioxidant enzymes and nucleotides [5]. The in vitro inhibition of NTPDase activity could be related to a susceptibility of these enzymes at higher methanol concentrations, which could interact with the membranes, modifying the protein structure of ecto-nucleotidases. Furthermore, considering these aspects, it is not possible to rule out oxidative damage induced by methanol in zebrafish brain after in vivo experiments.

In the literature, there is a correlation between apoptosis and methanol exposure in CNS from *Drosophila* embryos [32]. It is known that extracellular ATP can act on purinergic receptors P2X or P2Y. A subclass of P2X receptor, P2X7, has been already identified in zebrafish genome and it is usually associated with apoptosis events [30]. Consequently, extracellular ATP and P2 receptors may play a crucial role in neuropathological events of brain injuries [38,2] and activate a response mediated by caspases [10]. The inhibition of NTPDase activity in zebrafish brain membranes could significantly increase extracellular ATP levels and stimulates apoptosis.

In summary, the results demonstrated that purinergic and cholinergic systems are affected by acute methanol exposure due to NTPDase and AChE inhibition in zebrafish brain. These phenomena could be elicited by different mechanisms: (i) regulation of these enzymes activities at transcriptional level, (ii) modification of transduction signal pathways and (iii) indirect effect promoted by methanol metabolites on NTPDase and AChE, regulating the levels of the ligands ATP, ADP and acetylcholine on their respective receptors. Our findings are important to elucidate possible events promoted by this alcohol on brain injuries. The use of high methanol concentrations in vitrification protocols should be reevaluated and studies with embryos are necessary to better understand if this organic compound can induce any damage on zebrafish CNS development.

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II.2. Capítulo 2 – RICO, E.P., ROSEMBERG, D.B., DIAS, R.D., BOGO, M.R., BONAN, C.D. Ethanol alters acetylcholinesterase activity and gene expression in zebrafish brain. (artigo submetido ao periódico *Neurotoxicology and Teratology*).

**Ethanol alters acetylcholinesterase activity and gene expression in zebrafish brain**

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**Abstract**

Acute ethanol administration exerts a variety of actions on the central nervous system (CNS). Zebrafish has been used as an attractive model system to investigate mechanisms promoted by alcohol intoxication. However, the interactions between ethanol consume and cholinergic neurotransmission still remain unclear. Here we investigated *in vitro* and *in vivo* effects promoted by ethanol and its metabolites on zebrafish brain acetylcholinesterase (AChE). AChE activity presented a significant increase (33%) at 1.0% ethanol after acute exposure. However, ethanol *in vitro* did not alter AChE activity. Acetaldehyde, the first metabolite of alcohol metabolism, promoted a dose-dependent decrease (15%, 27.5% and 46.5%) at 0.25%, 0.5% and 1%. Acetate, product of acetaldehyde degradation, did not change AChE activity. Ethanol was able to inhibit AChE transcripts at 0.5 and 1.0%. These findings suggest that the alterations on zebrafish AChE could reveal molecular mechanisms related to cholinergic signaling in alcoholism.

**Key words:** ethanol, acetaldehyde, acetylcholinesterase, zebrafish, alcoholism.

## 1. Introduction

Alcohol abuse is a health problem throughout the world. Alcohol consumption is linked to the occurrence of many pathological conditions, such as various forms of cancer, liver disease, brain damage and fetal injuries during pregnancy [25]. Furthermore, the presence of ethanol impairs motor coordination, sensory perception and cognition [13].

Molecular mechanisms have been postulated in order to explain the ethanol effects, including damage induced by ethanol and its metabolites and the production of oxygen reactive species (ROS) and oxidative stress [32]. The major metabolic pathway for alcohol metabolism is through a two-step enzymatic process that requires nicotinamide adenine dinucleotide ($NAD^+$) as a co-factor. In the first step, the enzyme alcohol dehydrogenase (ADH) converts ethanol to acetaldehyde, reducing $NAD^+$ in the process. In the second step, acetaldehyde is metabolized to acetate by the enzyme aldehyde dehydrogenase (ALDH), again reducing $NAD^+$ [33]. Phylogenetic analysis of two zebrafish ADHs indicates that they share a common ancestor with mammalian ADHs [27]. In addition, there are two minor oxidative pathways for metabolizing ethanol to acetaldehyde in brain, involving cytochrome P450 (CYP2E1) and the enzyme catalase. Although CYP2E1 is present in low levels in CNS, induction of this enzyme together with increased ROS production has been reported in rats after ethanol administration [20]. Furthermore, in the presence of peroxides, the enzyme catalase can also oxidize ethanol to acetaldehyde and acetate [33].

Previous studies in zebrafish demonstrated that ethanol leads to craniofacial abnormalities, cardiac malformations and developmental delays [3,7,27]. This specie is used to study several neurobehavioral parameters, such as locomotor activity, learning,

aggression and social interaction [14,30]. Moreover, different zebrafish strains permit to investigate the genetic determinants involved in regulating the responses to ethanol [10,36].

Ethanol administration leads to an imbalance in different excitatory and inhibitory neurotransmitters such as the GABA, glutamate, dopamine, noradrenaline and acetylcholine [15,12]. Two different types of cholinesterases are able to hydrolyze ACh: acetylcholinesterase (AChE) (E.C.3.1.1.7) and butyrylcholinesterase (BuChE) (E.C.3.1.1.8). It has been demonstrated that BuChE is not encoded by zebrafish genome, but AChE gene is already cloned, sequenced and functionally detected in zebrafish brain [1]. This enzyme can rapidly cleave ACh into choline and acetate and it has been described as a well known biomarker for several environmental contaminants. Previous studies have described that AChE can be affected by carbamate insecticides, methanol and heavy metals in different fish species, such zebrafish and goldfish [28, 31, 35].

Therefore, the aim of this study was to evaluate the *in vivo* and *in vitro* effects of ethanol on acetylcholinesterase activity on zebrafish brain, followed by expression pattern analysis after short-term ethanol treatment.

## 2. Methods

### 2.1. Animals

Adult zebrafish were obtained from commercial supplier. All fish were acclimated to their new environment for at least 2 weeks in 50-L conditioned at 25 ± 2°C under natural light-dark photoperiod. They were used according to the National Institute of Health Guide for Care and Use of Laboratory Animals, being healthy and free of any signs of disease.

The Ethics Committee of Pontifical Catholic University of the Rio Grande do Sul (PUCRS) approved the protocol under the number 477/05 – CEP.

2.2. Chemicals

Ethanol ($C_2H_6O$; CAS number 64-17-5) and acetate ($C_2H_4O_2$; CAS number 127-09-3) were purchased from Merck, and Acetaldehyde ($C_2H_4O$; CAS number 75-07-0) from Fluka. Trizma Base, EDTA, EGTA, sodium citrate, Coomasie Blue G, bovine serum albumin, acetylthiocholine, 5, 5´-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma (USA).

2.3. *In vivo* and *in vitro* treatments

For acute treatment, fish were introduced in 20-L aquariums containing three different concentrations of ethanol v/v (0.25, 0.5 and 1.0%). The animals were maintained during 1 hour and brains were dissected before biochemical and molecular analysis. For *in vitro* assays, ethanol, acetaldehyde and acetate were added to reaction medium before the enzyme preincubation and maintained throughout the enzyme assays. The final concentration of ethanol, acetaldehyde and acetate were in the range of 0.25 to 1.0%.

2.4. Determination of AChE activity

Zebrafish brains were homogenized on ice in 60 volumes (w/v) of 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 rate of hydrolysis of acetylthiocholine (ACSCh, 0.8 mM) in 2 ml assay solutions with 100 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB was determined as described previously [11]. Before the addition of substrate, samples

containing protein (10 μg) and the reaction medium mentioned above were preincubated for 10 min at 25°C. The hydrolysis of substrate was monitored by the formation of thiolate dianion of DTNB at 412 nm for 2-3 min (intervals of 30 s). Controls without the homogenate preparation were performed in order to determine the non-enzymatic hydrolysis of ACSCh. The linearity of absorbance towards time and protein concentration was previously determined. AChE activity was expressed as micromole of thiocholine (SCh) released per hour per milligram of protein. We performed four different replicate experiments.

2.5. Protein Determination

Protein was measured using Coomassie Blue as color reagent [4] and bovine serum albumin as a standard.

2.6. Molecular Analysis

In order to identify AChE zebrafish orthologous genes, the mouse protein sequence (AAH11278) was used as query. NCBI Blast searches of GenBank yielded one zebrafish sequence similar to AChE. Forward (5′-CCAAAAGAATAGAGATGC CATGGACG-3′) and reverse (5′-TGTGATGTTAAGCAGACGAGGCAGG-3′) primers were designed and optimal conditions for RT-PCR were determined. The β-actin primers forward (5′-GTCCCTGTACGCCTCTGGTCG-3′) and reverse (5′- GCCGGACTCATCGTACTCCTG -3′) were used as previously described [8].

Total RNA was isolated from zebrafish brain using Trizol reagent (Invitrogen) in accordance with manufacturer instructions. RNA was quantified by spectrophotometry and

all samples were adjusted to 160 ng/µl. cDNA species were synthesized with SuperScript™ First-Strand (Synthesis System for RT-PCR) Invitrogen Kit following the suppliers. One microliter of RT reaction mix was used as a template for each PCR. PCR for AChE was performed in a total volume of 25 µl using 0.08 µM of each primer, 0.2 µM dNTP, 2 mM $MgCl_2$ and 1 U Taq DNA polymerase (Invitrogen). PCR for β-actin gene was performed in a total volume of 20 µl using 0.1 µM of each primer, 0.2 µM dNTP, 2 mM $MgCl_2$ and 0.5 U Taq DNA polymerase (Invitrogen). PCR were conducted at 1 min at 94 °C, 1 min at 60°C (AChE) and at 54 °C (β-actin), and 1 min at 72 °C for 35 cycles. A post-extension cycle at 72 °C was performed for 10 min.

For each set of PCR, negative control was included. PCR products were analyzed on 1.0% agarose gel, containing ethydium bromide and visualized with ultraviolet light. The Invitrogen 1Kb ladder was used as molecular marker and normalization was performed employing β-actin as a constitutive gene.

### 2.7. Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA), being expressed as mean ± S.D. A Duncan multiple test range as post-hoc was performed, considering a level of significance of 5%.

## 3. Results

The acute ethanol treatment was evaluated on AChE activity in zebrafish brain. The experiments were performed after 1 hour *in vivo* ethanol exposure at varying concentrations 0.25, 0.5 and 1.0%. Ethanol, at 0.25 and 0.5%, did not alter AChE activity in zebrafish

brain membranes. However, this enzyme activity was significantly increased (33%) at 1.0% ethanol when compared to the control group (Fig.1). On the other hand, ethanol did not promote significant changes on zebrafish brain AChE activity when tested *in vitro* at 0.25, 0.5 and 1.0% (Fig 2A).

In order to verify if the metabolites acetaldehyde and acetate could influence AChE activity, these compounds were added directly to reaction medium at 0.25, 0.5, and 1.0%. Acetaldehyde presented a significant effect on AChE, inhibiting *in vitro* ASCh hydrolysis (15%, 27.5% and 46.5%) at 0.25, 0.5, and 1.0%, respectively (Fig.2B). Acetate was not able to promote any alteration in the AChE activity in the same concentrations tested (Fig.2C). Since acetate can mediate ethanol effects [16], we performed experiments testing acetate *in vivo* on AChE activity. The aquariums were treated in the range 0.1 to 1.0% sodium acetate and there were no significant changes on AChE activity (data not shown).

The increased of ASCh hydrolysis promoted by ethanol could be consequence of transcriptional control. RT-PCR analyses were performed when kinetic alterations have occurred. Therefore, the ethanol concentrations tested were 0.5 and 1.0%. The 0.5% ethanol was tested because a trend toward an increase of AChE activity was induced by the treatment with this ethanol concentration. The expression patterns were presented (Fig.3) and have shown that AChE transcription was reduced at 0.5% and 1.0% after acute ethanol treatment.

## 4. Discussion

In the present study, we have shown that ethanol can alter the activity and expression pattern of AChE in zebrafish brain. The relationship between cholinergic system and operant tasks, exposition to novel stimuli, locomotor activity and the performance of

spatial memory tasks is well established [24]. The influence of ethanol has been described in these behavioral parameters in zebrafish [14].

Acute ethanol exposure enhanced the AChE activity, but when the same concentrations have been tested *in vitro*, AChE activity was not modified. There are several mechanisms that can contribute for enzyme activities in biological systems, which include modifications at transcriptional or post-transcriptional levels. The neuronal responses to alcohol involve several hormone and neurotransmitter-activated signal transduction pathways, leading to short-term (acute) and long-term (chronic) changes in gene expression and neuronal function. [19]. In order to verify if the AChE gene could be modulated after ethanol exposure, we have performed semi-quantitative RT-PCR experiments after 0.5 and 1.0% treatments. Interestingly, the results demonstrated that AChE mRNA levels were decreased after 1.0% exposure, suggesting that the increase of AChE activity is not directly related to a higher AChE gene expression. The transcription machinery is continuously controlled by a complex signaling system, creating a set of signals able to adjust gene expression profile of the cell. This signal transduction can be exerted by proteins, products of enzyme reactions or even toxins able to regulate transcription factors [18]. The phenomena known as negative feedback loop [29,17], which is situated at the interface of genetic and metabolic networks, could explain the concomitant increase of ACh hydrolysis and the decrease of AChE mRNA levels in zebrafish brain after ethanol exposure. Furthermore, ethanol could exert an influence on post-translational modulation. Zebrafish AChE sequence presents a high predicted score of possible PKC phosphorylation sites, according to analysis in NetPhosk, a kinase-specific prediction of protein phosphorylation site tool. Therefore, the increase of AChE activity could be attributed to a possible indirect effect of ethanol mediated by a set of molecular and biochemical modulatory mechanisms.

Considering that ethanol metabolites could be involved in the observed effects, we carried *in vitro* assays testing acetaldehyde and acetate in order to verify the action of ethanol metabolites on zebrafish brain AChE activity. Acetaldehyde, first metabolite of ethanol catabolism pathway, inhibited AChE activity in a concentration-dependent manner. Studies have demonstrated the role of acetaldehyde in several neurochemical and pharmacological effects promoted by ethanol. It has been postulated that *in vivo* acetaldehyde production may play a role in the ethanol cytotoxicity, inducing neuronal degeneration [34]. It is not possible to exclude that an important mechanism for ethanol toxicity is lipid peroxidation through the induction of the formation of free radicals and/or acetaldehyde adducts, which could be associated with brain and others organs damage [22,21,32]

Acetate, another product of this pathway, is a short-chain fatty acid that increases after ethanol administration and readily crosses the blood-brain-barrier, being metabolized in brain. This metabolite can be destined to acetyl-CoA, which is formed from acetate well as can be metabolized for energy generation into $CO_2$ and water [5]. Furthermore, evidence has suggested that extracellular acetate can be accumulated and released by cholinergic nerve terminals after stimuli [6], resulting in the increase of ACh levels at synaptic cleft. Since acetate treatment did not alter AChE activity, it is possible to exclude the involvement of this metabolite in the effects induced by ethanol.

Ethanol is able to modulate the action of several neurotransmitters and neuromodulators, including adenosine. This alcohol has been proposed to stimulate adenosine receptors by two mechanisms. The first involves metabolism of acetate, which requires ATP and yields AMP. The latter compound is converted into adenosine by the enzyme 5'-nucleotidase [2]. The second mechanism involves an inhibition promoted by

ethanol on type I equilibrative nucleoside transporter (ENT1), which leads to the accumulation of extracellular adenosine [9]. The stimulation of adenosine $A_{2A}$ receptors leads to the activation of $G_{\alpha s}$-coupled adenyl cyclase, increasing the generation of cAMP and acetylcholine release from rat hippocampus [23]. Consequently, an increase of ACh levels promoted by adenosine $A_{2A}$ receptors could also induce an enhancement of AChE activity by a stoichiometric effect, which could represent an important compensatory mechanism in order to maintain the control of ACh levels during ethanol exposure.

The search for biological alterations on cholinergic system during alcohol exposure in zebrafish may not only render some important insights into the pathophysiology of alcohol dependence, but might also identify neurochemical and molecular mechanisms involved in the alcoholism.

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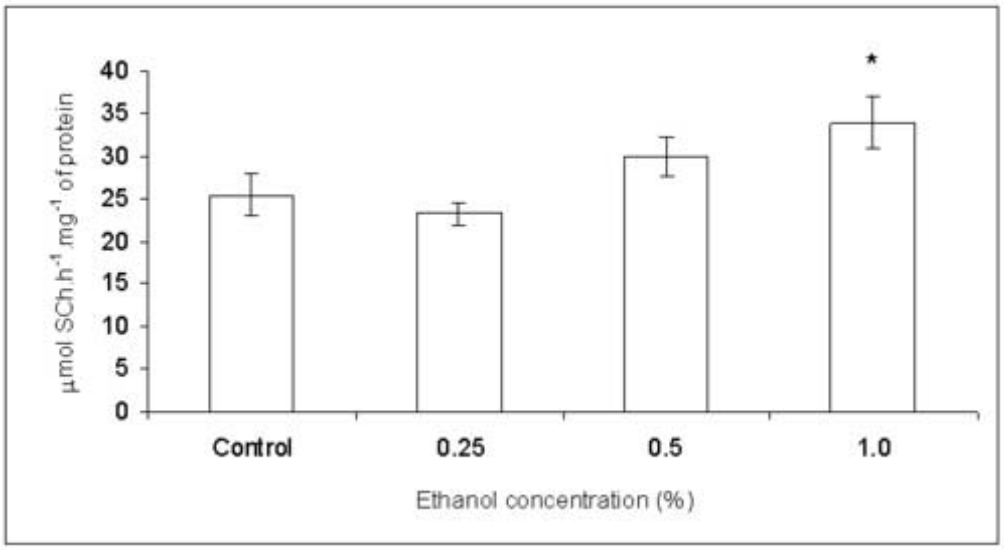
**Figure legends**

Fig.1: *In vivo* effect of ethanol on AChE activity in zebrafish brain homogenates. For acute treatment, fish were exposed to ethanol v/v (0.25, 0.5 and 1.0%) during 1 hour. Bars represent the mean ± S.D. of at least three different experiments. The control AChE activity was 25.5 ± 2.5 micromole of thiocholine released per hour per milligram of protein. (ANOVA followed by a Duncan multiple range test, considering $P \leq 0.05$ as significant). * Significantly different from control group.

Fig.2: *In vitro* effect of ethanol (A), acetaldehyde (B) and acetate (C) on AChE activity in zebrafish brain. ASCh hydrolysis was evaluated in different concentrations (0.25, 0.5 and 1.0%). Bars represent the mean ± S.D. of at least three different experiments. The control AChE activity was 24.82 ± 1.7, 30.47 ± 2.1, 32.33 ± 1.8 micromole of thiocholine released per hour per milligram of protein, respectively. (ANOVA followed by a Duncan multiple range test, considering $P \leq 0.05$ as significant). * Significantly different from control group.

Fig. 3: AChE and β-actin mRNA expression in the brain of adult zebrafish. Fish were exposed to ethanol concentrations (0.5 and 1.0%), the brains were excised and total RNA was isolated being subjected to RT-PCR for the indicated targets. RT-PCR products were subjected to electrophoresis on a 1.0% agarose gel. Three independent experiments were performed, with entirely consistent results.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µmol SCh.h-1 mg-1 of protein
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Control
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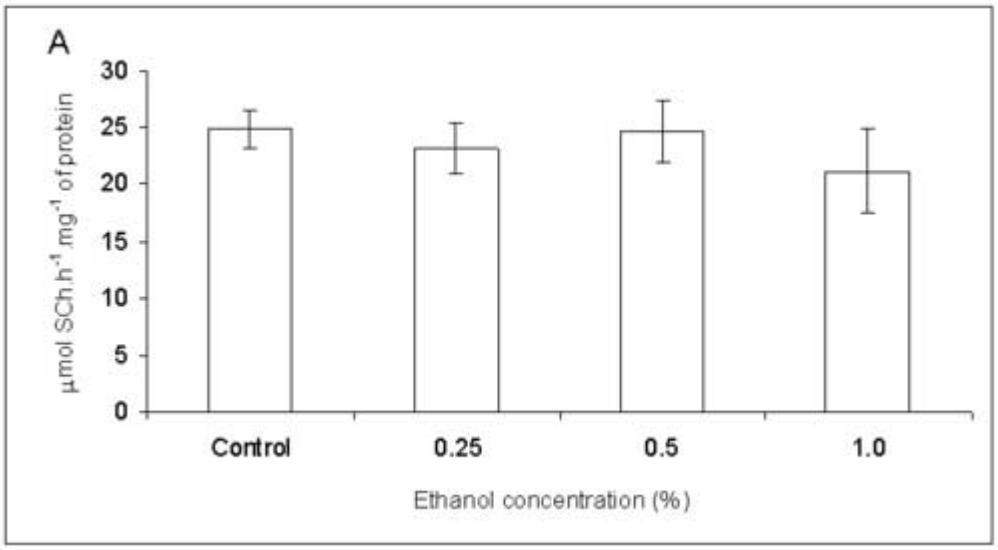
1.0
Ethanol concentration (%)

Fig.1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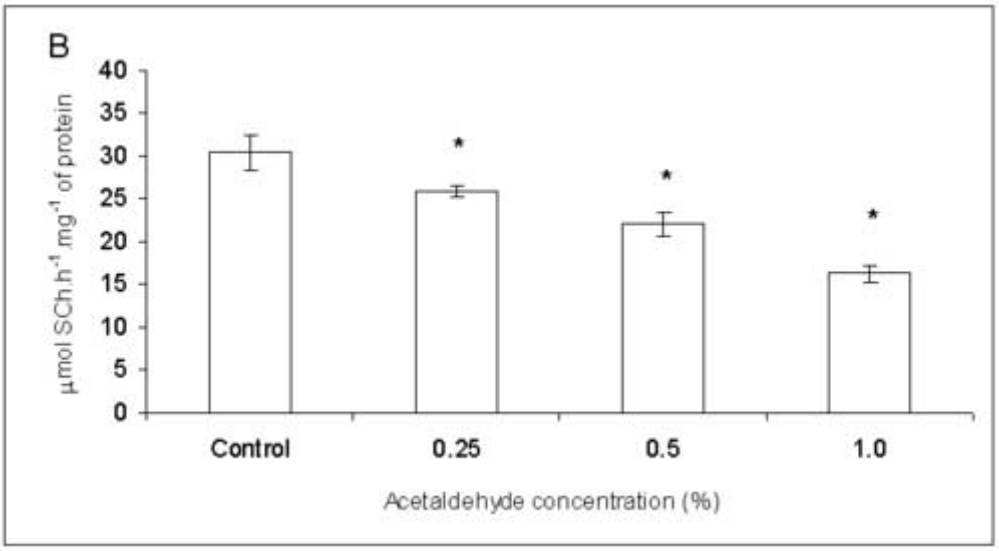
Ethanol concentration (%)

Fig.2A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0.5
1.0
Acetaldehyde concentration (%)

Fig.2B

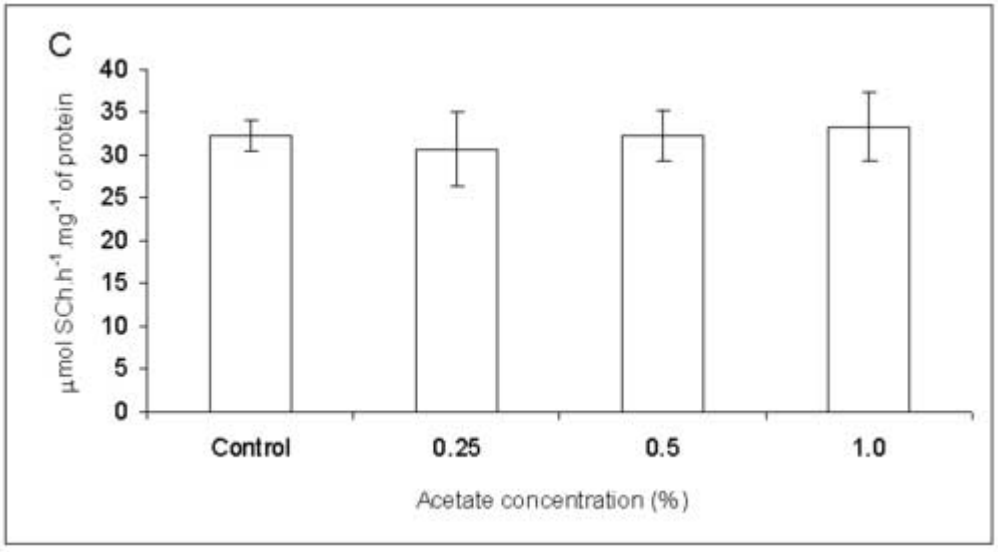
C
40
35
30
25
20
15
10
5
0
µmol SCh.h-1.mg-1 of protein
Control
0.25
0.5
1.0
Acetate concentration (%)

Fig.2C

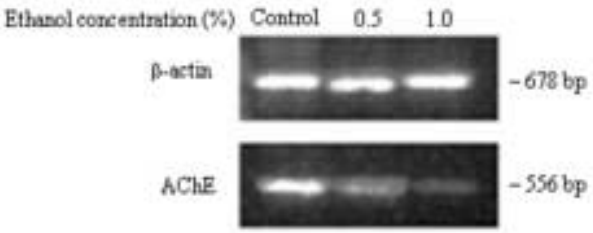
Ethanol concentration (%)
Control
0.5
1.0
β-actin
~678 bp
AChE
~556 bp

Fig.3

II.3. Capítulo 3 –RICO, E.P., ROSEMBERG, D.B., SENGER, M.R., ARIZI M. DE B., DIAS, R.D., SOUTO, A.A., BOGO, M.R., BONAN, C.D. Ethanol and acetaldehyde alters NTPDase and 5'-nucleotidase from zebrafish brain membranes. (artigo submetido ao periódico *Neurochemstry International*).

# Ethanol and acetaldehyde alter NTPDase and 5'-nucleotidase from zebrafish brain membranes

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**Running Title:** Ethanol alters zebrafish ecto-nucleotidases

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**Abstract**

Alcohol abuse is an acute health problem throughout the world and alcohol consumption is linked to the occurrence of several pathological conditions. Here we tested the acute effects of ethanol on NTPDases (nucleoside triphosphate diphosphohydrolases) and 5'-nucleotidase in zebrafish (*Danio rerio*) brain membranes. The results have shown a decrease of ATP (36.3% and 18.4%) and ADP (30% and 20%) hydrolysis after 0.5 and 1.0% (v/v) ethanol exposure during 60 minutes, respectively. In contrast, no changes on ecto-5'-nucleotidase activity were observed in zebrafish brain membranes. Ethanol *in vitro* did not alter ATP and ADP hydrolysis, but AMP hydrolysis was inhibited at 0.5, and 1.0% (23% and 28%, respectively). Acetaldehyde *in vitro*, in the range 0.5-1.0%, inhibited ATP (40%-85%) and ADP (28%-65%) hydrolysis, but AMP hydrolysis was reduced (52%, 58% and 64%) at 0.25, 0.5 and 1.0%, respectively. Acetate in vitro did not alter these enzyme activities. Semi-quantitative expression analysis of NTPDase and ecto-5´-nucleotidase was performed. Ethanol treatment reduced NTPDase1 and three isoforms of NTPDase2 mRNA levels. These findings demonstrate that acute ethanol intoxication may influence the enzyme pathway involved in the degradation of ATP to adenosine, which could affect the responses mediated by adenine nucleotides and nucleosides in zebrafish CNS.

**Keywords:** ethanol, ecto-nucleotidase, NTPDase, ecto-5'-nucleotidase, adenosine, zebrafish.

## 1. Introduction

Alcohol abuse is a significant public health problem. Its consumption affects wide proportion of the general population, being responsible for the occurrence of several mental disorders throughout the world. The acute effects are characterized by a variety of behavioral changes related to motor coordination, sensory perception and cognition (Fleming et al., 2001). Ethanol promotes several biochemical and physiological alterations on central nervous system, involving specific neurotransmitter systems and intricate signaling pathways (Chandler et al., 1997; Esel, 2006).

The zebrafish *Danio rerio* is a small freshwater teleost emerging as an important model in genetic and developmental neurobiology (Zon and Peterson, 2005). Zebrafish genes present a high degree of conservation and share a 70-80% homology to those of humans (Dooley and Zon, 2000). Therefore, zebrafish has become an excellent model system for identifying and understanding the genes responsible for normal vertebrate development, as well genes that regulate sensitivity and resistance to toxicants, including ethanol.

Recently, the terarogenic properties of ethanol have been established in zebrafish, through developmental abnormalities as Fetal Alcohol Syndrome (FAS) and induction of cell death in the CNS (Dlugos and Rabin, 2003). In addition, ethanol influences behavioral parameters, such as locomotor activity, aggression, group preference and pigment response in this specie (Gerlai et al., 2000; Reimers et al., 2004). The gene of alcohol dehydrogenase (ADH), the primary enzyme responsible for the degradation of alcohol, has been described and cloned in this specie (Reimers et al., 2004a, Dasmahapatra et al., 2001).

Extracellular ATP can play an important pivotal role in synaptic transmission, acting as a neurotransmitter and/or a neuromodulator (Cunha and Ribeiro, 2000; Burnstock, 2004). In literature, both ionotropic P2X and G protein-coupled P2Y receptors have already been characterized in this specie (Kucenas et al., 2003). Signaling events induced by extracellular adenine nucleotides are controlled by the action of a variety of surface-located enzymes known as ecto-nucleotidases (Zimmermann, 2001, Robson, 2006). There are important mechanisms involved in the control of ligand concentrations and hence regulate the activation of purinoceptors. Ecto-nucleotidases constitute a highly refined system for the regulation of nucleotide-mediated signaling, controlling the rate, amount and timing of nucleotide degradation and formation. The hydrolysis of ATP to AMP is catalyzed mainly by a family of ecto-nucleotidases named NTPDases (nucleoside triphosphate diphosphohydrolase). The nucleotide AMP is hydrolyzed to adenosine, an important neuromodulator, by the action of an ecto-5'-nucleotidase (Robson et al., 2006; Colgan et al., 2006). Adenosine can mediate different cellular functions by operating G-protein-coupled receptors ($A_1$, $A_{2A}$, $A_{2B}$, $A_3$), which can inhibit ($A_1$ and $A_3$) or facilitate ($A_{2A}$ and $A_{2B}$) neuronal communication (Fredholm et al., 2001).

Recently, we characterized the presence of NTPDase and ecto-5'-nucleotidase activities in brain membranes of zebrafish (Rico et al., 2003; Senger et al., 2004). Orthologous NTPDase1, three NTPDases2 and ecto-5'-nucleotidase genes were identified in zebrafish genome, presenting expression in brain (Rico et al, 2006). Thus, there are important mechanisms involved in the control of nucleotide and nucleoside concentrations, regulating the purinergic neurotransmission.

In order to understand the control of extracellular nucleotide levels in zebrafish brain and considering that: *(i)* Ethanol mediates actions in several excitatory or inhibitory

neurotransmitter systems; *(ii)* P2X receptors play a role in mediating cellular and behavioral effects of ethanol (Franke & Illes, 2006); *(iii)* ethanol activates signal transduction pathways, leading to changes in gene expression and neuronal function (Diamond & McIntire, 2002), the present study evaluated *in vivo* effects of ethanol on activities and the pattern of expression of the ecto-nucleotidase. In addition, *in vitro* effects of ethanol and its metabolites, acetaldehyde and acetate, were also investigated in zebrafish brain membranes.

## 2. Material and Methods

### 2.1.Animals

Adult zebrafish of both sexes were obtained from commercial supplier and acclimated for at least 2 weeks in a 50-L aquarium, with feeding done twice daily. The fish were kept between 25 $\pm$ 2$^{o}$C under a natural light-dark photoperiod. The use of animals was according to the National Institute of Health Guide for Care and Use of Laboratory Animals and the experiments were designed to minimize discomfort or suffering to the animals, as well the number used. The Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS) approved the protocol under the number 477/05 – CEP.

### 2.2. Chemicals

Ethanol ($C_2H_6O$; CAS number 64-17-5) and acetate ($C_2H_4O_2$; CAS number 127-09-3) were purchased from Merck, and acetaldehyde ($C_2H_4O$; CAS number 75-07-0) from Fluka. Trizma Base, malachite green, ammonium molybdate, polyvinyl alcohol, nucleotides, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin,

calcium and magnesium chloride were purchased from Sigma (USA). All other reagents used were of analytical grade.

### 2.3. *In vitro* assays

Ethanol, acetaldehyde and acetate were added to reaction medium before the preincubation with the enzyme and maintained throughout the enzyme assays. The compounds were tested in the following final concentrations: 0.25, 0.5, and 1%.

### 2.4. Acute ethanol exposure

For the *in vivo* treatments, animals were introduced to the test aquariums (20 L) containing solutions of ethanol at three different concentrations (0.25, 0.5 and 1.0%). The animals were maintained in the test aquarium during 1 hour. The acute ethanol exposure has been performed as described previously (Gerlai et al., 2000) and it was able to promote significant changes in zebrafish behavior.

### 2.5. Membrane preparation

The preparation of brain membranes was performed as described previously (Barnes, 1993). Zebrafish were sacrificed, their brains were removed of cranial skull by dissection technique and 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 1.000 *g* during 10 min and the pellet was discarded. The supernatant was centrifuged for 25 min at 40.000 *g*. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris-citrate buffer, and centrifuged

for 20 min at 40.000 *g*. This fresh-thaw-wash procedure was used to ensure the lysis of the brain membranes. The final pellet was resuspended and used in the enzyme assays. All samples were maintained at 2- 4°C throughout preparation.

2.6. Enzyme assays

The conditions of NTPDase and ecto-5'-nucleotidase assay were performed as described previously (Rico et al., 2003; Senger et al., 2004). Zebrafish brain membranes (3-10 □g 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 ecto-5'-nucleotidase activity) in a final volume of 200 □L. 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 200 □L 10% trichloroacetic acid and 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 determinate 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 nanomole 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 using Coomassie Blue as color reagent (Bradford et al., 1976) and bovine serum albumin as a standard.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

In order to obtain NTPDase1 and NTPDase2 zebrafish orthologous genes, the mouse proteins sequences (AAH11278 and NP_033979) were used as query. NCBI Blast searches of GenBank yielded one zebrafish sequence similar to NTPDase1 and three different isoforms of NTPDase2. PCR NTPDase1, NTPDase2_mg, NTPDase2_mq, NTPDase2_mv primers were designed based on sequences obtained throughout GenBank and the optimal conditions for primers annealing were determined (Table 1). The □-actin primers were designed as described previously (Chen et al., 2004).

Total RNA was isolated from zebrafish brain using Trizol reagent (Invitrogen) in accordance with manufacturer instructions. RNA was quantified by spectrophotometry and all samples were adjusted to 160 ng/µl. cDNA species were synthesized with SuperScript™ First-Strand (Synthesis System for RT-PCR) Invitrogen Kit following the suppliers. PCR reactions for different NTPDase2 and □-actin genes were performed in a total volume of 20 µl, 0.1 µM primers (Table 1), 0.2 □M dNTP, 2 mM $MgCl_2$ and 0.5 U Taq DNA polymerase (Invitrogen). The PCR conditions for NTPDase1 were similar to those described above, except that 1.5 mM $MgCl_2$ was employed. The following conditions were used for the PCR reactions: 1 min at 94 °C, 1 min for annealing temperature (see Table 1), 1 min at 72 °C for 35 cycles. Post-extension at 72 °C was performed for 10 min. For each set of PCR reactions, negative control was included. PCR products were analyzed on 1.5%

agarose gel, containing ethydium bromide and visualized with ultraviolet light. The Low DNA Mass Ladder (Invitrogen) was used as molecular marker and normalization was performed employing β-actin as a constitutive gene.

2.9. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), being expressed as means ± S.D. A Duncan multiple test range as post-hoc was performed, considering a level of significance of 5%.

## 3. Results

Ecto-nucleotidase activities were evaluated after ethanol acute treatment in zebrafish brain membranes. After 1 hour of ethanol exposure at varying concentrations at the range of 0.25 to 1.0%, this alcohol was able to inhibit NTPDase activity. There was a significant decrease of ATP (36.3% and 18.4%) and ADP hydrolysis (30% and 20%) at 0.5 and 1.0%, respectively (Fig.1A and 1B). Ecto-5'-nucleotidase was not altered in the concentrations tested (Fig.1C).

Ethanol *in vitro* did not promote a significant effect on ATP and ADP hydrolysis (Fig.2A and 2B), while the AMP hydrolysis was reduced (23.3% and 28.1%) at 0.5 and 1.0%, respectively (Fig.2C). Therefore, our results have shown that ethanol was able to inhibit ATP and ADP hydrolysis *in vivo*, but not *in vitro*. These findings lead us to the investigation of possible indirect effects of ethanol, which could be mediated by its metabolites. Thus, we tested the *in vitro* effect of the metabolites, acetaldehyde and acetate, produced through ethanol degradation pathway (Table 2). Acetaldehyde, the first product of ethanol catabolism, presented a significant effect on ecto-nucleotidases, inducing a decrease

of ATP hydrolysis in a dose-dependent manner (40.5%, 65.8% and 85.5%) at the concentrations 0.25, 0.5 and 1.0%, respectively. When acetaldehyde was added to the enzyme assay, ADP hydrolysis also presented an inhibitory effect (28.5%, 44.7% and 64.7%) in the concentrations 0.25, 0.5 and 1.0%, respectively. There was a significant decrease of AMP hydrolysis in all concentrations of acetaldehyde tested (52.2%, 58.7% and 64.4% in the concentrations 0.25, 0.5 and 1.0%, respectively). In the next step of ethanol catabolism, acetaldehyde is metabolized to acetate. Acetate *in vitro* did not induce alterations on NTPDase and 5'-nucleotidase activities in the concentrations tested (Table 2).

The inhibitory effect promoted by ethanol could be a consequence of transcriptional control. The semi-quantitative RT-PCR analyses were performed when kinetic alterations had occurred. For this reason, 5'-nucleotidase and the concentrations of 0.25% ethanol for NTPDase1 and NTPDases2 isoforms were not analyzed. The constitutive gene was normalized to β-actin expression to allow the comparison in different experimental conditions. Ethanol exposure decreased the expression of NTPDases in zebrafish brain (Fig.3). NTPDase1, NTPDase2_mg and NTPDase2_mv presented a decrease in the level of transcripts after exposure to ethanol 0.5%, while that NTPDase2_mq transcription apparently was not affected. Interestingly, all NTPDases demonstrated a reduction in the transcript levels at 1% of ethanol treatment.

## 4. Discussion

The results presented demonstrate the influence of ethanol on ecto-nucleotidase activities and expression patterns in zebrafish brain. Acute treatment significantly inhibited

NTPDase activity at higher concentrations tested. However, ecto-5'-nucleotidase did not present any significant change after *in vivo* exposure.

To verify if the inhibition observed in ATP and ADP hydrolysis from zebrafish brain could be due to alteration on gene expression, NTPDase and ecto-5'-nucleotidase primers were synthesized and RT-PCR experiments were conducted. The results have shown a decrease on NTPDase1, NTPDase2_mg, and NTPDase2_mv transcript levels after ethanol treatment.

Since these enzymes contribute to maintenance of physiological effects of extracellular ATP, ADP, AMP and adenosine, the influence of the enzymatic cascade involved in the control of these nucleotides and nucleosides have been proposed in several pathophysiological situations (Agteresch et al., 1999). Our results suggest that the inhibitory effect on ATP and ADP hydrolysis observed after ethanol exposure could induce a increase in the extracellular ATP levels and a consequent decrease of adenosine levels. Considering that ATP is an important excitatory neurotransmitter in CNS (Di Iorio et al., 1998), the inhibition of ATP hydrolysis could promote several processes related to brain excitability. As the massive release of extracellular ATP leads to excitotoxicity and cell death via activation of P2X7 receptor in neuronal cells, studies have related this nucleotide to neuropathological events targeting the CNS (Le Feuvre et al., 2002).

In order to verify the direct effect of ethanol on ecto-nucleotidase activities, *in vitro* assays were performed in zebrafish brain membranes. The NTPDase was not altered, but 5'-nucleotidase activity was inhibited at 0.25, 0.5, and 1.0%. Alcohol is believed to interact with biological membranes due to its lipophilic nature. The direct interaction of the ethanol molecule, *per se*, with membrane structures is complex and may involve such membrane components (Lovinger et al., 1989). Ethanol treatment was able to inhibit the NTPDase

activity while alterations were not observed on *in vitro* assays. These results permit to conclude that ethanol could not act directly, but indirectly through its metabolites, acetaldehyde and acetate. Generation of oxygen free radicals and reactive aldehydes as a result of excessive ethanol consumption has been well established and have indicated that acetaldehyde, and the aldehydic products of lipid peroxidation can bind to proteins in tissues forming stable adducts (Niemela O, 2001). Moreover, neuronal responses to alcohol involve several hormone- and neurotransmitter-activated pathways, leading short-term (acute) and long-term (chronic) changes in gene expression and neuronal function (Diamont & McIntire, 2002). Thus, the *in vivo* inhibitory effect on transcriptional and kinetic parameters could be associated to toxicity formation of adducts and oxidation stress.

After alcohol consumption, ethanol is metabolized in the liver through several mechanisms (Ramchandani et al., 2001), including alcohol dehydrogenase (ADH), cytocrome P4502E1 (CYP2E1) and catalase. Acetaldehyde hydrolysis is mainly mediated by aldehyde dehydrogenase (ALDH), producing acetate. It is already known that there are two oxidative pathways for metabolizing ethanol to acetaldehyde in brain: catalase may be responsible for about 60% of the process (Zimatkin et al., 2006) while cytocrome P450 (CYP2E1) is involved in the metabolic conversion of ethanol to reactive oxygen species (Sun & Sun, 2001, Yadav et al., 2006). Furthermore, it is consolidated that acetaldehyde and acetate play a key role in the brain mediating some of the actions of ethanol (Israel, 1994; Dietrich 2004). It is well established that acetaldehyde mediates the toxic effects of ethanol, and studies were aimed at unraveling its effects in pathological conditions (Quertermont et al., 2006). In order to understand the possible effect of products of ethanol metabolism, acetaldehyde and acetate were tested *in vitro* on ecto-nucleotidase activities. Acetaldehyde promoted an inhibition on NTPDase activities in a dose-dependent manner

ranging from 0.25 to 1.0%, while the activity of 5'-nucleotidase was equally inhibited at all concentrations tested.

Acetate is a molecule that promotes significant effects on CNS that can either potentiate or antagonize the effects of ethanol molecule (Carmichael et al., 1991). Acetate *in vitro* was not able to modify ecto-nucleotidase activities. Ethanol has been proposed to stimulate adenosine receptors by two mechanisms. The first involves metabolism of ethanol by liver, which generates acetate that can be metabolized to adenosine in the brain (Carmichael et al., 1991). The main entry point for acetate is its conversion to Acetyl-CoA that requires ATP and yields AMP. This AMP is converted to adenosine by the 5'-nucleotidase (Bianchi and Spychala, 2003). The second mechanism has been demonstrated through the inhibition of the type I equilibrative nucleoside transporter (ENT 1), which leads to accumulation of extracellular adenosine (Choi et al, 2004). The association of NTPDase and 5'-nucleotidase can promote the hydrolysis of ATP, ADP and AMP, leading to the formation of adenosine. To prevent adenosine accumulation, the inhibitory responses promoted by ethanol on NTPDases could be a compensatory mechanism to avoid a significant increase of adenosine levels, which can lead to desensitization of the adenosine receptors (Kiselevski et al., 2003). The action of ethanol on neuromodulatory function of adenosinergic system regulates the release of several neurotransmitters (Fredholm et al., 2005).

Recent evidence indicates that ethanol modulates the function of specific intracellular signaling cascades, including those that contain cyclic adenosine 3', 5'-monophosphate (cAMP)-dependent, protein kinase A (PKA) and protein kinase C (PKC) (Newton and Messing, 2006). Zebrafish NTPDase1 and all NTPDases2 isoforms protein sequences present possible PKC phosphorylation sites, according to analysis performed in

NetPhosk, a kinase-specific prediction of protein phosphorylation site tool. Furthermore, PKC phosphorylates numerous proteins, including transcription factors, which regulate the activity of many genes in the cell nucleus (Dohrman et al., 1997). Besides the decrease in NTPDase transcript levels, the inhibition on these enzyme activities could also be attributed to ethanol effect on signaling pathways involved in the possible post-translational modulation of these enzymes.

In summary, these findings demonstrate the actions induced by ethanol and its metabolites on ecto-nucleotidases in zebrafish brain. This investigation evaluated the relationship between ethanol, recognized for acting in neurotransmission, and the enzymes responsible for the hydrolysis of the neurotransmitter ATP to adenosine. The changes induced by ethanol acute treatment on ecto-nucleotidases suggest that the purinergic system is an interesting target for potential pharmacological studies. Our results could help to clarify the importance of neurochemical effects on purine metabolism associated to alcohol consumption.

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**Figure legends**

Fig.1: Effect of acute ethanol treatment on ecto-nucleotidase activities in zebrafish brain. The ATP (A), ADP (B) and AMP (C) hydrolysis were evaluated in three different concentrations (0.25, 0.5 and 1.0%). Data are expressed as mean ± S.D. of at least three different experiments. The control ATP, ADP and AMP hydrolysis (without ethanol) were 680.3 ± 38, 137.1 ± 11, 22.4 ± 1.6 nmol Pi $min^{-1}$ $mg^{-1}$ of protein, respectively. Asterisk (*) indicates significantly different from control group ($P \leq 0.05$).

Fig.2. *In vitro* effect of varying concentrations of ethanol on ATP (A), ADP (B) and AMP (C) hydrolysis in zebrafish brain membranes. Bars represent the means ± S.D. of at least three different experiments. The control ATPase, ADPase and AMPase activities (no ethanol added) were 559.6 ± 63.5, 114.7 ± 8.9 and 18.9 ±1.8 nmol Pi $min^{-1}$ $mg^{-1}$ of protein, respectively. Asterisk (*) indicates significantly different from control group ($P \leq 0.05$).

Fig.3: Gene expression patterns after acute ethanol exposure. The figure shows □-actin, NTPDase1, NTPDase2_mg, NTPDase2_mq and NTPDase2_mv mRNA expression in the brain of adult zebrafish. Fish were exposed to ethanol concentrations (0.25, 0.5 and 1.0%), the brains were excised and total RNA was isolated being subjected to RT-PCR for the indicated targets. RT-PCR products were subjected to eletrophoresis on a 1.5% agarose gel. Three independent experiments were performed, with entirely consistent results.

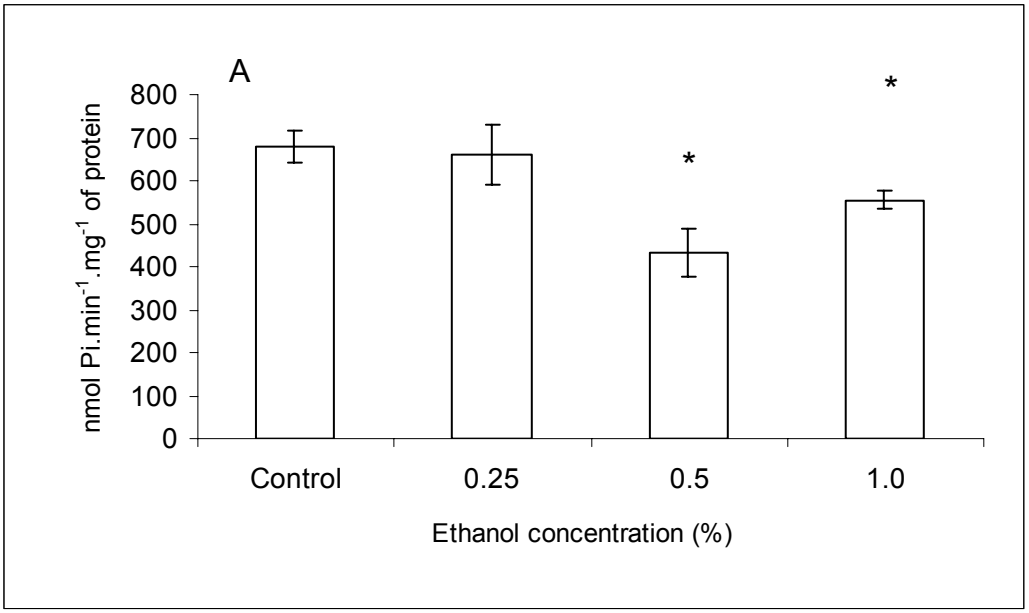
A
800
700
600
500
400
300
200
100
0
nmol Pi.min-1.mg-1 of protein
*
*
Control
0.25
0.5
1.0
Ethanol concentration (%)

Fig.1A

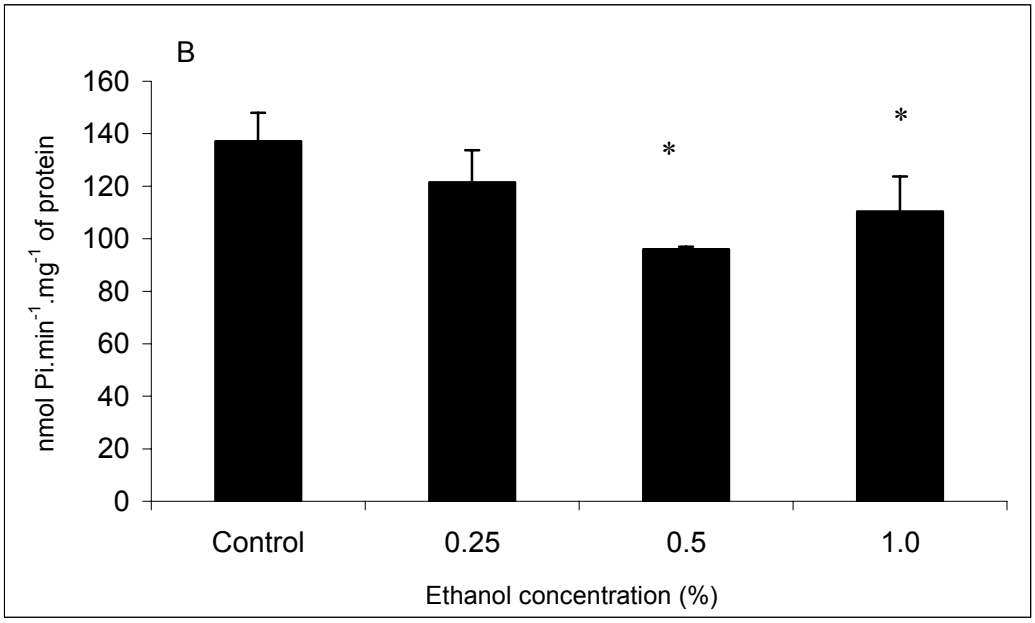
B
160
140
120
100
80
60
40
20
0
nmol Pi.min-1.mg-1 of protein
*
*
Control
0.25
0.5
1.0
Ethanol concentration (%)

Fig. 1B

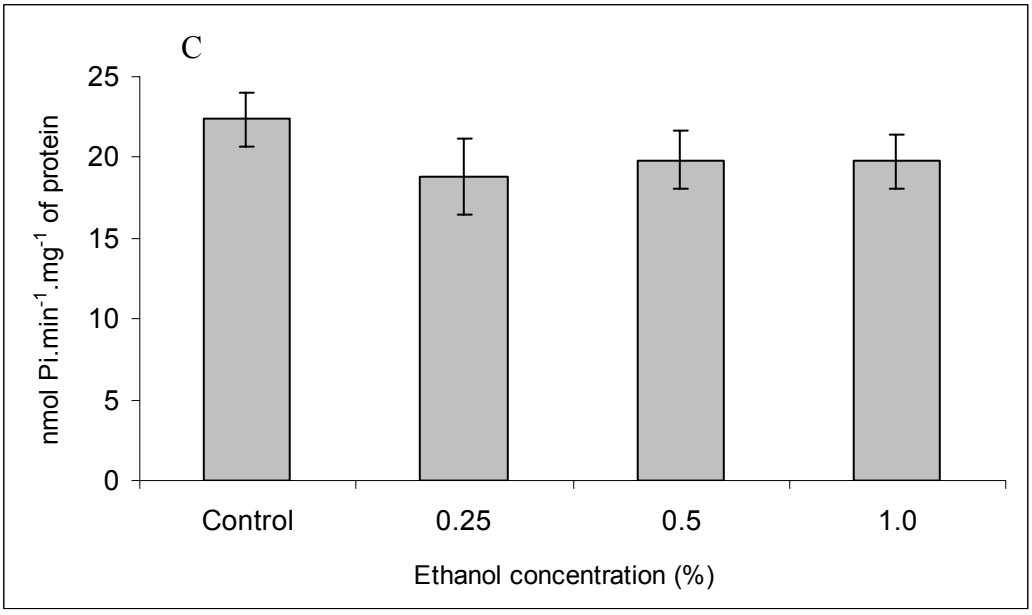
C
25
20
15
10
5
0
nmol Pi.min-1.mg-1 of protein
Control
0.25
0.5
1.0
Ethanol concentration (%)

Fig. 1C

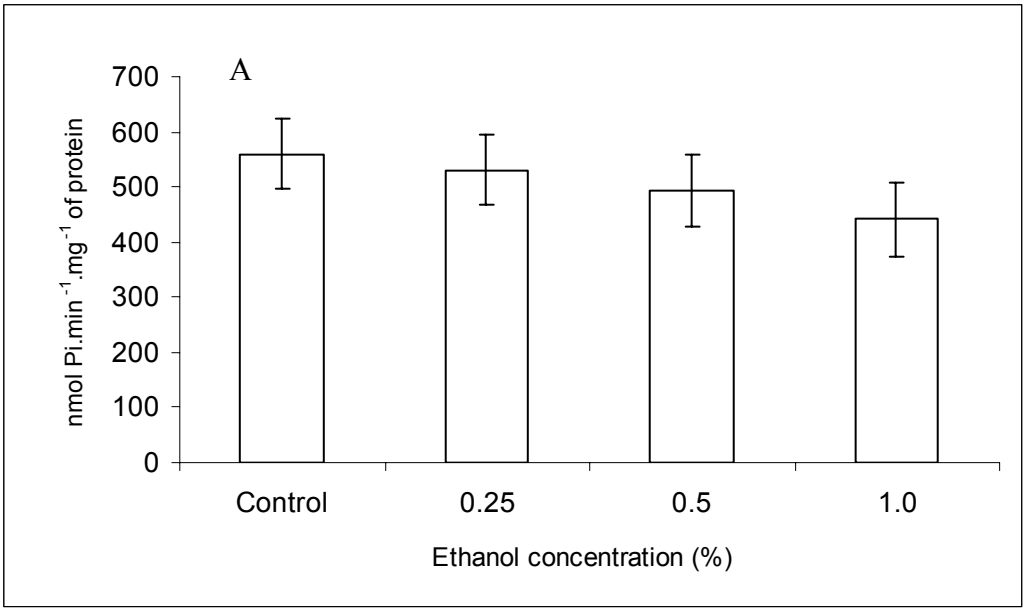
A
700
600
500
400
300
200
100
0
nmol Pi.min -1.mg -1 of protein
Control
0.25
0.5
1.0
Ethanol concentration (%)

Fig. 2A

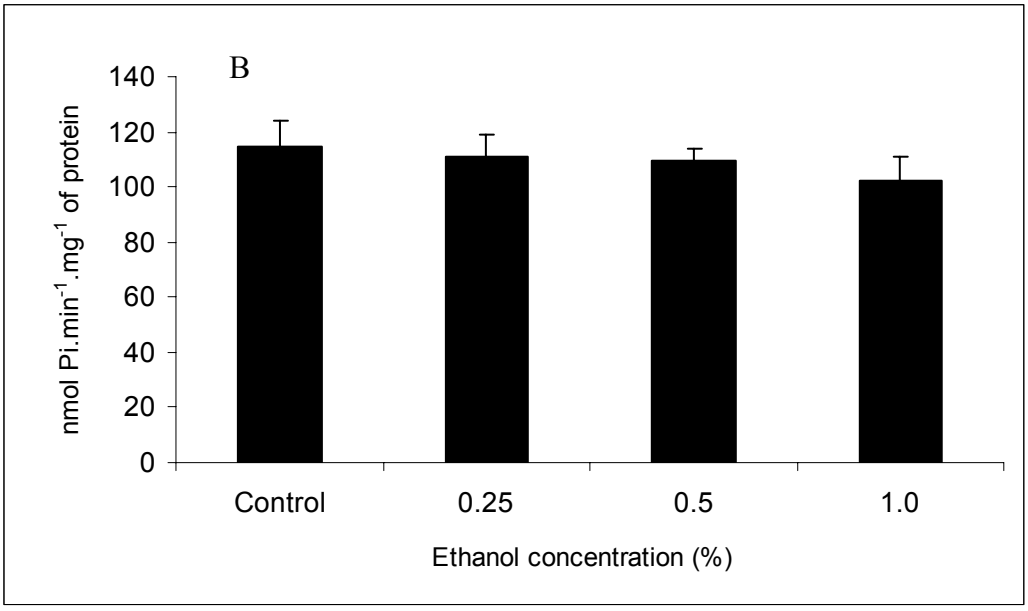
B
140
120
100
80
60
40
20
0
nmol Pi.min-1.mg-1 of protein
Control
0.25
0.5
1.0
Ethanol concentration (%)

Fig.2B

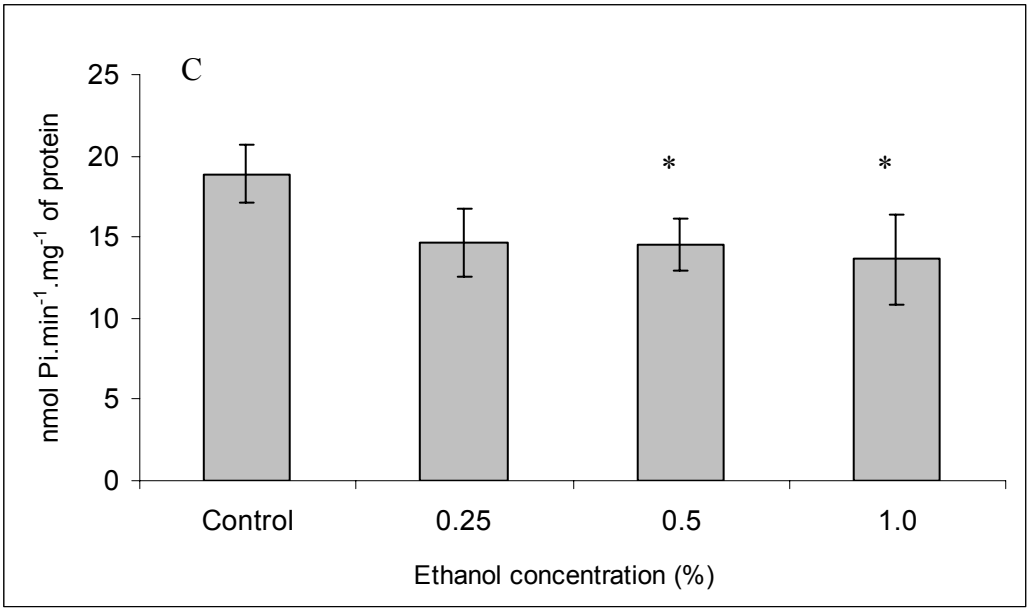
C
25
20
15
10
5
0
nmol Pi.min-1.mg-1 of protein
*
*
Control
0.25
0.5
1.0
Ethanol concentration (%)

Fig.2C

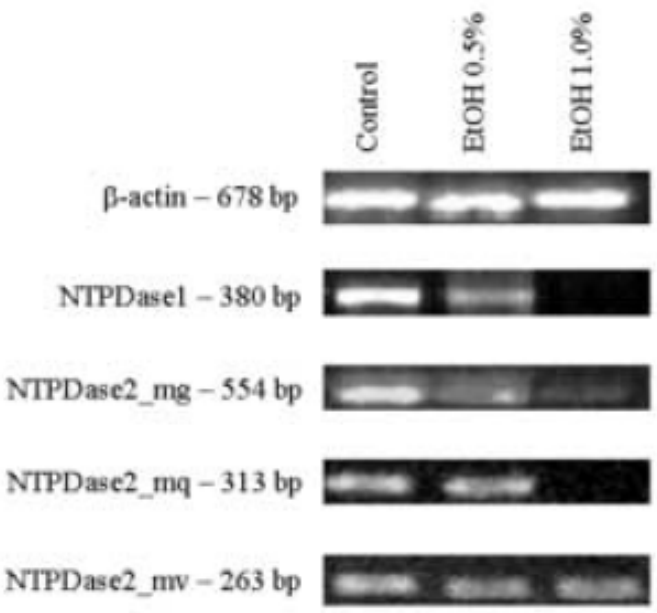
Control
EtOH 0.5%
EtOH 1.0%
β-actin – 678 bp
NTPDase1 – 380 bp
NTPDase2_mg – 554 bp
NTPDase2_mq – 313 bp
NTPDase2_mv – 263 bp

Fig.3

Table 1 : PCR primer design

| Enzymes | Sequences (5'-3') | Annealing temperature (°C) | PCR product (bp) | GenBank Accession number |
|---|---|---|---|---|
| NTPDase1 | CCCATGGCACAGGCCGGTTG (forward) | | | |
| | GCAGTCTCATGCCAGCCGTG (reverse) | 54 | 380 | AAH78240 |
| NTPDase2_mg* | GGAAGTGTTTGACTCGCCTTGCACG (forward) | | | |
| | CAGGACACAAGCCCTTCCGGATC (reverse) | 64 | 554 | XP_697600 |
| NTPDase2_mq* | CCAGCGGATTTAGAGCACGCTG (forward) | | | |
| | GAAGAACGGCGGCACGCCAC (reverse) | 64 | 313 | XP_687722 |
| NTPDase2_mv* | GCTCATTTAGAGGACGCTGCTCGTG (forward) | | | |
| | GCAACGTTTTCGGCAGGCAGC (reverse) | 64 | 263 | AAH78419 |
| β-actin | GTCCCTGTACGCCTCTGGTCG (forward) | | | |
| | GCCGGACTCATCGTACTCCTG (reverse) | 54 | 678 | AAC13314 |

* Correspond to the two first aminoacids residues of the protein sequence.

Table 2. *In vitro* effects of acetaldehyde and acetate on ATP, ADP and AMP hydrolysis in zebrafish brain membranes.

| | Acetaldehyde | | | Acetate | | |
|---|---|---|---|---|---|---|
| | **ATP** | **ADP** | **AMP** | **ATP** | **ADP** | **AMP** |
| Control | 613.1 ± 66.0 | 123.5 ± 16.1 | 21.3 ± 2.6 | 630.1 ± 125.2 | 114.3 ± 6.6 | 22.2 ± 2.3 |
| 0.25 | 365.2 ± 71.1* | 88.3 ± 2.5* | 10.2 ± 1.7* | 598.7 ± 115.1 | 128.8 ± 20.6 | 22.4 ± 4.9 |
| 0.5 | 210.2 ± 57.6* | 68.4 ± 8.8* | 8.7 ± 2.5* | 586.8 ± 144.3 | 124.2 ± 19.4 | 19.3 ± 3.9 |
| 1.0 | 89.5 ± 22.3* | 43.7 ± 6.1* | 7.6 ± 3.2* | 553.1 ± 116.6 | 103.9 ± 4.8 | 18.4 ± 3.5 |

Data represent the mean ± S.D. of at least three different experiments. The control ATPase, ADPase and AMPase activities for acetaldehyde were 613.1 ± 66.0, 123.5 ± 16.1, 21.3 ± 2.6 nmol Pi $min^{-1}$ $mg^{-1}$ of protein, respectively. The control ATPase, ADPase and AMPase activities for acetate were 630.1 ± 125.2, 114.3 ± 6.6, 22.2 ± 2.3nmol Pi $min^{-1}$ $mg^{-1}$ of protein, respectively.

* Significantly different from control group ($P \leq 0.05$) using ANOVA followed by a Duncan multiple range test.

# Parte III

## III.1. Discussão

### *III.1.1. Considerações gerais*

Os resultados apresentados neste estudo demonstram o efeito agudo do metanol e do etanol na atividade e expressão gência e padrão de expressão das ectonucleotidases e acetilcolinesterase em cérebro de zebrafish. Com o objetivo de investigar no genoma do zebrafish a presença dos genes de NTPDase1 e NTPDase2, genes ortólogos de camundongo foram utilizados como molde para busca. No banco genômico do zebrafish, foram encontradas uma seqüência similar a NTPDase1 e três diferentes isoformas de NTPDase2. Uma análise filogenética foi realizada mostrando que no genoma do zebrafish há um gene para NTPDase1 consistentemente agrupado às NTPDases1 humana e de camundongo. Ao comparar os genes de NTPDase2 entre zebrafish, camundongos e humanos, observa-se que estas seqüências estão agrupadas em um mesmo clado com alto valor de suporte, mostrando uma proximidade filogenética para NTPDases2. Para investigar se os genes para estas enzimas estão sendo expressos em cérebro de zebrafish, as seqüências obtidas serviram para a construção de primers específicos. A análise da expressão semi-quantitativa mostrou que estas NTPDases são expressas em cérebro de zebrafish.

### *III.1.2. Efeito in vivo do metanol e etanol sobre ectonucletidases e AChE*

Os tratamentos *in vivo* mostraram que tanto o metanol quanto o etanol são capazes de inibir a hidrólise dos nucleotídeos ATP e ADP, bem como promover redução nos níveis de transcritos na maioria dos genes das NTPDases. Desse modo, é evidente o potencial destes álcoois em alterar estas enzimas tanto em nível molecular quanto cinético nesta

espécie. Sabe-se que estas enzimas contribuem para a manutenção dos níveis extracelulares de ATP, ADP, AMP e adenosina. Diversas situações patofisiológicas podem influenciar esta cascata enzimática (AGTERESCH et al., 1999, BONAN et al., 2001). Assim, nossos resultados sugerem que o efeito inibitório observado na hidrólise de ATP e ADP após a exposição aguda ao metanol e ao etanol poderia induzir um aumento nos níveis extracelulates de ATP e a conseqüente diminuição nos níveis de adenosina.

O ATP extracelular pode agir nos receptores purinérgicos P2X ou P2Y. A subclasse P2X7, já identificada em zebrafish, participa de eventos relacionados a apoptose (KUCENAS et al., 2003). Estudos mostram que o ATP extracelular e receptores P2 podem estar associados em eventos neuropatológicos e injúria cerebral (RYU et al., 2002). A inibição das NTPDases poderia promover um aumento extracelular dos níveis de ATP e estimular a apoptose. Além disso, considerando que o ATP é um importante neurotransmissor excitatório no SNC (Di IORIO et al., 1998), a inibição da hidrólise do ATP poderia promover diversos processos relacionados com a excitabilidade cerebral. Após os tratamentos, não foram observadas alterações na reação de hidrólise do AMP até adenosina, mostrando que a ecto-5´-nucleotidase não foi sensível aos efeitos destes álcoois.

A atividade da AChE pode ser usada como um marcador da função colinérgica e mudanças na atividade da enzima podem indicar alterações na disponibilidade de ACh e do nível de seus receptores (FERNANDES & HODGES-SAVOLA, 1992). O número de moléculas de AChE pode ser aumentado (MOUDGIL & KANUDO, 1973) ou diminuído (BATTIE & MORAN, 1990), dependendo do estímulo recebido. Os níveis de AChE parecem ser controlados pela interação de ACh com seus receptores; quando a interação é acentuada, aumentam os níveis de AChE. A exposição aguda ao metanol promoveu uma significativa inibição na atividade da AChE e nos níveis de mRNA desta enzima. No

entanto, a exposição ao etanol promoveu um aumento na atividade da AChE e uma redução nos níveis de transcritos.

Neurotoxinas como methylazoxy-metanol e etanol, são capazes de influenciar a transdução de sinal, modificando a atividade da PKC (Di LUCA, et al., 1994; NEWTON & MESSING, 2006). AChE, NTPDases1 e as três isoformas de NTPDase2 apresentam possíveis sítios de fosforilação para PKC. As sequências das proteínas foram analisadas através do NetPhosk, uma ferramenta que prediz possíveis sítios de fosforilação para diversas proteínas quinase. A alteração da atividade das ecto-nucleotidases e AChE poderia também ser atribuída ao efeito destes álcoois na via de sinalização envolvida e na possível modulação pós-traducional destas enzimas. Além das modulações promovidas por xenobióticos, o maquinário de transcrição é continuamente controlado por um complexo sistema de sinalização, criando um ajuste no perfil de expressão gênica na célula. Assim, esta transdução pode ser exercida por proteínas e produtos de reações enzimáticas capazes de regular os fatores de transcrição (KRISHNA et al., 2006). Este fenômeno é conhecido como "negative feedback loop", na qual ocorre uma interface nas vias metabólica e gênica e poderia explicar o aumento na atividade com a concomitante redução na nos níveis de mRNA para a AChE em cérebro de zebrafish após aguda exposição ao etanol.

O acetato produzido a partir da degradação do etanol rapidamente é capaz de ultrapassar a barreira-hemato-encefálica, sendo metabolizado no cérebro. Este metabólito pode ser destinado à formação de Acetil CoA utilizado na geração de energia até $CO_2$ e $H_2O$ (CARMICHAEL et al., 1991). Evidências têm sugerido que o acetato extracelular pode ser acumulado e liberado através dos nervos terminais colinérgicos após o estímulo (CARROL, 1997), resultando em um aumento nos níveis de ACh na fenda sináptica. Conseqüentemente, este aumento de ACh poderia induzir um aumento na atividade da

AChE por um efeito estequiométrico, que poderia representar um importante mecanismo compensatório no sentido de manter o controle dos níveis de ACh durante a exposição de etanol. Desde que o tratamento com acetato não alterou a atividade da AChE, é possível excluir a ação deste metabólito em mediar efeitos induzidos pelo etanol.

Além disso, o etanol está envolvido na ação de diversos neurotransmissores e neuromoduladores, incluindo a adenosina. A conversão de acetato em Acetil CoA requer ATP e forma AMP. Este AMP é convertido a adenosina através da 5'-nucleotidase (BIANCHI & SPYCHALA, 2003). Outro mecanismo envolve uma inibição promovida pelo etanol no transportador bidirecional de adenosina (ENT1), levando a um acúmulo de adenosina no espaço extracelular (CHOI et al., 2004). Portanto, as respostas inibitórias promovidas pela exposição aguda ao metanol e ao etanol na atividade e expressão das NTPDases poderiam induzir a uma menor formação de adenosina, uma vez que o prolongado aumento dos níveis de adenosina durante o consumo de etanol pode levar a uma alteração da sensibilidade aos receptores de adenosina (KISELEVSKI, et al., 2003).

### *III.1.3. Efeito in vitro do metanol e etanol sobre ectonucletidases e AChE*

Metanol e etanol também foram testados *in vitro* com o objetivo de verificar se estes compostos podem modificar a atividade das ecto-nucleotidases e AChE diretamente. As enzimas AChE e ecto-5´-nucleotidase não tiveram alterações induzidas por metanol nas suas atividades. Por outro lado, a hidrólise do ATP e do ADP apresentou uma inibição significativa em altas concentrações, que representam exatamente as doses em que o metanol é empregado nos protocolos de vitrificação. Embora o metanol seja um importante crioprotetor (ZHANG, et al., 2005), este álcool foi capaz de inibir outras atividades

enzimáticas, tais como LDH e G6PD (ROBLES et al, 2004). Metanol, quanto testado *in vitro* na concentração de 3%, promoveu uma inibição na hidrólise de ATP e ADP. Entretanto, a hidrólise de AMP e AChE não foram alteradas após a exposição *in vitro*. Baseado nesses dados é possível sugerir que o metanol não atue diretamente nas NTPDases e AChE cerebrais de zebrafish nas baixas concentrações testadas, levando a investigar um possível mecanismo indireto que este composto foi capaz de induzir após a exposição *in vivo*. Álcoois, devido as suas propriedades lipofílicas, podem ligar-se fortemente a proteínas, alterando suas estruturas terciárias (NEUHAUS-STEINMETZ & RENSING, 1997), bem como características das membranas biológicas (CARMICHAEL, et al., 1991).

A atividade NTPDásica após o tratamento in vivo com etanol apresentou uma inibição significativa, enquanto que alterações não foram observadas nos ensaios *in vitro.* Estes resultados permitem concluir que o etanol pode não agir diretamente, mas indiretamente através dos seus metabólitos, acetaldeído e acetato. A geração de radicais livres do oxigênio e de aldeídos reativos, como conseqüência do consumo excessivo do etanol, tem sido bem estabelecida e indica que o acetaldeído e produtos aldeídicos da peroxidação lipídica podem ligar-se às proteínas nos tecidos dando forma a aductos estáveis (NIEMELA, 2001). Além disso, as respostas neuronais ao álcool envolvem diversas vias ativadas por hormônios e neurotransmissores, conduzindo mudanças sobre a expressão gênica e na função neuronal a curto (agudo) e a longo prazo (crônico) (DIAMONT & McINTIRE, 2002). Assim, o efeito inibitório *in vivo* observado nos parâmetros transcricional e cinético podem estar associados à formação de toxicidade através de aductos e dano oxidativo. Após o consumo de álcool, o etanol é metabolizado primariamente no fígado através de diversos mecanismos, incluindo ADH, CYP2E1 e catalase. Acetaldeído é hidrolidado através da ALDH formando acetato. Além disso, sabe-

se que o acetaldeído e o acetato têm um papel chave em mediar as ações do etanol no cérebro (ISRAEL et al., 1994; DEITRICH, 2004; QUERTERMONT et al., 2005). Com o objetivo de entender o possível efeito dos produtos da degradação do etanol, o efeito do acetaldeído e acetato foram testados *in vitro* sobre a atividade das ecto-nucleotidases e AChE. Acetaldeído promoveu uma inibição na atividade NTPDásica de maneira concentração-dependente, enquanto que a atividade da ecto-5'-nucleotidase foi inibida igualmente em todas as concentrações testadas. A atividade da AChE também foi inibida de maneira concentração-dependente. O acetato é uma molécula que promove significativos efeitos no SNC e pode tanto potenciar ou antagonizar os efeitos da molécula de etanol (CARMICHAEL et al., 1991). Entretanto, acetato *in vitro* não foi capaz de modificar as atividades das ecto-nucleotidases e da AChE.

Assim, estes resultados mostram as ações induzidas por metanol e etanol nas ecto-nucleotidases e acetilcolinesterase em cérebro de zebrafish. Esta investigação avaliou a relação entre estes álcoois, reconhecidos por agir na neurotransmissão, e as enzimas responsáveis pela hidrólise nos neurotransmissores ACh e ATP. As alterações transcricionais e cinéticas observadas nestas enzimas após o tratamento agudo com metanol e etanol sugerem que os sistemas purinérgico e colinérgico são interessantes alvos para potenciais estudos farmacológicos. Nossos resultados poderiam ajudar a esclarecer a importância dos efeitos neuroquímicos desses influentes sistemas de neurotransmissão associados ao consumo destes compostos.

## III.2. Conclusão final

Com os resultados apresentados nesta Dissertação de Mestrado, nós podemos concluir que as ectonucleotidases e a acetilcolinesterase são alteradas tanto na sua atividade quanto na expressão gênica no padrão de expressão em cérebro de zebrafish. Essas modificações mostram que essas enzimas são sensíveis à ação de metanol e etanol, sugerindo que os sistemas purinérgico e colinérgico parecem estar envolvidos na neurotoxicidade mediada por estes álcoois.

Portanto, nosso trabalho contribui para um melhor esclarecimento sobre a farmacologia destes álcoois e o papel destas enzimas nas respostas induzidas pelo tratamento agudo do metanol e etanol no SNC de zebrafish.

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# Anexos

**Lista de Figuras:**