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Arginine exposure alters ectonucleotidase activities and morphology of zebrafish larvae (*Danio rerio*)

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ABSTRACT

Hyperargininemia is an inborn error of metabolism (IEM) characterized by tissue accumulation of arginine (Arg). Mental retardation and other neurological features are common symptoms in hyperargininemic patients. Considering purinergic signaling has a crucial role from the early stages of development and underlying mechanisms of this disease are poorly established, we investigated the effect of Arg administration on locomotor activity, morphological alterations, and extracellular nucleotide hydrolysis in larvae and adult zebrafish. We showed that 0.1 mM Arg was unable to promote changes in locomotor activity. In addition, 7-day-post-fertilization (dpf) larvae treated with Arg demonstrated a decreased body size. Arg exposure (0.1 mM) promoted an increase in ATP, ADP, and AMP hydrolysis when compared to control group. These findings demonstrated that Arg might affect morphological parameters and ectonucleotidase activities in zebrafish larvae, suggesting that purinergic system is a target for neurotoxic effects induced by Arg.

1. Introduction

ATP and adenosine play a crucial role from the early stages of development. Their levels are controlled by a complex cell surface-located group of enzymes called ectonucleotidases, such as nucleoside triphosphate diphosphohydrolase (NTPDase) family and ecto-5′-nucleotidase (Schetinger et al., 2007; Zimmermann, 2008). The importance of the purinergic signaling throughout the development was demonstrated in several systems from amphibians, birds, and mammals, including humans (Robson et al., 2006).

Extracellular ATP promotes increased permeability in the membrane of fertilized eggs through the activation of P2 receptors (Kupitz and Atlas, 1993; Abbracchio et al., 2009), proving to be crucial in the fertilization process. Furthermore, during the complex phase of initial development, adenosine plays differential role from that observed in adults. This nucleoside regulates many physiological and pathophysiological processes through activation of G protein-coupled receptors called A_1, A_{2A}, A_{2B}, and A_3 (Ribeiro et al., 2003; Burnstock, 2007). In addition, adenosine plays an important role during pre- and postnatal development. During prenatal life, adenosine is the dominant humoral regulator of embryonic brain and heart function (Fredholm, 2003). In the forming central nervous system, activation of adenosine A_1 receptors potently inhibits the development of axons and can lead to leukomalacia (Rivkees et al., 2001). Several studies have shown the involvement of adenosine and ectonucleotidase activities in inborn errors of metabolism (IEM), such as phenylketonuria (Wyse et al., 1994), Lesch-Nyhan disease (Bertelli et al., 2006), hyperhomocysteinemia (Tasatargil et al., 2006), hyperprolinemia (Delwing et al., 2007), and hyperargininemia (Delwing et al., 2005). Hyperargininemia is a rare IEM, which affects the activity of the enzyme arginase, which is the final enzyme of urea cycle and catalyzes the conversion of arginine (Arg) to urea and ornithine (Ash, 2004). Tissue accumulation of Arg is the biochemical hallmark of hyperargininemia. Patients affected by this disease show progressive dementia, epilepsy, ataxia, and spasticity, as well as cortical and pyramidal

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tract deterioration and memory impairment (Buchmann et al., 1996; Iyer et al., 2002; Reis et al., 2002). The hyperargininemia is typically present in early childhood (between 2 and 4 years of age) with developmental delay associated with progressive spastic paraparesis (Brusilow and Horwich, 2001; Scaglia and Lee, 2006).

Some studies have concerned about the effect of Arg accumulation in early life through a mouse model that mimics human arginase deficiency and becomes a tool for studying potential treatments for hyperargininemia (Iyer et al., 2002). Other promising model for studying IEM is zebrafish, which is a small freshwater teleost widely used in Developmental Biology, Genetic, Behavioral, and Neurochemical studies (Lieschke and Currie, 2007; Gerlai et al., 2009; Grossman et al., 2010). In addition, the genome of this species is well-characterized and shares many similarities with the human genome (Barbazuk et al., 2000). In addition, several neurotransmitter systems have been already described in this species (Rico et al., 2011), such as the purinergic system. P2 purinoceptors have been identified (Kucenas et al., 2003) and has been characterized the presence of NTPDases and ecto-5′-nucleotidase in zebrafish brain membranes (Rico et al., 2003; Senger et al., 2004; Rosemberg et al., 2010).

Considering that ATP and adenosine are important signaling molecules involved in several pathological conditions observed in hyperargininemic patients, such as epilepsy, cognitive deficit, and motor disorders, and zebrafish might be an important model for studying morphological and developmental aspects related to IEM, we evaluated the influence of Arg administration on extracellular nucleotide hydrolysis, morphological and behavioral parameters in larvae and adult zebrafish.

2. Materials and methods

2.1. Zebrafish embryos and larvae

Zebrafish embryos were obtained from natural mating of adult zebrafish (wild type) breeds and maintained in an automated re-circulating tank system. After the posture, eggs were collected and kept in an incubator at 28.5 °C on 14:10 light/dark cycle until 7 days post-fertilization (dpf) and used for subsequent experiments.

2.2. Adult zebrafish

Zebrafish (*Danio rerio*) adult "wild type" (Tübingen background; 3–5 cm) of both sexes were acclimated for at least 14 days in the experimental room. Animals were housed in groups of 20 fish in 15-L thermostated (28 ± 2 °C) tanks kept under constant chemical and mechanical water filtration and aeration (7.20 mg O_2/L). Fish were maintained under a 14–10 h day/night photoperiod cycle and fed three times a day with commercial flakes (TetraMin®, NC, USA) and supplemented with live brine shrimp. All protocols were approved by the Institutional Animal Care Committee (10/00188–CEUA PUCRS) and followed Brazilian legislation, the guidelines of the Brazilian Collegiums of Animal Experimentation (COBEA), and the Canadian Council for Animal Care (CCAC) "Guide on the Care and Use of Fish in Research, Teaching, and Testing".

2.3. Chemicals

L-Arginine, Trizma Base, EDTA, EGTA, sodium citrate, Coomassie blue, bovine serum albumin, malachite green, ammonium molybdate, polyvinyl alcohol, nucleotides, calcium, and magnesium chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were from analytical grade.

2.4. In vivo treatments

2.4.1. Arg administration in zebrafish larvae

A pilot experiment was conducted to choose the Arg concentrations for larval exposure. In this experiment, fertilized eggs were treated with 0.1, 0.5, 1.0, and 1.5 mM Arg in order to use Arg concentration able to induce similar effects those found in hyperarginemic patients. Relative survival rate, hatching, and larvae morphology were monitored. Arg-treated animals started their treatment from 3 up to 7 dpf in 400 mL-tanks. Arg was diluted in the same living water of control embryos. We observed a very pronounced mortality in zebrafish larvae treated with 0.5, 1.0, and 1.5 mM Arg. Thus, for subsequent experiments, fertilized eggs were collected and treated with 0.1 mM Arg from 3 to 7 dpf or kept in living water (water from reverse osmosis plus marine salt) as control animals. The medium containing 0.1 mM Arg was changed once a day during the treatment period, in order to maintain the Arg concentration. The behavioral, morphological, and biochemical experiments were performed at the 7 dpf.

2.4.2. Arg administration in adult zebrafish

Groups of five adult animals were maintained in 5-L aquariums containing different Arg concentrations (0.1, 1.0, and 1.5 mM) during 1 h (Schetinger et al., 2007). Control group animals were maintained in 5-L aquariums with tank water for the same period and conditions as the Arg-administered groups. The behavioral and biochemical experiments were performed immediately after the 1 h-exposure to Arg.

2.5. Behavioral parameters

2.5.1. Zebrafish larval locomotor activity

All tests were performed with 7 dpf-larvae in the 96-well plate. After the Arg administration, a single larvae was placed in fresh 10% Hanks solution and transferred to 96-well plate. Larvae were housed individually for ease of tracking and statistical analysis. Temperature in the testing room was kept at 26 °C. The behavioral parameters analyzed were distance traveled, mean speed, and absolute turn angle.

2.5.2. Zebrafish adult locomotor activity

Immediately after Arg administration (0.1, 1.0, and 1.5 mM), control and treated animals were carefully placed individually into the novel tank, representing a 1.5 L rectangular tank (30 cm length × 15 cm height × 10 cm width) as previously described (Gerlai et al., 2000). The behavioral test was performed during the same time frame each day (between 10:00 am and 4:00 pm). Animals were first habituated to the apparatus for 30 s and then behavioral activity was recorded over a period of 5 min. The testing tank was virtually divided into one horizontal and four equally vertical areas in order to evaluate the exploratory activity. All videos were recording with a digital webcam (Quick cam Pro 9000, LOGITECH) and movement of each larva was monitored by ANY-Maze recording software (Stoelting Co., Wood Dale, IL, USA).

2.6. Morphological evaluation

Morphological evaluation was performed in larvae from 7 dpf under stereomicroscopy to search for body defects. Measure of body length was performed individually for each animal by photographical registration followed by measurement by an image analysis program NIS-Elements D 3.2. The body length was assumed as the distance of the center of an eye to tail bud.

2.7. Preparation of zebrafish brain membranes

Zebrafish larvae were cryoanesthesized and euthanized by decapitation. Eyes and taila were removed and the brain-enriched portion of the larvae was dissected (pool of eighty larvae for each sample). Immediately after Arg administration, adult zebrafish were euthanized and their whole brains (five brains for each sample) were dissected. Brains of adult zebrafish and larvae were homogenized in 60 vol. (v/w) of chilled Tris–citrate buffer (50 mM Tris–citrate, 2 mM EDTA, 2 mM EGTA, and pH 7.4 adjusted with citric acid) for NTPDase and ecto-5′-nucleotidase assays (Rico et al., 2003; Senger et al., 2004). Briefly, brain homogenates were centrifuged at 800 × *g* for 10 min and the supernatant fraction was subsequently centrifuged for 25 min at 40,000 × *g*. The pellet of membrane preparations was frozen in liquid nitrogen, thawed, and ressuspended in buffer. This freeze–thaw–wash procedure was used to ensure the lyses of brain vesicles membranes. For zebrafish larvae membranes, this was the last step to obtain the brain membrane. For adult zebrafish membranes, after lyses procedure, an additional centrifugation at 40,000 × *g* for 20 min was performed. The final pellet was ressuspended and used for biochemical analyses. All cellular fractions were maintained at 2–4 °C throughout preparation and immediately used for enzyme assays.

2.8. Protein determination

Protein concentration was measured by the Coomassie blue method with bovine serum albumin as a protein standard (Bradford, 1976).

2.9. Determination of ectonucleotidase activity

The ectonucleotidase activities were determined as previously described (Rico et al., 2003; Senger et al., 2004). Zebrafish larvae and adult brain membranes (3–5 μg protein) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for NTPDase activities) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for ecto-5′-nucleotidase activity) in a final volume of 200 μL. Samples were preincubated for 10 min at 37 °C before starting the reaction with the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was stopped after 30 min with the addition of 200 μL of trichloroacetic acid at a final concentration of 5%. The samples were chilled on ice for 10 min and 1 mL of a colorimetric reagent composed of 2.3% polyvinyl alcohol, 5.7% ammonium molybdate, and 0.08% malachite green was added in order to determine the inorganic phosphate

Table 1
Primer sequences and PCR amplification conditions.

Enzymes	Primer sequences (5′–3′)	GenBank accession number (mRNA)	Amplicon size
β-Actin[a]	F-CGAGCTGTCTTCCCATCCA R-TCACCAACGTAGCTGTCTTTCTG	ENSDART00000055194	
EF1α[a]	F-CTGGAGGCCAGCTCAAACAT R-ATCAAGAAGAGTAGTACCGCTAGCATTAC	NSDART00000023156	
entpd1[b]	F-TTATGGCCTACATTTATTTCCGTCG R-GATTCTTTGAAATGTAAAACCGCTTG	BC078240.1	176
entpd2a.1[b]	F-TTAAATCCAATGCTATATGCCGGTG R-TCTGTGATGGATGTGTCGGACAAAGG	BC078419.1	103
entpd2a.2[b]	F-AAAGTTGAAGACACCTCTGTCGGCTG R-CCATTCTTTTGGTAGCTTCGCAAC	XM_682630.2	188
entpd2-like[b]	F-AGGCGTCTGTTGGCTGGGCTC R-GAAACATCAAACCAGTCCATGCTGC	XM_692508.3	117
entpd3[b]	F-GCTACAATACCTCCATACCTGCAGAGG R-GATACTCCTGACCAAGGCTTTGCAC	EF446129.1	146
entpd8[b]	F-GTTGCAGATACAGATATTGGTTGGACG R-GTAGAGTGAGGAAGAGGGCAAATGC	NM_001002379.2	154
Ecto-5′-nucleotidase[b]	F-TGGACGGAGGAGACGGATTCACC R-GGAGCTGCTGAACTGGAAGCGTC	BC055243.1	149
casp-3[b]	F-TCCTGGAGAAACACAATGACCGGCTC R-TCTTGGCATCAAAGCCCGGCATG	NM_131877	166
bcl-2[b]	F-TTGCATTCTTCGAGTTTGGTGGGACC R-TCTGCTGACCGTACATCTCCACGAAGG	NM_001030253	174

[a] According to Tang et al. (2007).
[b] Designed by the authors.

released (Pi) (Chan et al., 1986). The quantification of Pi released was determined spectrophotometrically at 630 nm and the specific activity was expressed as nmol of Pi min^{-1} mg^{-1} of protein. In order to correct non-enzymatic hydrolysis of the substrates, controls with the addition of the enzyme preparation after the addition of trichloroacetic acid were used. All enzyme assays were performed in at least five different experiments, each one performed in triplicate.

2.10. Gene expression analysis by quantitative real time RT-PCR (RT-qPCR)

Gene expression analysis was carried out only when kinetic alteration occurred. Total RNA was isolated with Trizol® reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The total RNA was quantified by spectrophotometry and the cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 μg of total RNA, following the manufacturer's instructions. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were done in a volume of 25 μL using 12.5 μL of diluted cDNA (1:50 for *EF1α*, *β-actin*, *entpd2a.1*, *entpd2a.2*, *entpd2-like*, *entpd3*, *entpd8*, *ecto-5′-nucleotidase*, and *caspase-3* and 1:20 *entpd1* and *Bcl-2*), containing a final concentration of 0.2× SYBR® Green I (Invitrogen), 100 μM dNTP, 1× PCR Buffer, 3 mM $MgCl_2$, 0.25 U Platinum® Taq DNA Polymerase (Invitrogen) and 200 nM of each reverse and forward primers (Table 1). The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing and 15 s at 72 °C for elongation. At the end of cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 to 99 °C. Relative expression levels were determined with 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR 11.0 Software (http://LinRegPCR.nl) and the stability of the references genes, *EF1α*, and *β-actin* (*M-value*) and the optimal number of reference genes according to the pairwise variation (*V*) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). Relative RNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

2.11. Statistical analysis

All assays were run in triplicate and expressed as means ± S.E.M. Data were analyzed by one-way analysis of variance (ANOVA). *Post hoc* comparisons were made using Tukey's test considering $p \leq 0.05$ as statistically different for biochemical experiments in adult zebrafish. For biochemical experiments, morphological aspects and locomotor activity of the zebrafish larvae, data were analyzed using Student *t*-tests. Statistical significance was considered when the *p* value was less than 0.05.

3. Results

3.1. Morphological and behavioral parameters in zebrafish larvae

First, we observed the survival rate of zebrafish larvae after 0.1, 0.5, 1, 1.5 mM Arg during 4 days of treatment (from 3 to 7 dpf). Our results demonstrated that there is a significant mortality for 0.5, 1.0, 1.5 mM and only animals submitted to 0.1 mM Arg survived during the treatment (Fig. 1). For the subsequent experiments, we evaluated whether administration of 0.1 mM Arg could alter the morphology and size of zebrafish larvae during the development. Our results demonstrated that body length was decreased (4%) in larvae treated with Arg ($t = 6.570$, df = 28 and $p < 0.0478$; $n = 15$) in comparison with the control group (7 dpf – Control: 3.996 ± 0.014 mm; Arg: 3.846 ± 0.035 mm; $n = 15$) (Fig. 2). Therefore, we investigated if these changes could affect the locomotor activity. The evaluation of larval locomotor activity showed no differences in distance traveled ($p = 0.4238$; $n = 12$), medium speed ($p = 0.3836$; $n = 12$), and absolute turn angle ($p = 0.2995$) between control and Arg groups (Fig. 3).

3.2. Nucleotide hydrolysis in zebrafish larvae

We evaluate the influence of Arg on NTPDase and ecto-5′-nucleotidase activities, which are involved in the control of nucleotide and nucleoside levels in larval zebrafish brain. Fig. 4A–C

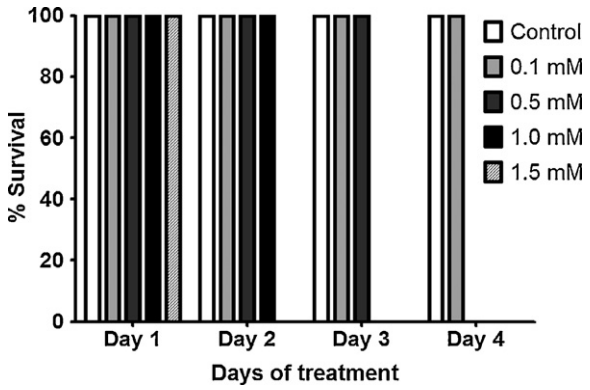

Fig. 1. Effect of Arg exposure on survival curve. Each group contains 25 animals submitted to different arginine concentrations (0.1, 0.5, 1.0, and 1.5 mM) and the survival was followed during 4 days of treatment (from 3 dpf to 7 dpf). Control group was not submitted to Arg treatment and was maintained in similar conditions when compared to treated groups. The survival was expressed as % of live animals during treatment period.

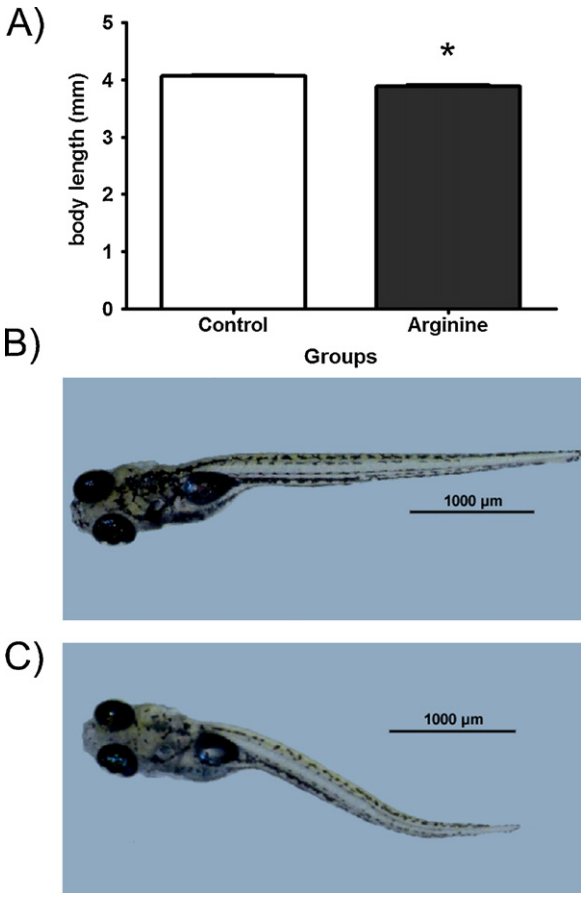

Fig. 2. Effect of exposure 0.1 mM Arg on body length in 7-dpf-zebrafish larvae. (A) The graph represents the larvae size (in mm) comparing treated and control groups; (B) representative images from control zebrafish larvae and (C) 0.1 mM Arg-treated zebrafish larvae. Body length was estimated using program NIS-Elements D 3.2. Data was expressed as mean ± S.E.M. from 15 animals for each group and analyzed by Student's *t* test. $^{*}p < 0.05$ denotes a significant difference from control group.

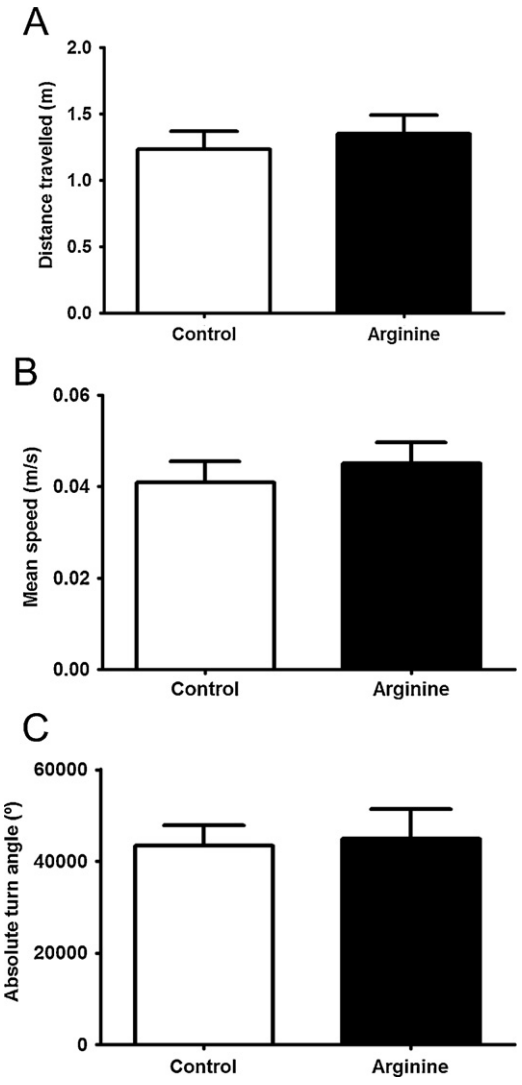

Fig. 3. Effect of 0.1 mM Arg administration on locomotor activity in 7-dpf-zebrafish larvae on distance traveled (A), mean speed (B), and absolute turn angle (C) during 5 min of videorecording. Data were expressed as mean ± S.E.M. from 12 animals for each group and analyzed by Student's *t* test.

shows that zebrafish larvae exposed to 0.1 mM Arg significantly increased the ATP (25%) ($t = 2.310$, df = 10, $p < 0.0435$, $n = 6$), ADP (32%) ($t = 2.480$, df = 6, $p < 0.0478$, $n = 6$), and AMP (55%) ($t = 3.167$, df = 10, $p < 0.0100$, $n = 6$) hydrolysis when compared to control groups. To evaluate whether the exposure of Arg in larvae could alter the gene expression of nucleotidases, *real-time quantitative PCR* analyses were performed when kinetic alterations were observed. We also evaluate if Arg treatment was able to alter the gene expression of apoptotic markers Bcl-2 and caspase-3. The results demonstrated that the *entpd2a.1* and *entpd3* expression levels were significantly increased after treatment with Arg. However, there were no changes in *entpd1*, *entpd2a.2*, *entpd2al*, *entpd8*, *ecto5′-nucleotidase*, *caspase-3* and *Bcl-2* gene expression of animals treated with 0.1 mM Arg when compared to control group (see Fig. 5).

3.3. Nucleotide hydrolysis in zebrafish adults

After verifying that Arg administration modifies morphological, biochemical, and molecular parameters in zebrafish larvae, we observed whether treatment was able to induce changes on locomotion and ectonucleotidase activities in adult zebrafish. Our results demonstrate that the exposure to different Arg concentrations (0.1, 1.0, and 1.5 mM) were unable to induce changes on locomotor activity (data not shown). In addition, adult zebrafish exposed to the same Arg concentration mentioned above did not alter ATP [$F(3,20) = 0.219$; $p = 0.8817$; $n = 6$], ADP [$F(3,20) = 1.096$; $p = 0.3739$; $n = 6$], and AMP [$F(3,19) = 0,118$; $p = 0.9483$; $n = 6$] hydrolysis when compared to control group (data not shown).

4. Discussion

In the present study, we have shown that Arg can alter *ex vivo* ectonucleotidase activities in brain zebrafish larvae. Arg administration activated ATP, ADP, and AMP hydrolysis in larval zebrafish brain and decreased the body size of 7 dpf-larvae. Furthermore, molecular analysis demonstrated that *entpd2a.1* and *entpd3* mRNA levels were increased in zebrafish brain larvae. In contrast, changes were not observed in ATP, ADP, and AMP hydrolysis after *in vivo* exposure to Arg in adults.

The hyperargininemia is an illness related to the absence or deficiency of the arginase enzyme, resulting in accumulation of high levels of Arg. Some studies have shown that arginase is very important in providing ornithine as a precursor to the synthesis of polyamines, which are involved in the control of DNA, RNA, and protein synthesis during cell growth, differentiation, and development (Fozard et al., 1980; Pegg et al., 1982; Hougaard, 1992) and are important in embryonic development (Kusunoki and Yasumasu, 1978).

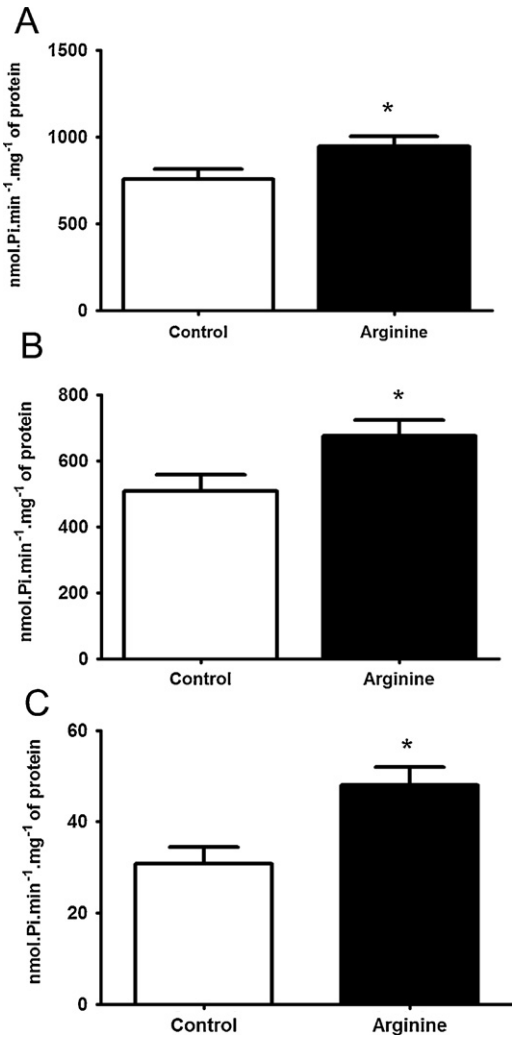

Fig. 4. Effect of 0.1 mM Arg administration on ATP (A), ADP (B), and AMP (C) hydrolysis in larval zebrafish brain membranes. Data were expressed as mean ± S.E.M. ($n = 6$) and were analyzed by Student's t test. $^{*}p < 0.05$ denotes a significant difference from control group.

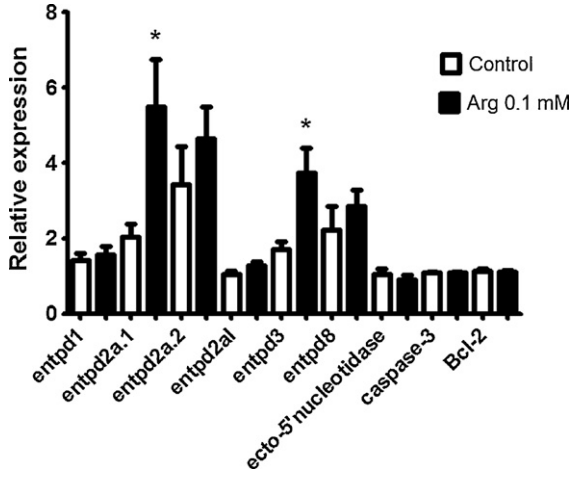

Fig. 5. Effect of 0.1 mM Arg treatment on *entpd1*, *entpd2a.1*, *entpd 2a.2*, *entpd2-like*, *entpd3*, *entpd8*, *ecto-5′-nucleotidase*, *bcl-2*, *casp-3* gene expression in 7-dpf-zebrafish larvae. Data are expressed as mean ± S.E.M. of four independent experiments performed in quadruplicate. Data were analyzed statistically by Student's t test. $^{*}p < 0.05$ denotes a significant difference from control group.

Some studies reported that hyperargininemic patients have as their primary symptoms delayed development associated with progressive spastic paraparesis (Scaglia and Lee, 2006; Esquivel-Aguilar et al., 2011). The Arg doses chosen in this study are consistent with those levels achieved in plasma and cerebrum of hyperarginemic patients which ranges around of 1.3 mM and 0.3 nmol/kg, respectively (Buchmann et al., 1996). The present results showed that there was a decrease in body size of zebrafish larvae (7 dpf) treated with Arg and these data are in agreement with those reported in literature, which demonstrated that children with hyperargininemia have development delay (Brusilow and Horwich, 2001). Studies showed that acute treatment with Arg did not alter locomotion of rats (Iyer et al., 2002). Our data reproduced the results found in literature, since there were no changes in locomotor activity in zebrafish larvae.

The purinergic signaling plays complex roles such as inhibitory or excitatory neurotransmission, besides acting as a trophic factor in long-term effects on cell proliferation, growth, and development (Abbracchio and Burnstock, 1998; Burnstock and Ulrich, 2011). The purinergic signaling was altered in rats submitted to hyperargininemia. Studies demonstrated that extracellular nucleotide hydrolysis is decreased in serum of rats subjected to acute administration of Arg (Delwing et al., 2005). However, intraperitoneal administration of Arg did not alter the ectonucleotidase activities in hippocampal synaptosomes (Delwing et al., 2007). A decrease was observed in the nucleotide hydrolysis when the Arg was administrated intracerebroventriculary (Delwing et al., 2007). Action of NTPDases and ecto-5′-nucleotidase may regulate the concentrations of ATP, ADP, and AMP by increasing/decreasing their hydrolysis with a consequent increase/decrease in adenosine levels, a natural protective metabolite (Robson et al., 2006). Our results demonstrated that there was an increase of ATP, ADP, and AMP hydrolysis in membrane-enriched fraction from larval zebrafish brain. However, we did not observe significant changes on nucleotide hydrolysis treated with Arg in adults, indicating that the changes observed are not due to an acute toxic effect of arginine, but are related to exposure during the early stages of development. Such changes observed in larval stage could alter adenosine levels in larval zebrafish brain. Therefore, the differences observed in nucleotide hydrolysis in larval and adult zebrafish as well as in rodents indicate that Arg could induce different effects depending on the route of administration, animal model used in the study, and stage of development in which the treatment was administered.

The alterations promoted by Arg on ectonucleotidase activities could be a consequence of transcriptional control. Interestingly, the results demonstrated that the *entpd2a.1* and *entpd3* expression levels were significantly increased after treatment with Arg. Therefore, the increase of ATP and ADP hydrolysis in zebrafish larvae induced by Arg administration is probably related to a higher *entpd2a.1* and *entpd3* gene expression. NTPDase2 almost exclusively hydrolyses ATP producing ADP and NTPDase3 degrades ATP to AMP with a transient ADP accumulation (Zimmermann, 2001; Kukulski and Komoszynski, 2003; Lavoie et al., 2004). Both activities ATPase and ADPase are increased in early stages of development with a gradual decrease throughout development (Childs and Valle, 2000), suggesting that NTPDase2 and NTPDase3 might play an important role in control of nucleotide levels in brain development. However, we observed an increase in AMP hydrolysis whereas *ecto-5′-nucleotidase* gene expression levels were not altered, suggesting that this enzyme activity could be regulated by post-translational events.

We questioned if the purinergic system also is affected in adults treated with Arg acutely, since these rare diseases and their manifestations can occur at any stage of development even in adulthood (Childs and Valle, 2000; Sedel et al., 2007). Our data demonstrated

that treatment with Arg did not alter the ectonucleotidase activities in adult zebrafish brain, suggesting that a short-term Arg exposure is not able to induce neurotoxic effects on central nervous system.

In order to investigate if the changes induced by Arg in larval developmental stage are related to induction of apoptosis, we evaluate the gene expression of Bcl-2 and caspase-3, which are involved in the activation of caspase cascade. Both the extrinsic and intrinsic pathways converge at caspase-3, which together with other caspases cause the dismantle of cellular structures (Earnshaw et al., 1999). Our results demonstrate that Arg treatment did not induce changes in gene expression pattern for these markers, suggesting that death cell induced by apoptosis is not involved in the alterations observed in morphological and neurochemical parameters analyzed. Therefore, we suggested that this modulatory effect on purinergic signaling may be related with a critical period of development in which the brain reestablishes compensatory mechanisms in order to achieve homeostasis in adulthood.

5. Conclusion

The present study demonstrated that Arg administration significantly increased ATP, ADP, and AMP hydrolysis, as well decreased the body size of 7 dpf zebrafish larvae. Considering treatment did not alter any parameters tested in adult zebrafish, it is possible to suggest that Arg is able to alter nucleotide hydrolysis, which could influence the brain development. Such findings present promising evidence that zebrafish serves as a good model for studies of IEM, since it permits *extra utero* development evaluation and these diseases seem to be well connected with the early stages of development.

Disclosure statement

No competing financial interests exist.

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