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# Assessment of mercury chloride-induced toxicity and the relevance of P2X7 receptor activation in zebrafish larvae

Fernanda Fernandes Cruz [a,b], Carlos Eduardo Leite [a,c], Talita Carneiro Brandão Pereira [e], Maurício Reis Bogo [d,e], Carla Denise Bonan [d], Ana Maria Oliveira Battastini [c,f], Maria Martha Campos [a,g], Fernanda Bueno Morrone [a,b,*]

[a] Instituto de Toxicologia e Farmacologia, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, 90619-900 Porto Alegre, RS, Brazil
[b] Faculdade de Farmácia, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, 90619-900 Porto Alegre, RS, Brazil
[c] Programa de Pós-Graduação em Ciências Médicas, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2400, 90035-003 Porto Alegre, RS, Brazil
[d] Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, 90619-900 Porto Alegre, RS, Brazil
[e] Laboratório de Biologia Genômica e Molecular, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, 90619-900 Porto Alegre, RS, Brazil
[f] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[g] Faculdade de Odontologia, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, 90619-900 Porto Alegre, RS, Brazil

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ABSTRACT

Zebrafish (*Danio rerio*) has been adopted as a model for behavioral, immunological and toxicological studies. Mercury is a toxic heavy metal released into the environment. There is evidence indicating that heavy metals can modulate ionotropic receptors, including the purinergic receptor P2X7. Therefore, this study evaluated the in vivo effects of acute exposure to mercury chloride ($HgCl_2$) in zebrafish larvae and to investigate the involvement of P2X7R in mercury-related toxicity. Larvae survival was evaluated for 24 h after exposure to $HgCl_2$, ATP or A740003. The combination of ATP (1 mM) and $HgCl_2$ (20 μg/L) decreased survival when compared to ATP 1 mM. The antagonist A740003 (300 and 500 nM) increased the survival time, and reversed the mortality caused by ATP and $HgCl_2$ in association. Quantitative real time PCR showed a decrease of P2X7R expression in the larvae treated with $HgCl_2$ (20 μg/L). Evaluating the oxidative stress our results showed decreased CAT (catalase) activity and increased MDA (malondialdehyde) levels. Of note, the combination of ATP with $HgCl_2$ showed an additive effect. This study provides novel evidence on the possible mechanisms underlying the toxicity induced by mercury, indicating that it is able to modulate P2X7R in zebrafish larvae.

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## 1. Introduction

In the recent years, the use of zebrafish (*Danio rerio*) has been adopted in behavioral, immunological and toxicological studies, establishing its importance as a model for human diseases, and as a tool for screening of new drugs (Sullivan and Kim, 2008). Compared to in vivo rodent models, the benefits of using this teleost embryos and larvae include the possibility of analyzing a greater number of animals in a given time, besides to the transparency, size of the animal (only small amounts of drugs are needed), its favorable absorption and the fact that the embryos develop outside the mother's body (Weinstein, 2002; Langheinrich, 2003; Berghmans et al., 2008). According to Rico et al. (2011), zebrafish has proven to be an excellent model for the study of toxins in the environment, including heavy metals and the effect caused by the accumulation of pollutants (Rico et al., 2011).

Mercury is classified as a heavy metal and it is considered a toxic metal released into the environment (Zhang et al., 2005; Vieira et al., 2009). It is used in electrical industries, chlorine and caustic soda, in nuclear reactors, dental offices, and in gold mining or pharmaceutical antifungal products (Moore, 2000). Fish contaminated with mercury suffer serious pathological changes, occurring inhibition of metabolic processes, blood disorders, and reduced fertility and survival (Mikryakov and Lapirova, 1997).

Oxidative stress is characterized by the increase in reactive oxygen species (ROS), decreased antioxidant defenses or inability to repair the cell damage (Dorval et al., 2003), and it can be caused by heavy metals, like mercury (Huang et al., 2010). Furthermore, phagocytes generate ROS during under infectious stimuli, directly affecting the host immune system (Martinon, 2010).

The extracellular nucleotides act as endogenous signaling molecules in tissue damage by exerting effects on inflammatory and immune response (Lazarowski et al., 1997; Burnstock, 2006). ATP is involved in the development of inflammatory process, being released as a result of cell damage, causing the liberation of histamine from mast cells and the production of cytokines from immune cells. The biological effects of nucleotides are exerted through different classes of purinoreceptors

* Corresponding author at: Laboratório de Farmacologia Aplicada, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, Partenon, 90619-900, Porto Alegre, RS, Brazil. Tel.: +55 51 3353 3512; fax: +55 51 3353 3612.
*E-mail addresses:* fernanda.morrone@pucrs.br, fbmorrone@gmail.com (F.B. Morrone).

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(Burnstock, 2006). There are two major families of purinergic receptors: the adenosine receptors or P1 (P1R), and the P2 receptors (P2R), which recognize primarily ATP, ADP, UTP, and UDP. Based on the different molecular structures and mechanisms of signal transduction, the P2R are divided into two subfamilies of receptors: ionotropic P2X (P2XR) and metabotropic P2Y (P2YR) receptors (Ralevic and Burnstock, 1998; Burnstock, 2006). Currently, seven P2XR subtypes (P2X1 to P2X7) have been identified (Burnstock, 2006). P2X receptors are cationic channels expressed in the brain and peripheral nerves, skeletal and smooth muscle, glands and connective tissues, as well as in immune cells (Ralevic and Burnstock, 1998). The expression of different purinergic receptors has been widely described in zebrafish (Egan et al., 2000; Kucenas et al., 2003; Appelbaum et al., 2007; Kucenas et al., 2009). P2X7 receptors (P2X7R) exist in a limited number of cell types, but they are easily detected in hematopoietic cell lines, including macrophages and lymphocytes (Labasi et al., 2002; Chessell et al., 2005; Yoon et al., 2007; Chen et al., 2010). Recent data obtained with knockout P2X7R mice or selective antagonists for this receptor suggest a crucial role in the onset and persistence of certain types of chronic inflammatory painful states (Labasi et al., 2002; Donnelly-Roberts and Jarvis, 2007).

In this context, the aim of this study was to further evaluate the effects of acute exposure and the involvement of oxidative stress in the toxicity induced by $HgCl_2$ in zebrafish larvae (*Danio rerio*), with special attempts to assess the relevance of the purinergic P2X7R in mercury-induced toxicity.

## 2. Materials and methods

### 2.1. Reagents

Mercury chloride ($HgCl_2$) was purchased from Vetec Química Fina (Brazil). A740003 and ATP were purchased from Santa Cruz Biotechnologies (USA). Coomassie blue and bovine serum albumin were obtained from Sigma-Aldrich (USA). Trizol LS reagent, SYBR Green I and Platinum Taq DNA polymerase were purchased from Invitrogen (USA). All reagents used were of analytical grade.

### 2.2. Animals

A total of 40 healthy 6-month-old adult zebrafish (*Danio rerio*) of both sexes were kept in an aquarium system with controlled temperature (25 ± 2 °C) (Zebtec, Tecniplast, Italy). The animals were subjected to a light/dark cycle of 14/10 h respectively, and received a balanced diet with commercial flocked ration, three times a day. The animals were mated as described by Westerfield (2000). The embryos selected were transferred and maintained in Petri dishes until treatments. They were kept in an incubator with controlled temperature (25 ± 2 °C) (Novoa et al., 2009). The experimental procedures reported in this manuscript followed the "Principles of Laboratory Animal Care" from the National Institutes of Health (NIH). The institutional animal care committee (CEUA-PUCRS, 09/00135) approved all the procedures performed in this study.

### 2.3. Treatments

We selected the final concentrations of 10, 20 and 30 μg/L of $HgCl_2$, since the concentration of 20 μg/L of mercury is found in the aquatic environment (Berzas Nevado et al., 2003; Jha et al., 2003; Senger et al., 2006). Moreover, previous studies have shown that the concentration of 20 μg/L of mercury alters the activity of ecto-nucleotidases in rats (Oliveira et al., 1994) and zebrafish (Senger et al., 2006). Treatments were performed in larvae of 7-days post-fertilization in six-well plates in a final volume of 3 mL for 4 h for oxidative stress assays, and 24 h for survival curve and real time PCR assay. The concentrations adopted for ATP (P2X7R agonist) were 1, 3, and 5 mM. Previous studies showed that such concentrations activate the P2X7R (Kucenas et al., 2003; Gehring et al., 2012). The A740003 concentrations used were 300, 500 and 2000 nM, based in previous studies (Honore et al., 2006; Gehring et al., 2012). ATP was diluted in phosphate buffered saline solution (PBS, pH 7.3) and A740003 was diluted in dimethyl sulfoxide (DMSO; final concentration of 0.01%). ATP and A70003 were incubated 30 min before exposure to $HgCl_2$.

### 2.4. Survival curve

For the evaluation of survival curves, 25 larvae per group were used, which were exposed to treatments for 24 h. The mortality was evaluated at 0, 2, 4, 8, and 24 h of exposure. To evaluate the larvae death we used the following parameters: mobility, larvae position and color.

### 2.5. Gene expression analysis by quantitative RT-PCR (RT-qPCR)

A pool of 15 zebrafish larvae with 7-days post-fertilization were exposed to $HgCl_2$ at the concentrations of 20 and 30 μg/L during 24 h. The total RNA was isolated with Trizol® reagent, according to the manufacturer's instructions. 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. 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). Relative RNA expression levels were 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*, *Rlp13α* and *β-actin* (M-value) and the optimal number of reference genes according to the pair wise variation (*V*) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/).

### 2.6. Oxidative stress assays

Larvae were exposed to $HgCl_2$ concentrations (10, 20 and 30 μg/L) for 4 h, using 20 larvae per treatment group. Larvae were stored at −80 °C in 500 μL of phosphate buffered saline solution (PBS, pH 7.3). After thawing, the samples were washed and homogenized with PBS buffer using a glass-Teflon homogenizer on ice bath. The samples were centrifuged at 13,500 *g* for 5 min at 4 °C and the supernatants

**Table 1**
Quantitative RT-PCR primers sequences.

| Proteins | Primer sequence (5′–3′) | Accession number (mRNA) |
|---|---|---|
| *EF1α*[a] | F-CTGGAGGCCAGCTCAAACAT<br>R-ATCAAGAAGAGTACCGCTAGCATTAC | NSDART00000023156[c] |
| *Rlp13α*[a] | F-TCTGGAGGACTGTAAGAGGTATGC<br>R-AGACGCACAATCTTGAGAGCAG | NM_212784[d] |
| *β-Actin*[a] | F-CGAGCTGTCTTCCCATCCA<br>R-TCACCAACGTAGCTGCTTTCTG | ENSDART00000055194[c] |
| P2X7R[b] | F-TCCTGCAATGTGGCCAAAGCAG<br>R-TCTGGGTTTTGTCTGCCATTGTGC | NM_198984[d] |

[a] According to Tang et al. (2007).
[b] Designed by the authors.
[c] Ensembl database.
[d] GenBank.

were used for the assays (Leite et al., 2012). The activity of the enzyme catalase (CAT) was determined by UV spectrophotometry (Cary 1E Varian), in accordance to the method described by Aebi (1984). High performance liquid chromatography (HPLC 1100 series Agilent Technologies) was used to quantify the malondialdehyde (MDA) levels (Cheng et al., 2008). Proteins were determined according to Bradford (1976) by Coomassie blue method, using bovine serum albumin as standard.

### 2.7. Statistical analysis

Statistical evaluation of the survival curve data was performed using the Kaplan–Meier method. For RT-qPCR and oxidative stress assays, the results were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. Data are expressed as means ± standard deviations and significance was set at $p < 0.05$ in all assays.

## 3. Results

### 3.1. Survival curve

In this study, the mortality induced by mercury (20 and 30 μg/L) alone or combined with ATP (1 and 3 mM) was evaluated at 24 h. No mortality was observed in the larvae treated with $HgCl_2$ at both concentrations. Otherwise ATP (3 mM) induced death of 100% of larvae, within 24 h of treatment. Interestingly, when mercury was associated with ATP, the larvae death was accelerated and mortality occurred following 8 h of exposure. The treatment with ATP (1 mM) caused a significant mortality when associated with mercury (20 μg/L), decreasing survival by 63% at 24 h (Fig. 1A). In Fig. 1B, we aimed to evaluate the A740003 antagonism. The mortality rate induced by ATP (3 mM) was completely reversed by the selective P2X7R antagonist A740003, at a concentration of 2000 nM, confirming the involvement of this receptor in the larvae death. The groups treated with ATP (3 mM) and A740003 (300 and 500 nM), at 24 h of exposure, displayed a reduced mortality rate, particularly for up to 8 h after exposure, when compared to the group treated only with 3 mM. To evaluate the additive effect of this treatment combination, we chose the concentrations of 1 mM ATP and 20 μg/L $HgCl_2$. The survival of the larvae was lower in the group exposed to ATP plus mercury, and it has been significantly prevented when the selective P2X7R antagonist A740003 was co-incubated (Fig. 1C).

### 3.2. Gene expression analysis by quantitative RT-PCR (RT-qPCR)

In order to evaluate the involvement of mercury in the modulation of P2X7R expression, we performed RT-qPCR assay within 24 h exposure to $HgCl_2$ (20 and 30 μg/L). The results did not show any alteration of the receptor expression at 20 μg/L. On the other hand, we observed a significant decrease in the receptor transcript levels in the larvae treated with 30 μg/L $HgCl_2$, when compared to control (1.33 ± 0.6) (Fig. 2).

### 3.3. Evaluation of oxidative stress

The evaluation of oxidative stress was accomplished by the determination of MDA and CAT activity, in order to check the damage caused by $HgCl_2$ and ATP. The results revealed a significant decrease in CAT activity (29 ± 6% and 38 ± 2%) and increased MDA levels (101 ± 4% and 205 ± 9%), in larvae treated with $HgCl_2$ at concentrations of 20 μg/L and 30 μg/L, respectively. $HgCl_2$ did not cause oxidative stress at the concentration of 10 μg/L (Fig. 3A and B).

The treatment with 5 mM ATP induced a reduction of 52 ± 4% in CAT activity, similar to that induced by the association of $HgCl_2$ (20 μg/L) and 3 or 5 mM ATP (53 ± 5% and 58 ± 1%, respectively) (Fig. 4A). In addition, there was an increase of MDA levels in the groups treated with 20 μg/L $HgCl_2$ (101 ± 7%), 3 mM (61 ± 3%) or 5 mM ATP (72 ± 4%). When these treatments were combined, we obtained an additive effect for both 3 mM ATP (187% ± 15%) and 5 mM ATP (199 ± 16%) (Fig. 4B). Comparing these groups, there was a significant increase in MDA amounts in the groups treated with 20 μg/L $HgCl_2$, 3 mM ATP and 5 mM ATP in relation to the co-treatment. We observed an increase of MDA levels when comparing the group treated with mercury in relation to the combination of $HgCl_2$ plus 3 mM ATP (43 ± 7%) or 5 mM ATP (48 ± 8%). Likewise,

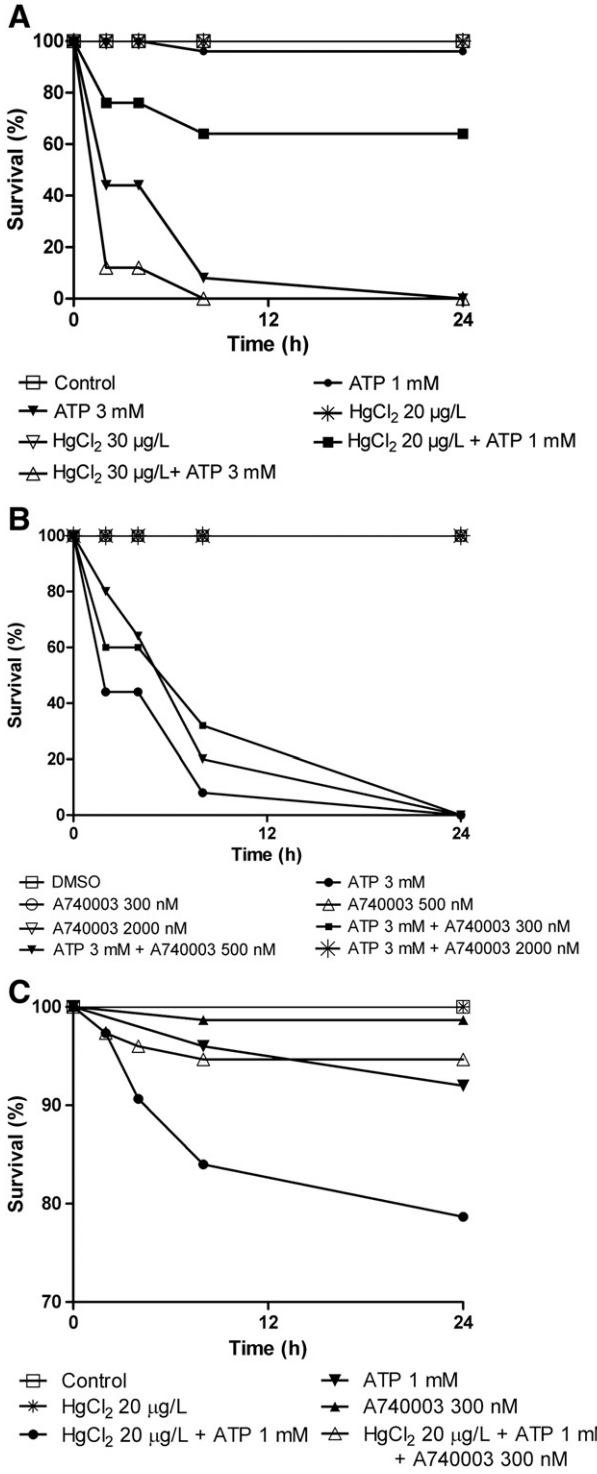


**Fig. 1.** Evaluation of the survival of the larvae treated with mercury and ATP (A). The larvae were treated with the P2X7R antagonist (A740003) and ATP (B). Effect on mortality was evaluated after exposure to $HgCl_2$, ATP and A740003 (C). Statistical evaluation of the results was performed using the Kaplan–Meier method. (25 zebrafish larvae each group of treatment.)

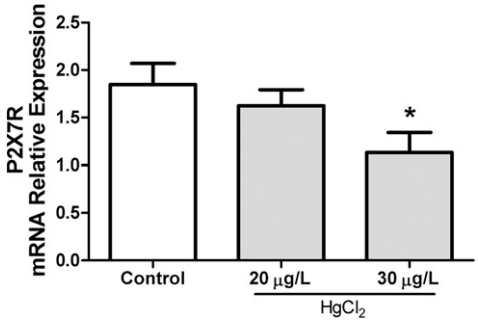


**Fig. 2.** Effect of 24 h $HgCl_2$ exposure on P2X7R gene expression in zebrafish larvae ($N = 4$). Each bar represents the mean ± standard deviations. The statistical analysis was determined by Student's *t* test and for comparison from the respective group, was determined by ANOVA with Tukey's post-hoc test. Significant difference between groups compared to control *$p < 0.05$.

the same pattern was observed by comparing the groups treated only with ATP (3 mM and 5 mM) in relation to the combination of $HgCl_2$ plus ATP (3 mM) (78 ± 9%), and $HgCl_2$ plus 5 mM ATP (74 ± 9%), respectively. No significant difference between the groups without association of treatment was observed.

## 4. Discussion and conclusion

Mercury can be found in the aquatic environment at a concentration of 20 µg/L (Berzas Nevado et al., 2003; Jha et al., 2003; Senger et al., 2006). In our study, this concentration of $HgCl_2$ did not alter the survival of the larvae, but it caused a marked change of the oxidative stress. The levels of MDA were increased, whereas the CAT activity was decreased, indicating the damage caused by this heavy metal at the concentrations of 20 and 30 µg/L. Ung et al. (2010) showed, by transcriptome analysis, the down-regulation of genes involved in electron transport chain, mitochondrial fatty acid beta-oxidation, and apoptotic nuclear receptor signaling pathways in zebrafish exposed to mercury, indicating that this heavy metal is involved in depletion of oxidative defenses (Ung et al., 2010).

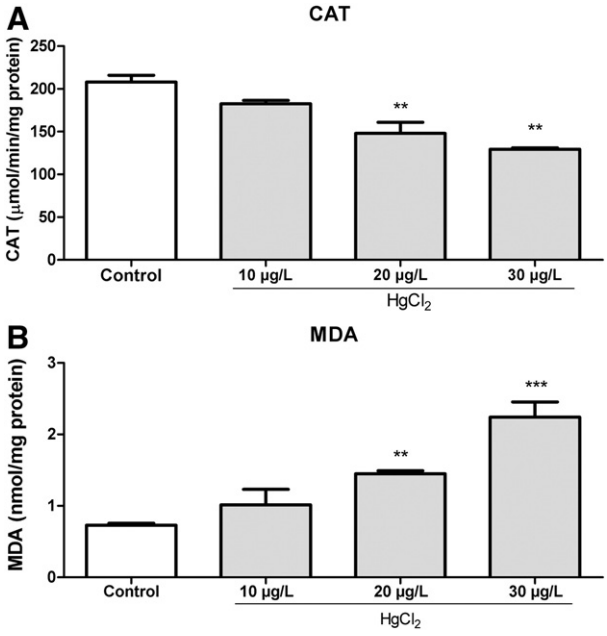


**Fig. 3.** Evaluation of oxidative stress in zebrafish larvae treated with $HgCl_2$. CAT activity (A) and MDA level (B). Significant difference between groups compared to control (without addition of $HgCl_2$). The results are expressed as mean ± standard deviations. The bars represent the mean of three homogenates (20 zebrafish larvae each). Significant difference between groups compared to control, **$p < 0.01$, ***$p < 0.001$.

During the inflammatory process, ATP and adenosine are released at the sites of inflammation, as a result of cell damage. In addition, nucleotides can be actively transported to the extracellular medium under cell activation. Activated platelets and endothelial cells secrete ATP and ADP under conditions of physiological stress (Luttikhuizen et al., 2004; Burnstock, 2006). The extracellular concentration of the nucleotides is controlled by ectonucleotidases, such as ecto-nucleoside triphosphate diperoxiphohydrolases (E-NTPDases) and the ecto-5′-nucleotidase (ecto-5′-NT) sequentially to catalyze the hydrolysis of ATP to adenosine, asserting control of the effects triggered by these signaling molecules. According to Baldissarelli et al. (2012), heavy metals, like arsenic, can alter brain ectocucleotidases activities in zebrafish and behavioral parameters (Baldissarelli et al., 2012). Another previous study showed that the same mercury concentration used in this work was able to decrease the activity of enzymes that break down ATP in adult zebrafish, after 96 h of exposure (Senger et al., 2006), by increasing the purinergic signaling and consequent development of inflammatory processes. Gonçalves et al. (2006) showed that P2X7R is involved in interstitial inflammation and collagen deposition in a model of unilateral obstruction of the urethra (Gonçalves et al., 2006). It has been proposed that prolonged exposure to P2X7R agonists leads to formation of a pore in cytolytic cell membrane, allowing entry of large particles and subsequent cell death (Burnstock, 2006; Di Virgilio, 2007; Donnelly-Roberts and Jarvis, 2007). Hattori and Gouaux (2012) studied the crystal structure of the P2X4 receptor in zebrafish, showing the trimeric conformation characteristic of P2X receptors, and this receptor opening pore when coupled to ATP (Hattori and Gouaux, 2012). It has been already described (Cruz et al., 2007) that treatment with 3 mM ATP, display an increased activation of purinergic receptors, stimulating the production of reactive oxygen species (ROS), thereby increasing oxidative stress, which is consistent with our results. Furthermore, there was a high and rapid mortality of larvae at 8 h and 100% mortality at 24 h of treatment with 3 mM ATP. A740003 is a selective antagonist of rat and human P2X7R (Honore et al., 2006). The activation of this receptor triggers the release of IL-1β and pore formation receptor and it was shown in the present work that the selective antagonist of this receptor, A740003 at a concentration of 2000 nM was able to reverse the mortality induced by 3 mM ATP and other concentrations increased the survival time of zebrafish larvae.

The two ATP concentrations (3 and 5 mM) tested herein presented similar effects in the experiments for the evaluation of oxidative stress, showing an additive effect when combined to $HgCl_2$ (20 µg/L). The effect of this association was significantly different when compared to groups treated with $HgCl_2$ or ATP separately, demonstrating a synergistic behavior of these combinations. Treatment with 1 mM ATP did not cause significant death, although it has been reported that this concentration is able to activate the P2X7R in zebrafish (Kucenas et al., 2003). Interestingly, the association of 1 mM ATP with 20 µg/L $HgCl_2$ demonstrated a significant mortality.

In order to further assess whether P2X7R is involved in decreased survival induced by this association, we tested the receptor antagonist A740003, and we observed a significant reversal of the zebrafish larvae mortality treated with the combination of $HgCl_2$ (20 µg/L) plus ATP (1 mM).

There is evidence that heavy metals can act as modulators of P2X purinergic receptors, in particular P2X2, P2X4, and P2X7 (Huidobro-Toro et al., 2008). In this study, the mortality caused by a lower mercury concentration (20 µg/L), when associated with ATP, was prevented by A740003, whereas this concentration did not affect P2X7R gene expression. Interestingly, the higher tested mercury concentration (30 µg/L) showed a decrease in the receptor gene expression. Supporting our data, a recent study evaluating the modulation generated by heavy metals of the activity of purinergic receptors in mice, showed that

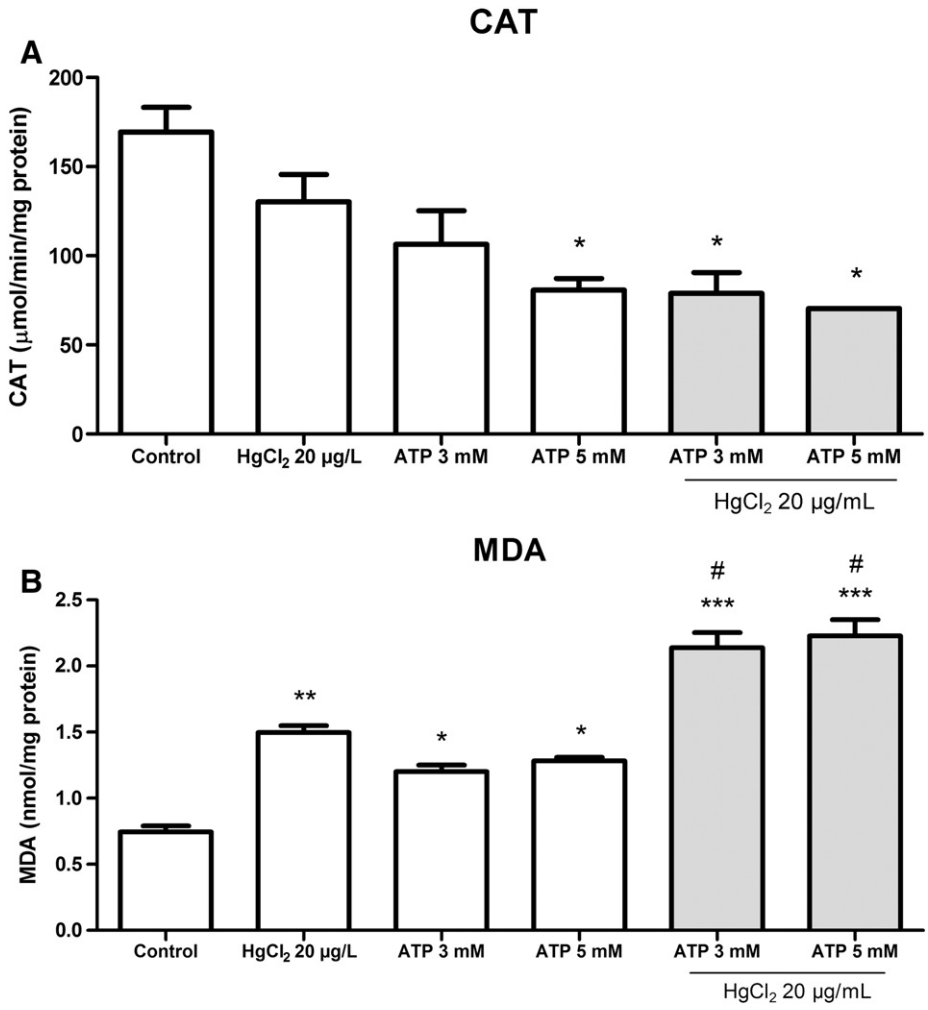


**Fig. 4.** Evaluation of oxidative stress in zebrafish larvae treated with $HgCl_2$ and ATP. (A) Effect of $HgCl_2$ and the ATP and their association on CAT activity. (B) Effect of $HgCl_2$ and the ATP and their association on MDA level. The results are expressed as mean ± standard deviations. The bars represent the mean of three homonegates (20 zebrafish larvae each). Significant difference between groups compared to control (without addition of $HgCl_2$) $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ for comparison versus control, $^{\#}p < 0.05$ for comparison from the respective groups.

mercury acts by inhibiting other P2X receptors (Coddou et al., 2005). Probably, lower concentrations of mercury do not totally inhibit P2X7R activation, allowing the action of ATP. The effects of mercury on the activation of P2X7R can occur indirectly, since the induction of oxidative stress leads to injury and cell death, releasing ATP and consequently activating the receptor. We believe that the association with P2X7R antagonist could decrease the oxidative stress caused by exposing the larvae to mercury and ATP, since A740003 demonstrated to reduce mortality caused by the exposure of this association. Further studies will be performed to elucidate these ideas. When analyzed in concert with literature data, our results bring novel evidence on the toxicity induced by mercury, suggesting that this heavy metal is involved in oxidative stress and is able to modulate the activity and expression of P2X7R in zebrafish larvae.

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