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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

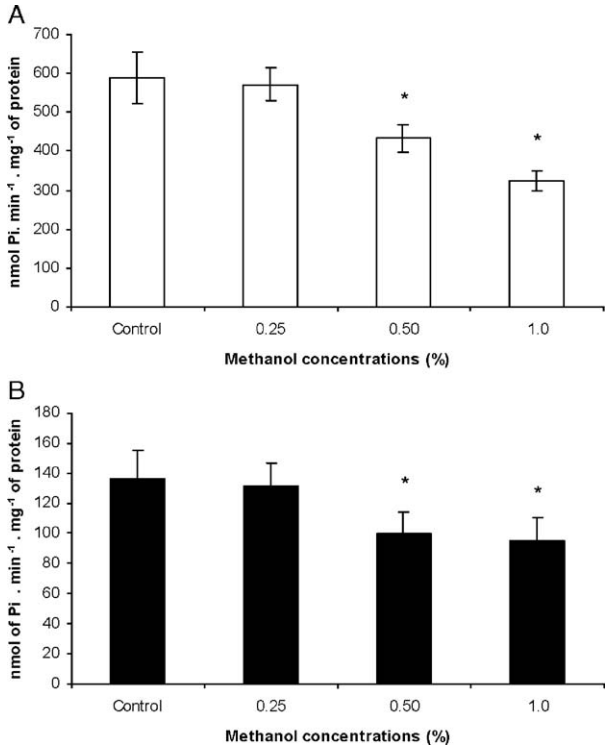

Fig. 1. Effect of acute methanol treatment on NTPDase activities in zebrafish brain membranes. ATP (A) and ADP (B) hydrolysis were evaluated in three different concentrations (0.25%, 0.5% and 1.0%). Bars represent the mean ± S.D. of at least three different experiments. Data were analyzed by ANOVA followed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

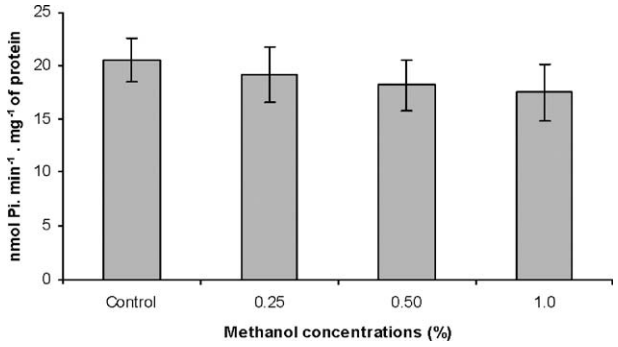

Fig. 2. Ecto-5′-nucleotidase activity from zebrafish brain membranes after acute methanol treatment. AMP hydrolysis was evaluated in three different concentrations (0.25%, 0.5% and 1.0%). Bars represent the mean±S.D. of at least three different experiments. Data were analyzed by ANOVA followed by a Duncan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

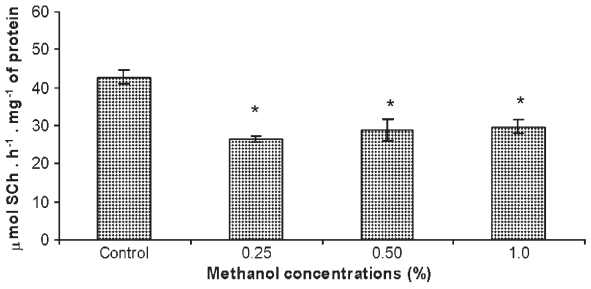

Fig. 3. Inhibition of zebrafish brain AChE activity after acute methanol treatment. ASCh hydrolysis was evaluated in three different concentrations (0.25%, 0.5% and 1.0%). Bars represent the mean±S.D. of at least three different experiments. Data were analyzed by ANOVA followed by a Duncan multiple range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

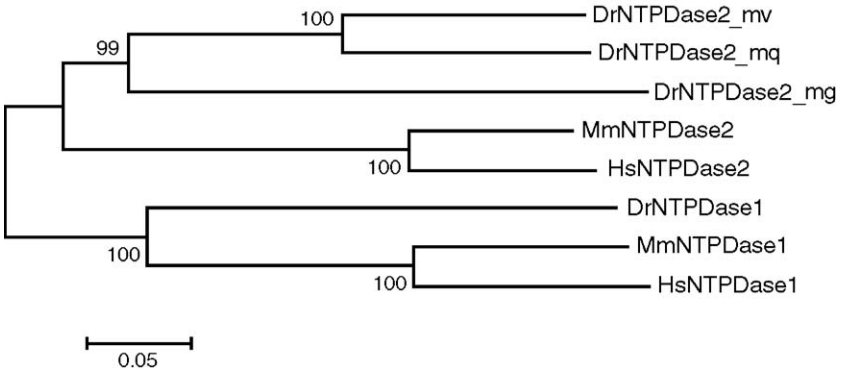

Fig. 4. Phylogenetic analysis of E-NTPDase family, demonstrating the existence of three different isoforms of NTPDase2 in zebrafish genome. The sequence was alignment by ClustalX program and the phylogenetic tree was constructed using neighbor-joining method, using proportional (*p*) distance with MEGA 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

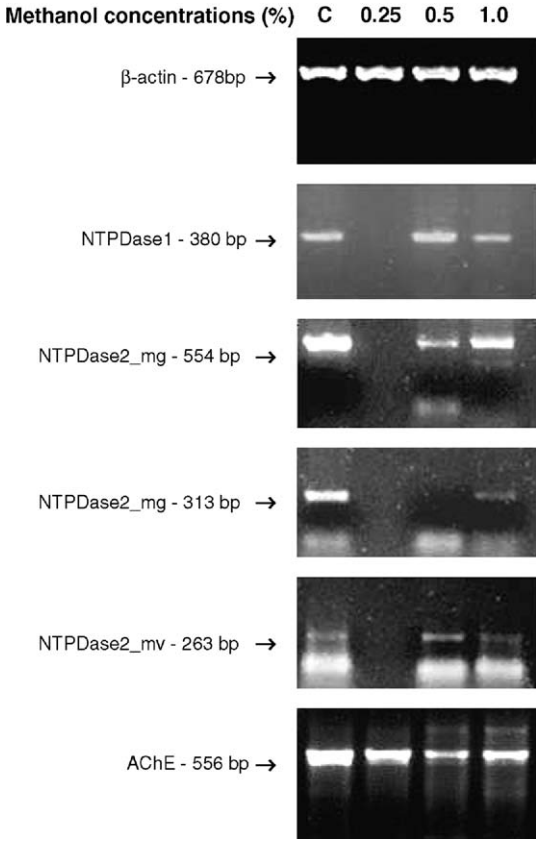

Fig. 5. Gene expression patterns after acute methanol exposure. The figure shows β-actin, NTPDase1, NTPDase2_mg, NTPDase2_mq, NTPDase2_mv and AChE mRNA expression in the brain of adult zebrafish. Fish were exposed to methanol concentrations (0.25%, 0.5% and 1.0%), the brains were excised and total RNA 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.

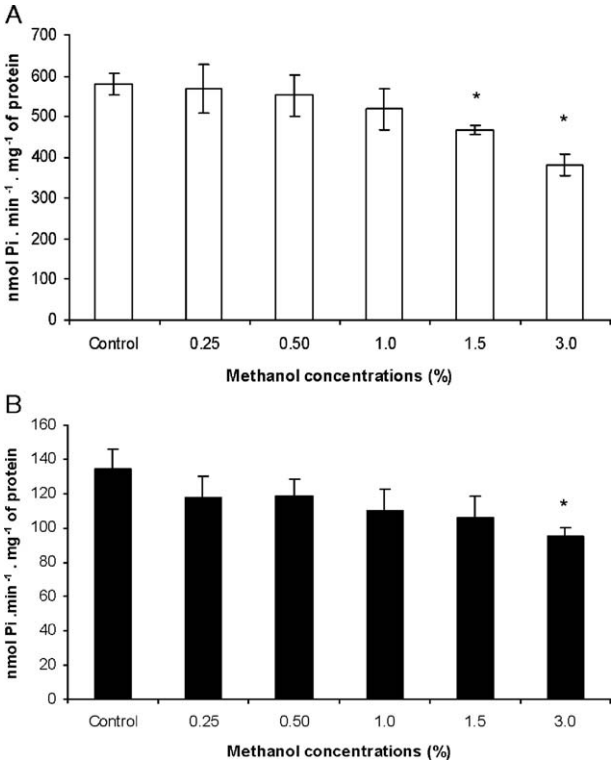

Fig. 6. In vitro effect of methanol on NTPDase activities in zebrafish brain membranes. ATP (A) and ADP (B) hydrolysis were evaluated in different concentrations (0.25–3.0%). Bars represent the mean±S.D. of at least three different experiments. Data were analyzed by ANOVA followed by a Duncan multiple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

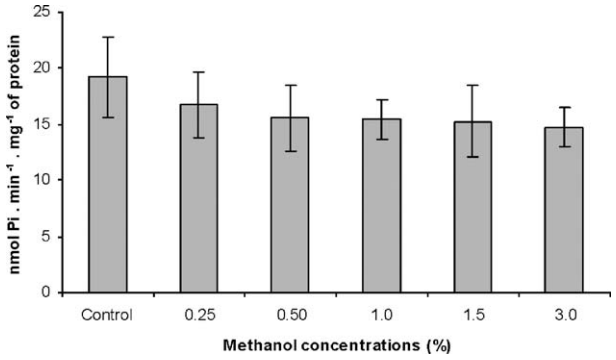

Fig. 7. In vitro effect of methanol on AMP hydrolysis in zebrafish brain membranes. The ecto-5′-nucleotidase activity was evaluated in different concentrations (0.25–3.0%). Bars represent the mean±S.D. of at least three different experiments Data were analyzed by ANOVA followed by a Duncan multiple range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

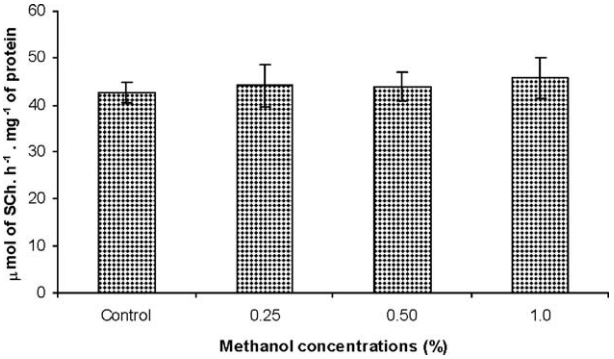

Fig. 8. Effect of methanol on AChE activity in zebrafish brain after in vitro assays. The AChE activity was evaluated in three different concentrations (0.25%, 0.5% and 1.0%). Bars represent the mean±S.D. of at least three different experiments. Data were analyzed by ANOVA followed by a Duncan multiple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

Acknowledgments

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