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Involvement of purinergic system in inflammation and toxicity induced by copper in zebrafish larvae

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ABSTRACT

The use of zebrafish (*Danio rerio*) is increasing as an intermediate preclinical model, to prioritize drug candidates for mammalian testing. As the immune system of the zebrafish is quite similar to that of mammals, models of inflammation are being developed for the screening of new drugs. The characterization of these models is crucial for studies that seek for mechanisms of action and specific pharmacological targets. It is well known that copper is a metal that induces damage and cell migration to hair cells of lateral line of zebrafish. Extracellular nucleotides/nucleosides, as ATP and adenosine (ADO), act as endogenous signaling molecules during tissue damage by exerting effects on inflammatory and immune responses. The present study aimed to characterize the inflammatory status, and to investigate the involvement of the purinergic system in copper-induced inflammation in zebrafish larvae. Fishes of 7 days post-fertilization were exposed to 10 μM of copper for a period of 24 h. The grade of oxidative stress, inflammatory status, copper uptake, the activity and the gene expression of the enzymes responsible for controlling the levels of nucleotides and adenosine were evaluated. Due to the copper accumulation in zebrafish larvae tissues, the damage and oxidative stress were exacerbated over time, resulting in an inflammatory process involving IL-1β, TNF-α, COX-2 and PGE_2. Within the purinergic system, the mechanisms that control the ADO levels were the most involved, mainly the reactions performed by the isoenzyme ADA 2. In conclusion, our data shed new lights on the mechanisms related to copper-induced inflammation in zebrafish larvae.

Introduction

Zebrafish (*Danio rerio*) is a vertebrate that has been widely used in studies of the immune system. Toxicology and safety pharmacology are broadly assessed by in vitro assays, but the results might not be predictive of in vivo effects. Thus, the use of zebrafish is increasing as an intermediate step, together with the cell culture evaluation, to prioritize drug candidates for mammalian testing, allowing a reduction in the number and cost of studies (McGrath and Li, 2008; Thienpont et al., 2013). Among other advantages of using this teleost, especially embryos and larvae, it is possible to remark the favorable absorption characteristics, small amounts of compounds necessary for testing and, animals and experimental groups that can be evaluated in a single trial (Berghmans et al., 2008).

The immune system of zebrafish is quite similar to that of mammals, including the presence of T and B lymphocytes, antigen presenting cells,

Abbreviations: ADA, adenosine deaminase; ADO, adenosine; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CAT, catalase; COX-2, cyclooxygenase 2; Ecto-5′-NT, ecto-5′-nucleotidase; E-NTPDase, ecto-nucleoside-trifosfo difosfoidrolases; GSH, reduced glutathione; HPLC-FLD, high performance liquid chromatography with fluorescence detector; ICP-MS, Inductively coupled plasma mass spectrometry; IL-10, Interleukin 10; IL-1β, Interleukin 1β; INO, Inosine; MPO, Myeloperoxidase; NO, Nitric oxide; PGE_2, Prostaglandin E_2; SOD, Superoxide dismutase; TNF-α, Tumor necrosis factor α; UHPLC, Ultra high performance liquid chromatography coupled with mass spectrometry; DAD, Diode array detector.

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phagocytic cells, and humoral immunity, together with the complement components. The similarities between the immune system of fishes and mammals, a fully sequenced genome and an increase in the production of transgenic and knockout zebrafish have encouraged many researchers to establish models of bacterial infections (Loynes et al., 2010; Vojtech et al., 2009) and chemical inflammation, such as induced by *Escherichia coli* and copper, respectively (d'Alencon et al., 2010; Leite et al., 2012).

Copper is an essential metal obtained from diet sources, chelated by amino acids, absorbed in the small intestine and transported in the blood in a binding form (Gaetke and Chow, 2003; Rosemberg et al., 2007b). Oxidative damage has been linked to chronic copper overload and/or exposure to excessive concentrations caused by accidents, occupational hazards, and environmental contamination. Additionally, oxidative damage induced by copper has been implicated in disorders associated with abnormal metabolism (Wilson disease) and neurodegenerative diseases (Alzheimer disease) (Brenner, 2013; Gaetke and Chow, 2003). Olivari et al. (2008) described events induced by copper via oxidative stress, such as cell death by apoptosis and necrosis in hair cells of lateral line of zebrafish. These events, also described in cell cultures, were related to the participation of this metal in Fenton chemistry (Olivari et al., 2008; Prousek, 2007). Recently, d'Alencon et al. demonstrated that copper exposure induces neutrophil migration to the inflammatory focus, due to the damage induced in hair cells of lateral line of zebrafish larvae, pointing out this model as a potential tool for screening of anti-inflammatory compounds (d'Alencon et al., 2010). Another interesting study described the toxicity induced by copper in the central nervous system of adult zebrafish, via inhibition of ecto-nucleoside-trifosfo difosfoidrolases (E-NTPDase) and ecto-5′-nucleotidase (ecto-5′-NT) (Rosemberg et al., 2007a). These enzymes belong to ecto-nucleotidase family and are responsible for controlling the levels of nucleotides and nucleosides by sequential hydrolysis of adenosine triphosphate (ATP) to adenosine monophosphate (AMP) and, sequentially the desamination of AMP to adenosine (ADO) is carried out by the enzyme adenosine deaminase (ADA) (Rico et al., 2003; Rosemberg et al., 2008, 2010; Senger et al., 2004).

The extracellular nucleotides act as endogenous signaling molecules in tissue damage by exerting effects on inflammatory and immune response (Burnstock, 2006; Lazarowski et al., 1997). The ATP is important in the secretion of cytokines and, recruitment and differentiation of immune cells, whereas ADO commonly displays immunosuppressive responses to protect healthy tissues from stressful stimuli (Bours et al., 2006). In the inflammatory process, ATP and ADO are released at the site of inflammation as a result of cell damage or through the release to extracellular medium in consequence to cell activation (Burnstock, 2006; Luttikhuizen et al., 2004).

Considering that: (I) zebrafish is a model widely used in toxicological studies; (II) copper model of inflammation has emerged as an important tool for drug screening; (III) purinergic system can play a key role on inflammation homeostasis, modulating ATP and ADO levels and; (IV) there is no data relating copper inflammation model in zebrafish larvae with purinergic system, the major aim of this study was to standardize the time-related biochemical and molecular markers related to inflammation, and to investigate the involvement of the purinergic system in the inflammatory status induced by copper in larvae of zebrafish.

Materials and methods

Animal. Zebrafish larvae were generated by natural pair-wise mating in aquariums with filtration system in series (Zebtec, Tecniplast®, Italy). They were staged and reared according to standard procedures (Westerfield, 2000). Because the embryo receives nourishment from an attached yolk sac, no feeding was required until 7 days post-fertilization (dpf) (Lewis, 2010). All protocols were approved by the Institutional Animal Care Committee (09/00135, CEUA–PUCRS).

Chemicals. Copper, added as copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) were purchased from Merck (Darmstadt, Hessen, Germany). All other reagents used were purchased from Sigma (St. Louis, MO, USA).

Treatments. All treatments were performed in 6-well culture plates and, the copper ($CuSO_4 \cdot 5H_2O$) was added directly to water. First, we used the oxidative stress as a parameter for evaluating the extent of damage caused by 1, 10 and 25 μM of copper during 24 h of treatment. The concentration of 10 μM of copper, considered harmful without excessive mortality, was used to characterize the inflammation model and, to determine its association with the purinergic system in zebrafish larvae. The observation times were tested and based on knowledge of the activation cascade of inflammation. The parameters assessed and times of observations were: oxidative stress (0, 4, 6 and 24 h) neutrophil migration to the damaged tissues (0, 0.5, 1, 4 and 24 h), release of prostaglandin E_2 (PGE_2) (0, 1, 2, 4 and 24 h), expression of inflammatory mediators (0, 4 and 24 h), extracellular ATP hydrolysis (0, 4 and 24 h) and ecto-nucleotidase and ADA activities (0 and 24 h). The enzyme gene expressions were assessed in 0 and 24 h. The temporal determination of copper absorption by zebrafish larvae was performed in 0, 1, 2, 4, 6 and 24 h to evaluate the relationship of the concentration of copper with the effects at different times.

Oxidative stress and antioxidative defenses. In order to determine the antioxidant defenses and oxidative stress, we have measured catalase (CAT), reduced glutathione (GSH), superoxide dismutase (SOD), and nitric oxide (NO). Initially, the experiments were performed using n = 6, containing a pool of 20 larvae each, after 24 h of treatment with 1, 10 and 25 μM of copper. The concentration of 10 μM of copper was chosen to continue the study and, it was evaluated in 0 h (control), 4, 6 and 24 h after copper treatment as described above. After the different concentrations and times of treatments the larvae were homogenized in 500 μl of phosphate buffered saline–PBS (pH 7.2–7.4). All samples were centrifuged at 13500 ×*g* for 5 min at 4 °C in 1.5 ml tubes, and the supernatants were collected for analysis. CAT activity was assessed through the hydrogen peroxide concentration decrease, according to the method described previously (Aebi, 1984). GSH levels were determined by using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), according to the method described previously (Shim et al., 2010). SOD activity was assayed by measuring the adrenaline auto-oxidation inhibition, according to the method described previously (Bannister and Calabrese, 1987). NO was determined by high performance liquid chromatography with fluorescence detector (HPLC-FLD) using 2,3-diaminonaphthalene to derivatize the samples, according to the method described previously (Leite et al., 2012).

Determination of PGE_2 by UHPLC-MS/MS. PGE_2 was tested at 0 h (control), 0.5, 1, 4 and 24 h, since it is one of the first mediators to be released during the inflammatory process. The experiments were conducted using n = 6, containing a pool of 35 larvae each. Homogenates were prepared in 500 μl of phosphate buffered saline — PBS (pH 7.2–7.4). A 400 μl aliquot of the homogenate was transferred into a 9 ml glass tube and subjected to extraction using a modified method described previously (Shim et al., 2010). Briefly, 80 μl of 1 M nitric acid was added to the samples. Fifty microliters of BHT 1% was added to each tube. PGE_2 was then extracted with 2 ml of hexane:ethyl acetate (1:1, v/v) and mixed for 1 min. Samples were centrifuged at 800 ×*g* for 5 min at 4 °C. The upper organic layer was collected, and the organic phases from three extractions were pooled and then evaporated to dryness under a stream of nitrogen at room temperature. Samples were then reconstituted in 100 μl of methanol before analysis by ultra high performance liquid chromatography coupled with mass spectrometry (UHPLC–MS/MS). Five microliters was injected into the UHPLC

1290/MS 6460 TQQQ — Agilent (all UHPLC components and software MassHunter were from Agilent Technologies®). Chromatographic separations were performed using a Zorbax Eclipse Plus Phenyl–Hexyl 4.6 × 50 mm 1.8 μm column. The flow rate of formic acid 0.1%:acetonitrile (formic acid 0.1%) 50:50 v/v mobile phase was 0.4 ml/min with a column temperature of 45 °C. PGE_2 was detected using electrospray negative ionization and multiple-reaction monitoring of the transition ions. The collision energy was 14 V for transition 351 > 271 (quantifier) and 6 V for 351 > 315 (qualifier). The results were expressed as nanograms of PGE_2 per mg protein.

Neutrophil myeloperoxidase assay. Neutrophil migration was assessed by means of myeloperoxidase activity (MPO), according to the method described previously, with slight modifications for zebrafish (Passos et al., 2004). MPO was tested at 0 h (control), 1, 2, 4 and 24 h following copper treatment. Experiments were conducted using n = 6, containing a pool of 20 larvae each. All samples were homogenized in 500 μl of EDTA/NaCl buffer (pH 4.7) and centrifuged at 5 000 ×*g* for 20 min at 4 °C. The pellet was suspended in 200 μl of hexadecyltrimethyl ammonium bromide buffer (pH 5.4), and the samples were frozen. Upon thawing, the samples were re-centrifuged and, the supernatant was used for MPO assay. The results were expressed as optical density per milligram of tissue.

Analysis of extracellular ATP metabolism by HPLC. ATP hydrolysis and its degradation products (ADP, AMD, ADO, and INO) were analyzed in the groups at 0 h (control), 4 h and 24 h after copper exposure. Experiments were conducted using n = 4 containing a pool of 35 larvae each. The membranes were prepared in 500 μl of chilled Tris–citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4 adjusted with citric acid), centrifuged at 800 ×*g* for 10 min and the pellet was discarded. The supernatant was centrifuged for 25 min at 40000 ×*g* and, the pellet was frozen in liquid nitrogen, thawed, suspended in 500 μl of Tris–citrate buffer and, used for analysis. All membranes were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$. All samples were preincubated for 10 min at 37 °C before starting the reaction by the addition of substrate ATP to a final concentration of 100 μM. To stop the reaction, an aliquot of the incubation medium was transferred to a pre-chilled tube and centrifuged at 4 °C for 15 min at 14000 ×*g*. Twenty microliters was injected into the HPLC system (all HPLC components and software ChemStation were from Agilent Technologies®). Chromatographic separations were performed using a reverse-phase column (150 mm × 4 mm, 5 μm Agilent® 100 RP-18 ec). The column was protected by a guard column (4 × 4 mm, 5 μm Agilent® 100 RP-18 ec), and was maintained at room temperature. The flow ramp of methanol: 60 mM KH_2PO_4 with 5 mM tetrabutylammonium chloride (pH 6.0) (13:87, v/v) mobile phase was maintained between 1.2 and 2.0 ml/min according retention time of each compound. The mobile phase was prepared daily due to low stability. Absorbance was monitored at 260 nm with diode array detector (DAD).

Ecto-nucleotidase assay. E-NTPDase and ecto-5′-NT assays were performed as described previously (Rico et al., 2003; Senger et al., 2004). Activities were analyzed in 0 h (control) and after 24 h of exposure to copper in zebrafish larvae. Zebrafish larvae membranes (3–5 μg protein) prepared as described above (analysis of ATP metabolism) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for the ATPase and ADPase activities) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for the AMPase activity) in a final volume of 200 μl. All membranes were preincubated for 10 min at 37 °C before starting the reaction by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was stopped after 30 min by the addition of 200 μl of trichloroacetic acid 10% (final concentration 5% w/v). Samples were chilled on ice for 10 min before assaying for the release of inorganic phosphate (Pi), according to malachite green method, using KH_2PO_4 as a Pi standard (Chan et al., 1986). The non-enzymatic Pi released from nucleotides into the assay medium was subtracted from the total Pi released during the incubation, giving net values for enzyme activity. Specific activity was expressed as nanomol of Pi released per minute per milligram of protein. All enzyme assays were run at least in triplicate.

Determination of total adenosine deaminase activity. Adenosine deaminase (ADA) activity was determined spectrophotometrically as described previously (Rosemberg et al., 2008). The activity was analyzed in control group (0 h) and 24 h after exposure the larvae to copper. To assess the total activity of ADA the homogenate was centrifuged to 1000 ×*g* and, the supernatant was used for activity assay. Zebrafish larvae supernatants (10 μg protein) were added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0) in a final volume of 200 μl. The samples were preincubated for 10 min at 37 °C and the reaction was initiated by the addition of adenosine to a final concentration of 1.5 mM. After incubation for 120 min, the reaction was stopped by adding the samples on 500 μl of phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/ml). Controls with the addition of the enzyme preparation after mixing with phenol-nitroprusside reagent were used to correct non-enzymatic deamination of substrate. The reaction mixtures were mixed to 500 μl of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125% available chlorine, in 0.6 M NaOH), vortexed and incubated at 37 °C for 15 min for colorimetric assay at 635 nm. The ADA activity was expressed as nmol of NH_3 released per minute per milligram of protein.

Quantitative real time RT-PCR (qRT-PCR). The gene expression of important elements in inflammation as IL-1β (interleukin 1β), IL-10 (interleukin 10), TNF-α (tumor necrosis factor α) and COX-2 (cyclooxygenase 2) and, expressions of ADA subfamilies (ADA 1, ADA 2.1, ADA 2.2) including an alternative splicing isoform (ADAasi) and, an adenosine deaminase like related gene (ADA L) were determined. The total RNA was isolated with Trizol® reagent, according to the manufacturer's instructions. Pools of 15 zebrafish larvae with 7 dpf were used. Total RNA was quantified by spectrophotometry and the cDNA was synthesized with ImProm-II™ Reverse Transcription System from 1 μg total RNA in accordance with the manufacturer's instructions. Quantitative PCR was performed using SYBR® Green I to detect double-strand cDNA synthesis. The reactions were done in a volume of 25 μl using 12.5 μl of diluted cDNA (1:50), containing a final concentration of 0.2× SYBR Green, 100 μl dNTP, 1× PCR Buffer, 3 mM $MgCl_2$, 0.25 U Platinum® Taq DNA Polymerase 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 for denaturation, 35 s at 60 °C for annealing and 15 s at 72 °C for elongation. At the end of the cycling protocol, a melting curve analysis was included and fluorescence measured from 60 °C to 99 °C. Relative expression levels were determined with 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems) and, determined using the $2^{-\Delta\Delta ct}$ method. The efficiency per sample was calculated using LinRegPCR 11.0 Software (http://LinRegPCR.nl) and the stability of the reference genes EF1α, RIp13α and β-actin (M-value) and its optimal number according to the pair wise variation (V) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/).

Analysis of temporal absorption of copper by ICP-MS. The levels of copper absorbed for 24 h were assessed by inductively coupled plasma mass spectrometry (ICP-MS), according to the method described previously, with minor modifications (Ashoka et al., 2009). ICP-MS (all ICP-MS 7700x series components and software MassHunter from Agilent Technologies®) were used. Briefly, a pool of 10 larvae was washed two times with the water of Zebtec system and three times with 1 ml of Ringer's solution (Ringer's) to eliminate any environmental contamination of copper. After cleaning procedures, the samples, in 100 μl of cold Ringer's solution, were digested with 0.3 ml of concentrated nitric acid and 0.2 ml of hydrogen peroxide in a screw cap polypropylene tube. The

Table 1
PCR primer design and PCR product.

Enzymes		Sequences (5′–3′)	PCR product (bp)	GenBank Accession number	ZFIN ID (ZBD-GENE)
IL-1β	F	TCGCCCAGTGCTCCGGCTAC	178	NM_212844	040702-2
	R	GCAGCTGGTCGTATCCGTTTGG			
IL-10	F	TTCAGGAACTCAAGCGGGATATGG	73	NM_001020785	051111-1
	R	GCTGTTGACTTCAAAGGGATTTTGG			
TNF-α	F	AGGAACAAGTGCTTATGAGCCATGC	157	NM_212859	050317-1
	R	AAATGGAAGGCAGCGCCGAG			
COX-2	F	AACTAGGATTCCAAGACGCAGCATC	207	ENSDART00000093609[a]	–
	R	AAATAAGAATGATGGCCGGAAGG			
Ecto-5′-NT	F	TGGACGGAGGAGACGGATTCACC	149	BC055243.1	040426-1261
	R	GGAGCTGCTGAACTGGAAGCGTC			
ADA 1	F	GCACAGTGAATGAGCCGGCCAC	168	AAH76532	040718-393
	R	AATGAGGACTGTATCTGGCTTCAACG			
ADA 2.1	F	TTCAACACCACACGTATCGGGCAC	161	AAL40922	030902-4
	R	ATCAGCACTGCAGCCGGATGATC			
ADA 2.2	F	TTGCAATTGTTCATCATCCCGTAGC	186	XP_687719	041210-77
	R	TCCCGAATAAACTGGGATCATCG			
ADAasi	F	CTTTGTGGTACTTCAAGGACGCTTTG	121	AAL40922	030902-4
	R	TTGTAGCAGATAAAAGAAGCGAGACG			
ADA L	F	CTCTAATGTGAAAGGTCAAACCGTGC	108	NP_001028916	050913-145
	R	AAGACGCCCTTATCATCCGTGC			

[a] Ensembl Data base.

cap was tightened and the tube was placed in a water bath at 85 °C. After digestion, each sample was diluted to 5 ml with a 1% solution of nitric acid in a volumetric flask for analysis.

Protein quantification. The total protein concentrations of zebrafish larvae homogenates were determined according to the Coomassie blue method (Bradford, 1976).

Statistical analysis. Data were expressed as mean ± standard deviation and were subjected to Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's test. The statistical comparison of data regarding extracellular ATP hydrolysis was carried out at each time-point of incubation and over-time of incubation. For assessing the global over-time changes, the area under the curve were obtained for each homogenate. The test used was one-way ANOVA followed by Tukey's test. Values of $P < 0.05$ were considered significant.

Results

The biological markers of oxidative stress were used as an initial tool to characterize and evaluate the inflammation model induced by copper in zebrafish larvae. Initially, we have made a curve using concentrations of 1, 10 and 25 μM of copper, challenging the larvae for a period of 24 h. As shown in Table 2, the concentrations of 10 and 25 μM induced a significant reduction in GSH and in the activities of CAT and SOD. Since 10 μM of copper showed a significant increase of oxidative stress with less mortality than 25 μM, as demonstrated in a previous report from our group (Leite et al., 2012), we chose the respective concentration for further experiments. After determining the optimal concentration, the oxidative stress was evaluated at different time-points of 0, 4, 6 and 24 h (Table 3). SOD and CAT activities displayed a significant reduction after 24 h of exposure to copper when compared to control, whereas GSH decrease was significant in 6 and 24 h of exposure to copper. NO levels were also significantly elevated after 24 h of copper exposure.

The release of PGE_2 and the neutrophil migration to the inflammatory site occurred before the increase of oxidative stress. Fig. 1 shows that copper promotes an increase in PGE_2 levels (29.2 ± 9% and 32.6 ± 5%, at 1 and 24 h, respectively) when compared to the control group. Neutrophil migration, as indirectly measured by MPO activity, displayed a significant increase of 69.3 ± 18% after 4 h of exposure to copper (Fig. 2).

The nucleotide hydrolysis revealed a rapid catabolism of ATP and ADP (Figs. 3A and B), in which both nucleotides were completely consumed after 1 h of incubation. No significant difference was found between the control and the exposed groups. However, a significant reduction in ADP hydrolysis was observed only at 30 min of incubation in the group exposed for 24 h with 10 μM of copper. The extracellular metabolism of AMP, ADO, and inosine (INO) (Figs. 3C, D, and E) occurred slowly during the incubation period of 2 h. The AMP and ADO were less metabolized in the group exposed to 10 μM of copper for 24 h when compared to the control groups, showing a significant difference from 10 min to 180 min of incubation for AMP and, from 30 min to 180 min for ADO. INO levels were significantly decreased according to evaluation after 24 h of challenge to copper, probably due to the inhibition of ADA activity. The areas under the curve were calculated for all groups and the statistical analysis confirmed the data described above (Fig. 3 — inset).

Considering the results of HPLC analysis, which showed that ATP and ADP hydrolysis presented a partial, but not significant decrease after copper treatment, the ATPase and ADPase activities were also assessed by Pi released to ensure the reliability of data. The results showed that there were no significant changes for ATP and ADP hydrolysis after 24 h of treatment with 10 μM of copper. Additionally, as AMP hydrolysis and ADO deamination (Fig. 3) were significantly reduced after the same treatment, ecto-5′-NT and ADA activities were also evaluated by measuring Pi and NH_3 released, respectively. As expected, ecto-5′-NT and ADA activities decreased by ~20% (Fig. 4), confirming data from HPLC hydrolysis assay.

The RT-qPCR quantification (Fig. 5) showed a significant increase of IL-1β and TNF-α expression at 4 and 24 h after copper exposure. IL-10 was found significantly decreased at 4 h, whereas COX-2 was

Table 2
Effect on oxidative stress after 24 h of exposure to 1, 10, and 25 μM of copper.

	CAT (μmol/min/mg protein) (Mean ± SD)	GSH (μmol/mg protein) (Mean ± SD)	SOD (U/mg protein) (Mean ± SD)
Control	127.4 ± 5.9	9.0 ± 0.9	1.7 ± 0.2
Copper – 24 h			
1 μM	119.2 ± 4.5	7.0 ± 0.5	1.6 ± 0.5
10 μM	64.1 ± 5.2[a]	4.2 ± 0.8[a]	0.9 ± 0.2[a]
25 μM	65.7 ± 6.7[a]	3.6 ± 1.2[a]	1.4 ± 0.3

Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test.
[a] Mean value was significantly decreased compared to control group ($P < 0.05$).

Table 3
Effect of copper 10 μM on oxidative stress after 4, 6 and 24 h of exposure.

	CAT (μmol/min/mg protein) (Mean ± SD)	GSH (μmol/mg protein) (Mean ± SD)	SOD (U/mg protein) (Mean ± SD)	NO (nmol/mg protein) (Mean ± SD)
Control	118.4 ± 16.9	9.0 ± 1.2	1.7 ± 0.1	758.2 ± 88.5
Copper – 10 μM				
4 h	95.2 ± 13.3	6.9 ± 0.6	1.8 ± 0.5	771.9 ± 28.71
6 h	90.2 ± 12.8	3.0 ± 0.2[a]	1.4 ± 0.3	894.1 ± 70.9
24 h	58.3 ± 7.9[a]	3.7 ± 0.4[a]	0.8 ± 0.3[a]	1373.0 ± 68.2[b]

Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test.
[a] Mean value was significantly decreased compared to control group ($P < 0.05$).
[b] Mean value was significantly increased compared to control group ($P < 0.05$).

significantly increased at 24 h treatment. Ecto-5′-NT gene expression showed a significant decrease following 24 h of copper exposure. The genes of ADA showed different expressions after 24 h of exposure to copper: expressions of ADA 1 and ADAasi were not affected by copper; nevertheless, ADA 2.1 showed decreased expression, while ADA 2.2 and ADA L demonstrated an increased expression when compared to control (Fig. 6).

To evaluate the absorption levels of copper over the exposure time, an absorption curve of copper until 24 h of treatment was performed. We verified that the copper uptake was significantly increased over time, from 4 to 24 h, as shown in Fig. 7.

Discussion

Inflammation is a physiological process that represents the body immediate response to cellular and tissue damage by pathogens and chemical or physical stimuli. In some cases, it is associated with chronic pathological states, including allergies, atherosclerosis, cancer, arthritis and autoimmune diseases. Both in acute and chronic responses, cytokines and other inflammatory mediators are involved in key cellular events such as the production of reactive oxygen species (ROS) (Dantzer et al., 2008; Martinon, 2010).

In a previous study from our group (Leite et al., 2012), we assessed the dose-related mortality induced by copper in zebrafish larvae, and it was demonstrated that copper kills 15–20% of the animals at 10 μM, after 24 h of exposure. In the same study, it was demonstrated that lower concentrations of copper did not induce mortality, whereas 25 μM induced 75–80% of deaths. Supporting these data, a previous study conducted by Olivari et al. (2008) revealed that 1 μM copper is able to induce injury of lateral line hair cells of zebrafish larvae. Furthermore, the authors demonstrated that the concentration of 10 μM also induced a partial damage of the supporting cell layer. The concentration of 10 μM of copper, assessed by oxidative stress in the present study, leads to moderate toxicity, what might be allied to the biochemical and molecular inflammatory changes, while the concentration of 1 μM was not effective and 25 μM induces a high mortality. Production of ROS is crucial in regulating the innate immune response. The activation of phagocyte cells generates ROS that drive their toxicity to phagocytized microorganisms. ROS are also important in signaling damage to the immune system, and its production regulates the activation of transcription factors and cytokine production (Martinon, 2010). Our results using 10 μM copper demonstrated that oxidative stress increases slowly during the first hours of exposure to copper, but it is rather evident after 24 h, presumably due to the continuous exposure and increased uptake of copper by zebrafish larvae. The oxidative stress showed herein can occur by two mechanisms: direct effect of copper, which induces DNA and cell damage, or the increase of ROS release due to the activation of phagocytic cells and increased tissue damage (Olivari et al., 2008). The antioxidant defenses, including CAT and SOD activation, may be reduced after copper exposure due to the high levels of ROS present. Reduced activity of CAT may also be related to excessive superoxide anion radical resulting from reduced activity of SOD (Liu et al., 2008). During the inflammation process, there are two important groups of cytokines: (i) the pro-inflammatory series, secreted primarily by activated macrophages, and (ii) the anti-inflammatory ones, which are involved in the reduction of the inflammatory response. Homologs for a variety of mammalian cytokines have been identified in zebrafish, such as: TNF-α, IL-1β, IL-8, IL-10, IL-11, IL-15, IL-22 and IL-26 (Sullivan and Kim, 2008). The immersion of zebrafish embryos in LPS induced the gene expression of pro-inflammatory cytokines TNF-α, IL-8 and IL-β (Novoa et al., 2009; Oehlers et al., 2010; Watzke et al., 2007). Our data showed that in zebrafish larvae of 7 dpf challenged to copper presented a significant increase in IL-1β and TNF-α expression, and a reduction of the expression of IL-10 when compared to the control groups. These altered expressions, associated with a significant increase of PGE_2,

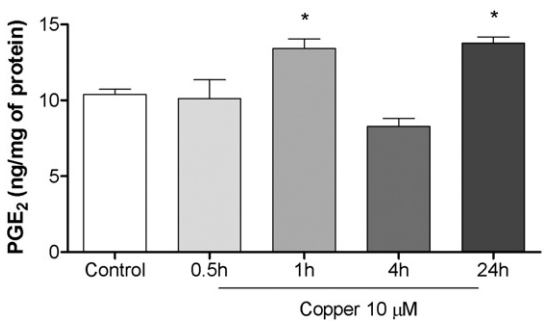

Fig. 1. Profile of PGE_2 release in 24 h of exposure to 10 μM of copper. The levels of PGE_2 were significantly higher at 1 and 24 h of exposure than controls. Each column represents the mean of six homogenates and, the vertical lines show the standard deviations. Asterisks (*) denote the significance level ($P < 0.05$) in comparison with control values. Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test.

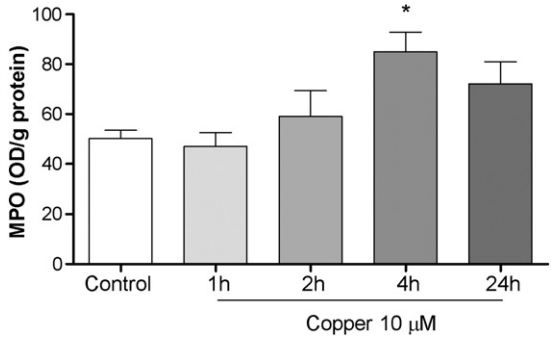

Fig. 2. MPO profile in 24 h of exposure to 10 μM of copper. The levels of MPO were significantly higher at 4 h of exposure than controls. Each column represents the mean of six homogenates and, the vertical lines show the standard deviations. Asterisks (*) denote the significance level ($P < 0.05$) in comparison with control values. Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test.

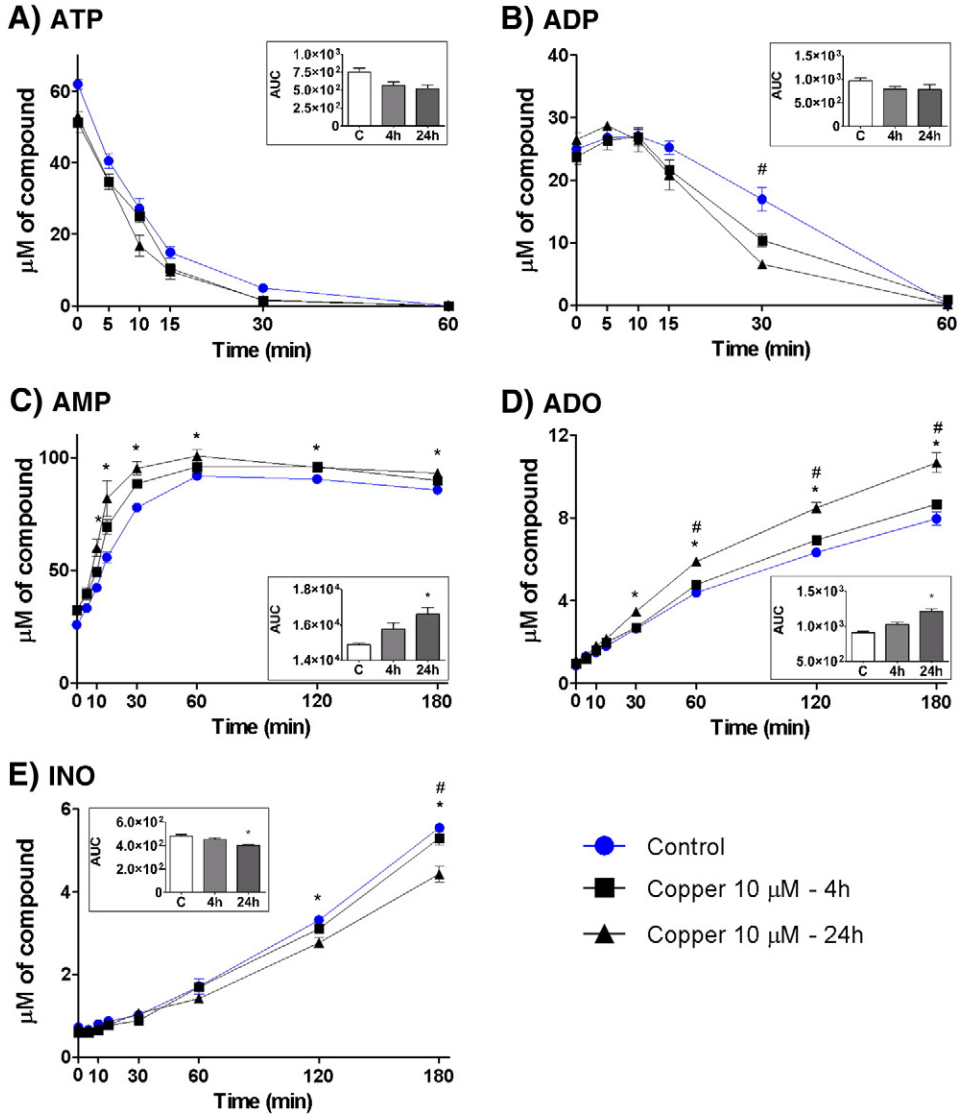

Fig. 3. Copper effects on extracellular ATP hydrolysis and, its degradation products. The zebrafish larvae were exposure to copper for 4 and 24 h. ATP (A), ADP (B), AMP (C), ADO (D) and INO (E) were assayed by HPLC-DAD. The data are mean ± S.D. of four homogenates. Asterisks (*) denote the significance ($P < 0.05$) in comparison with control values; pound signs (#) denote the significance ($P < 0.05$) in comparison with 4 h group. Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test. The groups were compared at each time of incubation (lines) and over time of incubation (inset). For assessment over time, the area under the curve was obtained for each homogenate.

MPO and ROS production provide a general scenario of the inflammatory response induced by copper.

PGE_2 is known for presenting multiple functions in the immune system. Its actions are related to the classical signals of inflammation, including vasodilation, increased vascular permeability, in addition to the chemotaxis and activation of neutrophils, macrophages, and mastocytes (Kalinski, 2012). Chronologically, the results of our research showed a remarkable increase of PGE_2 release after 1 h of exposure to copper, a return to normal levels, followed by a second peak after 24 h, where it is likely important for resolution. The first wave of PGE_2 production probably occurs due to the activation of the inflammatory process induced by copper, which is necessary for cell migration and subsequent activation. This migration was confirmed by increased MPO activity after 4 h of exposure. Furthermore, it preceded the increase in the expression of important inflammatory mediators released by activated cells (IL-1β and TNF-α).

The PGE_2 levels are controlled via the cyclooxygenase 1 and 2 (COX-1 and COX-2) pathways and its degradation by 15-hydroxyprostaglandin dehydrogenase (15-PGDH) (Kalinski, 2012). Expression of COX-2 showed a significant increased after 24 h of exposure to copper, which is likely responsible for the increase in the production of PGE_2 at this time-point. This second increase in PGE_2 can be related to its role in the late phases of inflammation, which might be linked to IL-10 release and suppression of pro-inflammatory cytokines (Kalinski, 2012). The elevation of IL-10 observed in our study at 24 h (in relation to 4 h), together with the second peak of PGE_2, might be indicative of the activation of pro-resolution mechanisms in the experimental model described herein.

The extracellular concentration of the nucleotides is controlled by E-NTPDase and ecto-5′-NT that sequentially catalyze the hydrolysis of ATP to ADO and thereby exert a tight control of the effects triggered by these important signaling molecules (Robson et al., 2006). ATP is implicated in the development of inflammation through a combination of actions, such as histamine release from mast cells (inducing the production of prostaglandins) and the production and release of cytokines from immune cells (Burnstock, 2006). To assess the ATP hydrolysis, the nucleotide hydrolysis was registered at different times and analyzed by HPLC. We observed that hydrolysis of either ATP or ADP was not altered by 24 h of exposure to copper. However, the hydrolysis of AMP was reduced at 24 h of exposure, with the consequent increase of AMP levels (the results were confirmed by decrease of ecto-5′-NT activity). In addition, RT-PCR analysis indicates that changes in the ecto-5′-NT activity could be related to the decrease of the expression of this

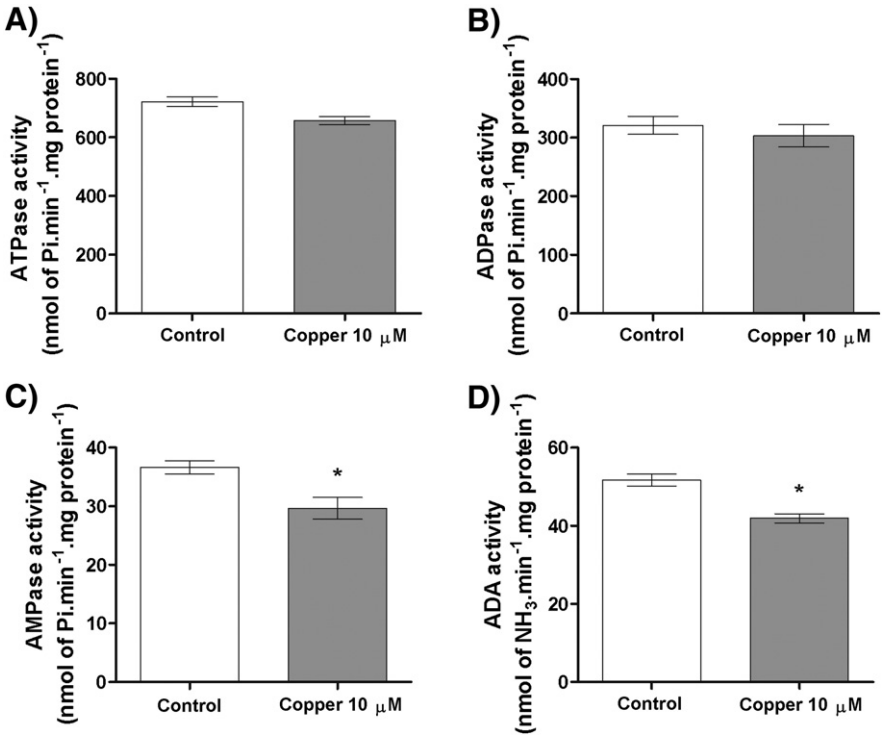

Fig. 4. Nucleotide hydrolysis analyzed by Pi liberation after 24 h of exposure to 10 μM of copper. ATPase (A), ADPase (B), AMPase (C) and ADA (D) activities were determined as described in Materials and methods. Asterisks (*) denote significant difference from control group by Student's *t*-test analysis ($P < 0.05$).

enzyme. Ramakers et al. (2012) demonstrated that the peak TNF-α production was coincident with the reduction of ecto-5′-NT activity in peripheral blood mononuclear cells, in an experimental model of endotoxemia.

Excessive collateral damage in healthy tissues threatens its functions, and should be stopped by resolution mechanisms, which are activated to avoid excessive cell damage. ADO shows anti-inflammatory effects and its extracellular levels are found increased in inflammatory pathologies such as asthma and sepsis (Ohta and Sitkovsky, 2009). Thus, ADO is an important pharmacological target during the resolution of inflammation. The concentrations of ADO can be regulated by reuptake and subsequent phosphorylation to AMP by adenosine kinase or deamination to INO by the enzyme ADA (Ramakers et al., 2012; Rosemberg et al., 2007a). The results presented in Fig. 3 show that there was an increase in ADO

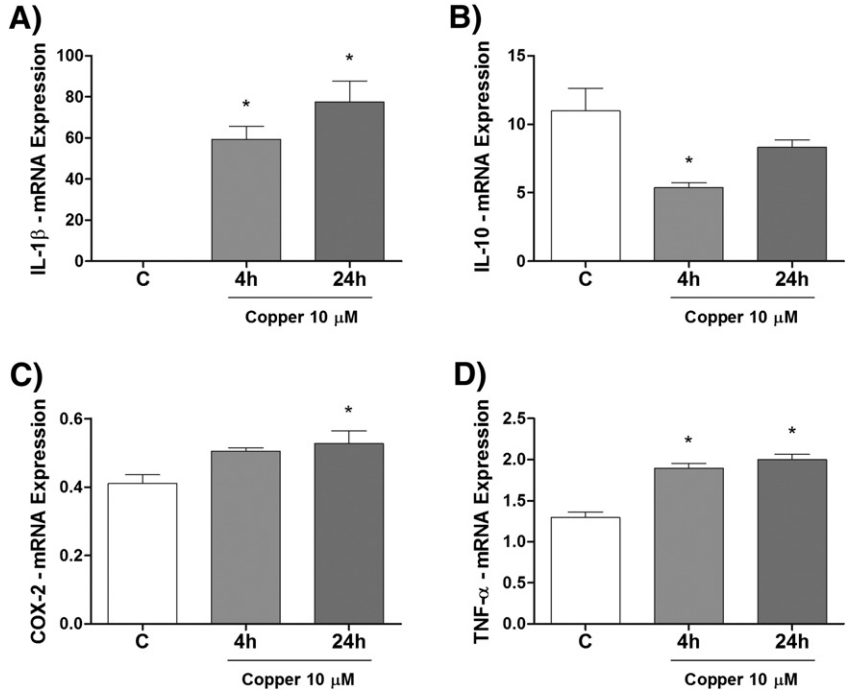

Fig. 5. Copper effect on inflammatory markers after 24 h of exposure. The figure shows IL-1β (A), IL-10 (B), COX-2 (C) and TNF-α (D) mRNA expression in zebrafish larvae. Asterisks (*) denote the significance level ($P < 0.05$) in comparison with control values. Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test.

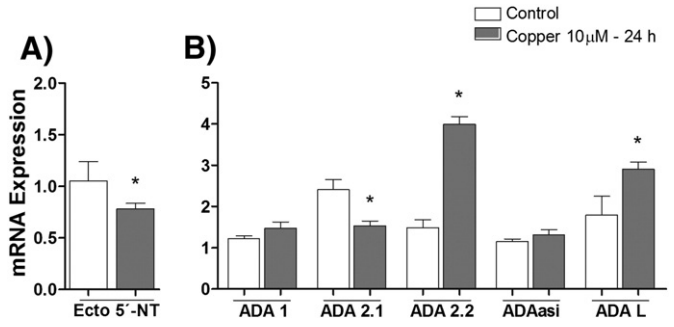

Fig. 6. Copper effect on enzymes transcripts after 24 h of exposure. The figure shows ecto-5′-NT (A) and ADA isoforms (B) mRNA expressions in zebrafish larvae. Asterisks (*) denote significant difference from control group by Student's *t*-test analysis ($P < 0.05$).

concentration which would be, at least in part, due to inhibition of ADA activity. We confirmed its reduction by determining the total activity of this enzyme, which controls the high systemic levels of ADO. Elevated ADO levels and reduction of ADA activity have been already described in LPS-induced endotoxemia in humans, demonstrating similarities of adenosinergic modulation mechanisms in these two models (Ramakers et al., 2012). Nevertheless, this modulation of ADO levels in inflammatory process had never been described in zebrafish larvae before.

To assess ADA subfamilies involved in the inflammatory process, we determined the gene expression of ADA 1, ADA 2.1, ADA 2.2 and ADAasi (the adenosine deaminase "alternative splicing isoform" of ADA 2.1). The expression of another similar group ADA L (adenosine deaminase "Like") was also investigated. Three of the assessed genes showed changes in their expression, with a reduction in the expression of ADA 2.1 and increase in the expression of ADA 2.2 and ADA L. This modulation in the gene expression is likely responsible for the resultant total ADA activity.

ADA 2 can be released at the sites of inflammation by macrophages, as demonstrated by Conlon and Law (2004) using "in vitro" experiments or "in vivo" model of sepsis in rodents. In humans, it was demonstrated that ADA 2 stimulates the proliferation of $CD4^+$ T cells, and induces the differentiation of monocytes into macrophages (Zavialov et al., 2010). Furthermore, a differential release of isoenzymes by macrophages might exist and ADA also may have distinct functional role according to the tissue in which they are expressed (Conlon and Law, 2004; Rosemberg et al., 2007a). Considering that macrophages release ADA at the sites of inflammation, a fine adjustment of the levels of different isoenzymes may occur. In the present study, we detected increased ADO levels, decreased ADA activity and significant different modulations in the expression of ADA 2.1 and ADA 2.2. Thus, we showed the first evidence that ADA 2 is closely linked to the control of ADO levels during inflammation induced by copper in zebrafish larvae. This event most likely occurs over the 24 h period and involves mainly ADA 2.1 (Fig. 5).

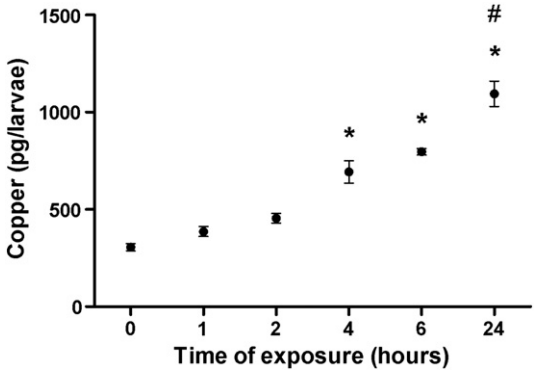

Fig. 7. Uptake of copper by zebrafish larvae over time in 24 h. Asterisks (*) denote the significance ($P < 0.05$) in comparison with control values; pound signs (#) denote the significance ($P < 0.05$) in comparison with 4 h group. Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test.

The functional role of ADA L still remains unclear, but it was demonstrated by Rosemberg et al. (2007a, 2007b) that ADA L mRNA levels were more abundant in the liver and kidney of zebrafish, suggesting that it has some physiological role in these tissues. Chronic exposure to copper primarily affects the liver, because this organ is the first site of copper deposition after entering the blood. The toxicity of copper in humans is typically manifested by the development of liver cirrhosis and damage of kidney and brain tissues (Gaetke and Chow, 2003). Considering that our results showed an increase in the expression of ADA L and, that the concentrations of copper in larvae exposed over 24 h were higher than in control samples, we might suggest that the increased expression of ADA L is related to liver and kidney toxicity induced by copper. However, additional studies should be conducted to elucidate the role of this ADA subtype in toxicity induced by copper.

In this study, we demonstrated that copper induces oxidative stress in zebrafish larvae and that this element is absorbed throughout the exposure period of 24 h. Due to the accumulation of copper, the damage and oxidative stress are exacerbated over the time, resulting in an inflammatory process involving COX-2 and PGE_2, and relying on the regulation of pro-inflammatory and anti-inflammatory cytokines. Concerning the purinergic system, the adenosinergic mechanisms might be the most involved in these events, and we could infer that this system is implicated in the resolution phase of inflammation induced by copper. In conclusion, this work brings novel advances to the characterization of inflammatory responses induced by copper in zebrafish larvae. Moreover, we provide additional knowledge on the role of purinergic system in copper-evoked inflammation.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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