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Effects of caffeine on behavioral and inflammatory changes elicited by copper in zebrafish larvae: Role of adenosine receptors

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ABSTRACT

This study investigated the effects of caffeine in the behavioral and inflammatory alterations caused by copper in zebrafish larvae, attempting to correlate these changes with the modulation of adenosine receptors. To perform a survival curve, 7 dpf larvae were exposed to 10 μM $CuSO_4$, combined to different concentrations of caffeine (100 μM, 500 μM and 1 mM) for up to 24 h. The treatment with copper showed lower survival rates only when combined with 500 μM and 1 mM of caffeine. We selected 4 and 24 h as treatment time-points. The behavior evaluation was done by analyzing the traveled distance, the number of entries in the center, and the length of permanence in the center and the periphery of the well. The exposure to 10 μM $CuSO_4$ plus 500 μM caffeine at 4 and 24 h changed the behavioral parameters. To study the inflammatory effects of caffeine, we assessed the PGE_2 levels by using UHPLC-MS/MS, and TNF, COX-2, IL-6 and IL-10 gene expression by RT-qPCR. The expression of adenosine receptors was also evaluated with RT-qPCR. When combined to copper, caffeine altered inflammatory markers depending on the time of exposure. Adenosine receptors expression was significantly increased, especially after 4 h exposure to copper and caffeine together or separately. Our results demonstrated that caffeine enhances the inflammation induced by copper by decreasing animal survival, altering inflammatory markers and promoting behavioral changes in zebrafish larvae. We also conclude that alterations in adenosine receptors are related to those effects.

1. Introduction

Zebrafish (*Danio rerio*) have been widely used in developmental biology and molecular genetic studies, as well as in high-throughput screening for toxicity of chemicals (Hill et al., 2005). Among the several characteristics that make zebrafish an useful animal model in research there are: high fecundity; low maintenance costs in comparison to mammals; rapid embryogenesis; larvae transparency that allows the visualization of tissues in vivo; absorption of compounds from water, and the high degree of similarity with the human genome (Chakraborty et al., 2009). Copper (Cu) is a trace metal present in living organisms that can cycle between oxidized Cu (II) and reduced Cu (I) states (Tapiero et al., 2003). This metal is important in several biological processes, such as photosynthesis and respiration, iron metabolism, connective tissue formation, free radical scavenging and neurological function (Kuo et al., 2001). The cellular damage promoted by copper is probably secondary to the production of reactive oxygen species (ROS) (Brown and Borutaite, 2001; Valko et al., 2006), and this element is likely implicated in reactions that generate the hydroxyl radical, which may be detrimental to lipids, proteins and DNA (Halliwell and Gutteridge, 1984; Puig and Thiele, 2002). The zebrafish copper-induced inflammation model has been previously used, and present several advantages for being a non-invasive and sterile method in relation to methods involving physical damage and use of infectious agents, besides the wispy manipulation of the larvae (Pereira et al., 2016). Adenosine is a product from the hydrolysis of adenosine triphosphate (ATP) and plays a series of pathophysiological functions throughout the body (Sheth et al., 2014). This purine nucleoside promotes its effects by binding and activating four P1 adenosine receptors: A_1, A_{2A}, A_{2B} and

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A_3, which are G-protein-coupled cell-surface receptors (Haskó et al., 2008; Ferrari et al., 2016). Adenosine binding to A_1 and A_3 receptor subtypes leads to an inhibition of adenylate cyclase enzyme, decreasing cyclic AMP levels, whereas the activation of A_{2A} and A_{2B} subtypes causes the stimulation of adenylate cyclase, resulting in increased cyclic AMP levels (Burnstock, 2007; Montinaro et al., 2013). During inflammation, excessive damage to healthy tissues can compromise the normal functions and it must be controlled by resolution mechanisms, and adenosine is involved in these processes through their anti-inflammatory effects. For instance, high extracellular levels of adenosine are observed in inflammatory diseases, such as asthma and sepsis, being an important target for the resolution of inflammation (Ohta and Sitkovsky, 2009). Caffeine is a natural alkaloid and one of the most used bioactive substances worldwide, being present especially in coffee beans, tea leaves, cola nuts and cocoa (Gonzalez de Mejia and Ramirez-Mares, 2014). This methylxanthine is well known mainly for stimulating the central nervous system (Porciúncula et al., 2013), affecting sleep, cognition, learning abilities, memory (Rivera-Oliver and Díaz-Ríos, 2014) and human behavior (Smith, 2002). It exerts most of its actions by antagonizing the four adenosine receptors subtypes, but also present several other targets such as calcium channels, phosphodiesterases (PDEs), GABA-A receptors (Ribeiro and Sebastião, 2010) and phosphatidylinositol-3-kinase (PI3K) (Foukas et al., 2002), triggering effects in immunomodulation, inflammation (Horrigan et al., 2006) and central mechanisms (Ribeiro et al., 2002; Kaster et al., 2004). Previous studies of our research group have demonstrated the effects of copper in inflammatory markers, such as IL-1β, COX-2, PGE_2 and IL-10, as well as the involvement of purinergic system in these processes (Leite et al., 2013), suggesting that the copper-induced inflammation model is suitable for the study of the role of adenosine receptors in inflammatory processes. Furthermore, evidences show that, by the signaling of cytokines in the brain, there is a relation of inflammation with neurochemical, neuroendocrine and neuroimmune processes that could culminate in behavioral changes (Hou and Baldwin, 2012). Given the high caffeine intake and the therapeutic potential of adenosinergic signaling, as well as its controversial outcome in inflammatory processes, the aim of the study was to evaluate the effects of caffeine in adenosine receptors expression, behavior and inflammation in a copper-induced inflammatory model in zebrafish larvae.

2. Materials and methods

2.1. Animals

Adult zebrafish were maintained in an aquarium system with controlled water temperature and pH (Zebtec, Tecniplast, Italy), under a light/dark cycle of 14/10 h, respectively. The animals were fed with balanced diet that combines commercial flake and artemia (*Artemia salina*). To obtain the embryos, fishes were mated as described by Westerfield (2000). All protocols used in this study were performed with the consent of the Institutional Animal Care Committee (09/00135, CEUA–PUCRS). To perform the experimental procedures described in this study, we followed the "Principles of Laboratory Animal Care" from the National Institutes of Health (NIH).

2.2. Chemicals

Copper, added as copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), was acquired from Merck (Darmstadt, Hessen, Germany), and caffeine was purchased by Sigma (St. Louis, MO, USA).

2.3. Treatments

Seven dpf (days post-fertilization) larvae were treated with 10 μM $CuSO_4$, a concentration capable to induce inflammation and alter the adenosinergic signaling of zebrafish larvae, according to the work of Leite et al. (2012, 2013). Capiotti et al. (2011) demonstrated that 100 μM of caffeine alters the expression of adenosine receptors in zebrafish embryos. From this data, larvae were exposed to 3 different concentrations of caffeine: 100 μM, 500 μM and 1 mM. In the combined treatments, copper (10 μM) was added to the medium 30 min before caffeine for induction of inflammation.

2.4. Survival curve

For evaluation of the survival rates, larvae mortality was verified after 0, 2, 4, 8 and 24 h after treatments as described above. Twenty five larvae were used per group and the experiments were performed in triplicate ($n = 3$). The parameters observed to determine the mortality were color, locomotion, position and heart rate, using a microscope (Nikon® SMZ 1500).

2.5. Larvae behavior

The zebrafish larvae behavior was evaluated according to Colwill and Creton (2011). A 6-well plate was illuminated from above and filmed from below, using a high-resolution digital camera (Logitech®). The acquired recordings were analyzed by the software ANY-Maze (Stoelting Co., Wood Dale, IL, USA). After 4 and 24 h of exposure to treatments, the animals were selected and transferred one per well to shoot the plate. In the videos, larvae have undergone a period of one minute for acclimation and five minutes for behavior analysis. This experiment required at least 12 larvae per group (Capiotti et al., 2013). The parameters evaluated were distance, number of entries in the center, time spent in the center and time spent in the peripheral area of the well. The last three parameters are indicative of thigmotaxis, which is related to anxious behavior (Kalueff et al., 2013).

2.6. Determination of PGE_2 by UHPLC-MS/MS

PGE_2 (Prostaglandin E_2) levels were determined at 4 and 24 h after copper and caffeine treatments. To conduct these set of experiments, a pool of 35 larvae was required for each group of treatment ($n = 6$). The methodology employed was similar to that described by Leite et al. (2013). Larvae homogenates were prepared in 500 μL of phosphate buffered saline - PBS (pH 7.2–7.4). An aliquot of 400 μL of the homogenate was transferred into a 9 mL glass tube to carry out the extraction. Eight μL of 1 M nitric acid were added to the samples and 50 μL of BHT 1% were added to each tube. PGE_2 extraction was made using 2 mL of hexane: ethyl acetate (1:1, v/v) and mixing for 1 min. The samples were centrifuged at 800 ×*g* for 5 min at 4 °C. The organic phases from three extractions were collected, and under a stream of nitrogen at room temperature, were evaporated to dryness and reconstituted in 100 μL of methanol. The samples were analyzed by ultrahigh performance liquid chromatography coupled with mass spectrometry (UHPLC–MS/MS). Five microliters was injected into the UHPLC 1290/MS 6460 TQQQ - Agilent Technologies® (UHPLC components and software Mass Hunterwere from Agilent Technologies®). Chromatographic separations were executed using a Zorbax Eclipse Plus Phenyl–Hexyl 4.6 × 50 mm 1.8 μm column. The flow rate of formic acid: 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 detection was performed using an electrospray negative ionization and multiple-reaction monitoring of the transition ions. The collision energy was 14 V for transition 351 N 271 (quantifier) and 6 V for 351 N 315 (qualifier). The results were expressed as nanograms of PGE_2 per mg of protein.

2.7. Protein quantification

For quantification of total protein in the homogenates of the zebrafish larvae, the method using Coomassie Blue as described by Bradford (1976) was adopted.

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

Zebrafish gene expression of adenosine receptor subtypes (A_1, A_{2A1}, A_{2A2} and A_{2B}), and inflammatory markers TNF (tumor necrosis factor), COX-2 (cyclooxygenase 2), IL-6 (interleukin-6) and IL-10 (interleukin-10) was determined by RT-qPCR. Using Trizol® reagent (Invitrogen, Carlsbad, CA, USA), the total RNA was isolated from pools of 20 zebrafish larvae ($n = 5$) with 7 dpf, after 4 and 24 h of exposure to treatments. Total RNA quality and concentration was estimated by A_{260}/A_{280}, and it was used to Deoxyribonuclease I (Invitrogen) eliminate genomic DNA. Following the manufacturer's instructions, the cDNA was synthesized using ImProm-II™ Reverse Transcription System (Promega) from 1 μg total RNA. SYBR® Green I (Invitrogen) was used to detect double-strand cDNA synthesis in the quantitative PCR. Reactions were done in a volume of 25 μL using 12.5 μL of diluted cDNA, with 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). PCR cycling conditions were: polymerase activation 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 the cycling protocol, a melting-curve analysis was included and fluorescence was measured from 60 to 99 °C, showing one single peak in all cases. *EF1α* and *Rpl13α* were used as reference genes for normalization. Relative mRNA expression levels were determined with 7500 Real-Time Systems Software v.2.0.6 (Applied Biosystems). The assays were carried out in quadruplicate ($n = 4$). A reverse transcriptase negative control was included. The efficiency per sample was calculated using LinRegPCR 2012.3 Software (http://LinRegPCR.nl) and the stability of the references genes, and the optimal number of reference genes according to the pairwise variation (V) was analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). Relative levels of mRNA expression were determined using the $2^{-\Delta\Delta Cq}$ method (Bustin et al., 2013).

2.9. Statistical analysis

The statistical analysis of the survival curve was performed using the Kaplan-Meier method; a decrease of 20% of survival at the end of exposure time was considered as significant. In the behavior evaluation, RT-qPCR and determination of PGE_2 the results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. Data are expressed as mean ± standard error, and P values <0.05 were considered as indicative of significance.

3. Results

For survival assessment, the animals were treated for 24 h and the survival rates were determined at 2, 4, 8 and 24 h. There was no significant decrease in larvae survival, in the groups treated with 10 μM $CuSO_4$, 100 μM caffeine, 500 μM caffeine, 1 mM caffeine, or 10 μM $CuSO_4$ plus 100 μM caffeine, at the end of 24 h of exposure. However, the treatment with 10 μM $CuSO_4$ reduced the larvae survival to 60%, when combined with 500 μM caffeine, and caused death of 100% of larvae, when associated with 1 mM caffeine after 24 h of exposure (Fig. 1). We tested the combination of 10 μM $CuSO_4$ with the intermediate concentration of 500 μM caffeine after 4 and 24 h of exposure, to evaluate larvae behavior, to determine PGE_2 levels, and to access cytokines and adenosine receptors gene expression. In the evaluation of larvae behavior, 4 h treatment caused a significant reduction in the travelled distance by the animals exposed to 10 μM $CuSO_4$ (0.091 ± 0.017), 500 μM caffeine (0.027 ± 0.005) or 10 μM $CuSO_4$ plus 500 μM caffeine (0.043 ± 0.017), when compared to the control group (Fig. 2A). The number of entries in the center was also significantly decreased in the groups treated with 500 μM caffeine (0.250 ± 0.193) or 10 μM $CuSO_4$ plus 500 μM caffeine (0.083 ± 0.083), in comparison to the control group (Fig. 2B). The time spent at the center was significantly decreased when compared to the control group and 500 μM caffeine (0.737 ± 0.661), or 10 μM $CuSO_4$ and 500 μM caffeine (0 ± 0) (Fig. 2C). The relative time spent at the peripheral ring area of the well was increased in the groups treated with 500 μM caffeine alone (299.3 ± 0.661) or with 10 μM $CuSO_4$ combined to 500 μM caffeine (300 ± 0) (Fig. 2D). After 24 h treatment, the groups treated with 10 μM $CuSO_4$, 500 μM caffeine and 10 μM $CuSO_4$ plus 500 μM caffeine, showed a decrease of the distance travelled by the larvae in the well when compared to control (0.089 ± 0.230; 0.137 ± 0.041; 0.113 ± 0.033, respectively) (Fig. 3A). The number of entries in the center was also decreased when compared to the control, in the groups treated with 10 μM $CuSO_4$ (0.538 ± 0.143), 500 μM caffeine (0.153 ± 0.104) or 10 μM $CuSO_4$ combined to 500 μM caffeine (0.250 ± 0.130) (Fig. 3B). Similarly, the time spent at the center was significantly decreased in the groups 10 μM $CuSO_4$ (0.846 ± 0.338), 500 μM caffeine (0.284 ± 0.232) or 500 μM caffeine plus 10 μM $CuSO_4$ (0.725 ± 0.351) (Fig. 3C). There was no significant difference among the experimental groups in the time at the periphery of the well (Fig. 3D). To evaluate the inflammation outline, we selected the following inflammatory markers: PGE_2, COX-2, TNF, IL-6 and IL-10. PGE_2 levels were measured after 4 and 24 h of treatment. The results showed a significant increase of PGE_2 levels in larvae treated with 10 μM $CuSO_4$ plus 500 μM caffeine (32.19 ± 10.67%) in comparison to the control, whilst the

Table 1
Primer sequences used in RT-qPCR experiment.

Gene	Primer sequences (5′–3′)	Accession number (mRNA)	Reference
EF1α	F-CTGGAGGCCAGCTCAAACAT R-ATCAAGAAGAGTAGTACCGCTAGCATTAC	ENSDART00000023156	Tang et al. (2007)
Rpl13α	F-TCTGGAGGACTGTAAGAGGTATGC R-AGACGCACAATCTTGAGAGCAG	NM_212784	Tang et al. (2007)
A_1 (adora1)	F-GTTCCTCATTTACATTGCCATTCTGC R-TGGTTGTTATCCAGTCTCTCGCTCG	NM_001128584.1	Altenhofen et al. (2015)
A_{2A1} (adora2aa)	F-GCGAACTGTACGCCGAGCAGAG R-TTATTCCCAGTGAGCGGCGACTC	NM_001039815.1	Altenhofen et al. (2015)
A_{2A2} (adora2ab)	F-GGATTGGGTCATGTACCTGGCCATC R-GCTGTTTCCAATGGCCAGCCTG	NM_001040036.1	Altenhofen et al. (2015)
A_{2B} (adora2b)	F-GTTTGTTCGCTCTCTGTTGGCTGC R-CTAAAAGTGACTCTGAACTCCCGAATG	NM_001039813.2	Altenhofen et al. (2015)
IL-6	F-TCAACTTCTCCAGCGTGATG R-TCTTTCCCTCTTTTCCTCCTG	NM_001261449.1	Varela et al. (2012)
IL-10	F-TCACGTCATGAACGAGATCC R-CCTCTTGCATTTCACCATATCC	BC163031	Faikoh et al. (2014)
TNF	F-AGGAACAAGTGCTTATGAGCCATGC R-AAATGGAAGGCAGCGCCGAG	NM_212859	Leite et al. (2013)
COX-2	F-AACTAGGATTCCAAGACGCAGCATC R-AAATAAGAATGATGGCCGGAAGG	ENSDART00000093609	Leite et al. (2013)

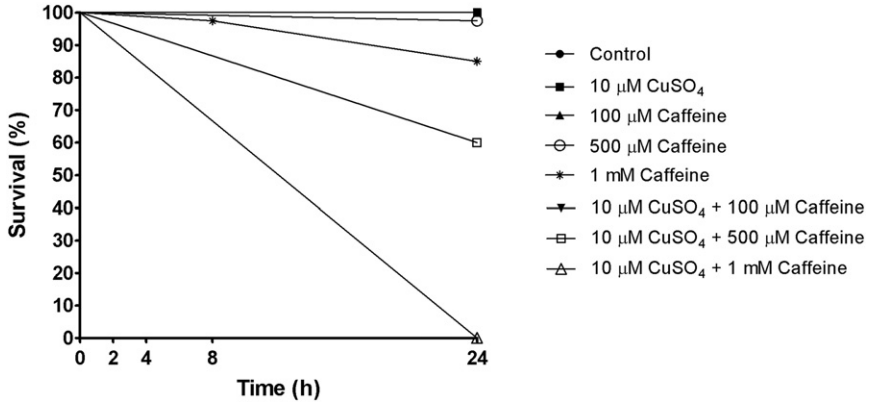

Fig. 1. Survival curve: Evaluation of the survival of the larvae treated with copper and different caffeine concentrations. The statistical analysis of the results was performed using the Kaplan–Meier method (25 larvae per group of treatment $n = 3$).

groups treated with 10 μM $CuSO_4$ demonstrated lower levels of PGE_2 when compared to 500 μM caffeine group, after 4 h of exposure (Fig. 4A). The treatment of 24 h with 10 μM $CuSO_4$ elicited an increase of PGE_2 levels (70.67 ± 17.03%) in comparison to control (Fig. 4B). COX-2, TNF, IL-6 and IL-10 gene expression was also evaluated after 4 and 24 h of exposure. After 4 h treatment, there was an increase in

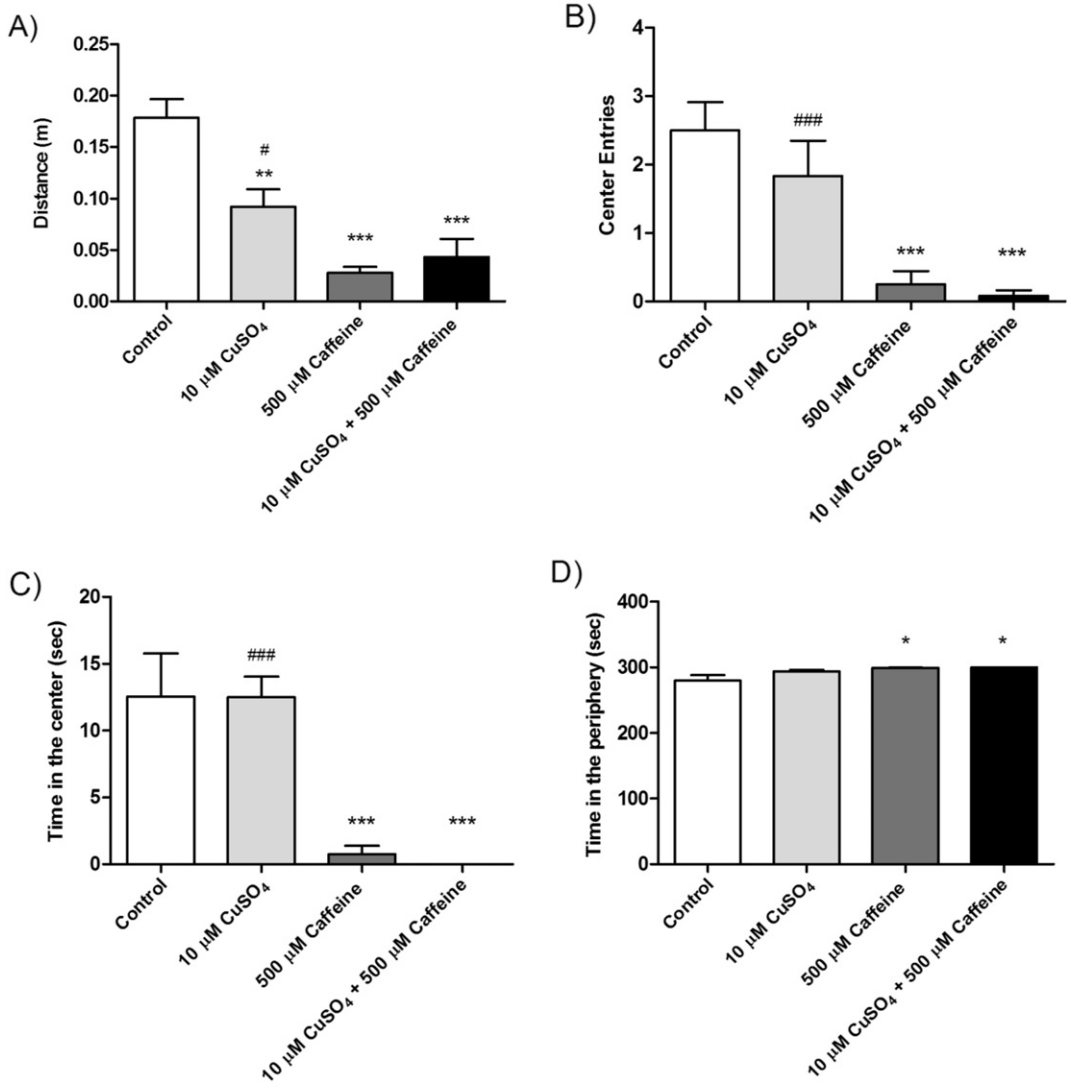

Fig. 2. Larvae behavior after 4 h exposure: Effect of 10 μM $CuSO_4$ and 500 μM caffeine in larvae behavior after 4 h treatment. The parameters analyzed were travelled distance (A), center entries (B), time spent in the center (C) and time spent in the periphery of the well (D). Each bar represents the mean of at least 12 larvae per group. Values are expressed as mean ± SEM. Mean values significantly different from control group $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Statistical analysis was performed by one-way ANOVA followed by Tukey's test.

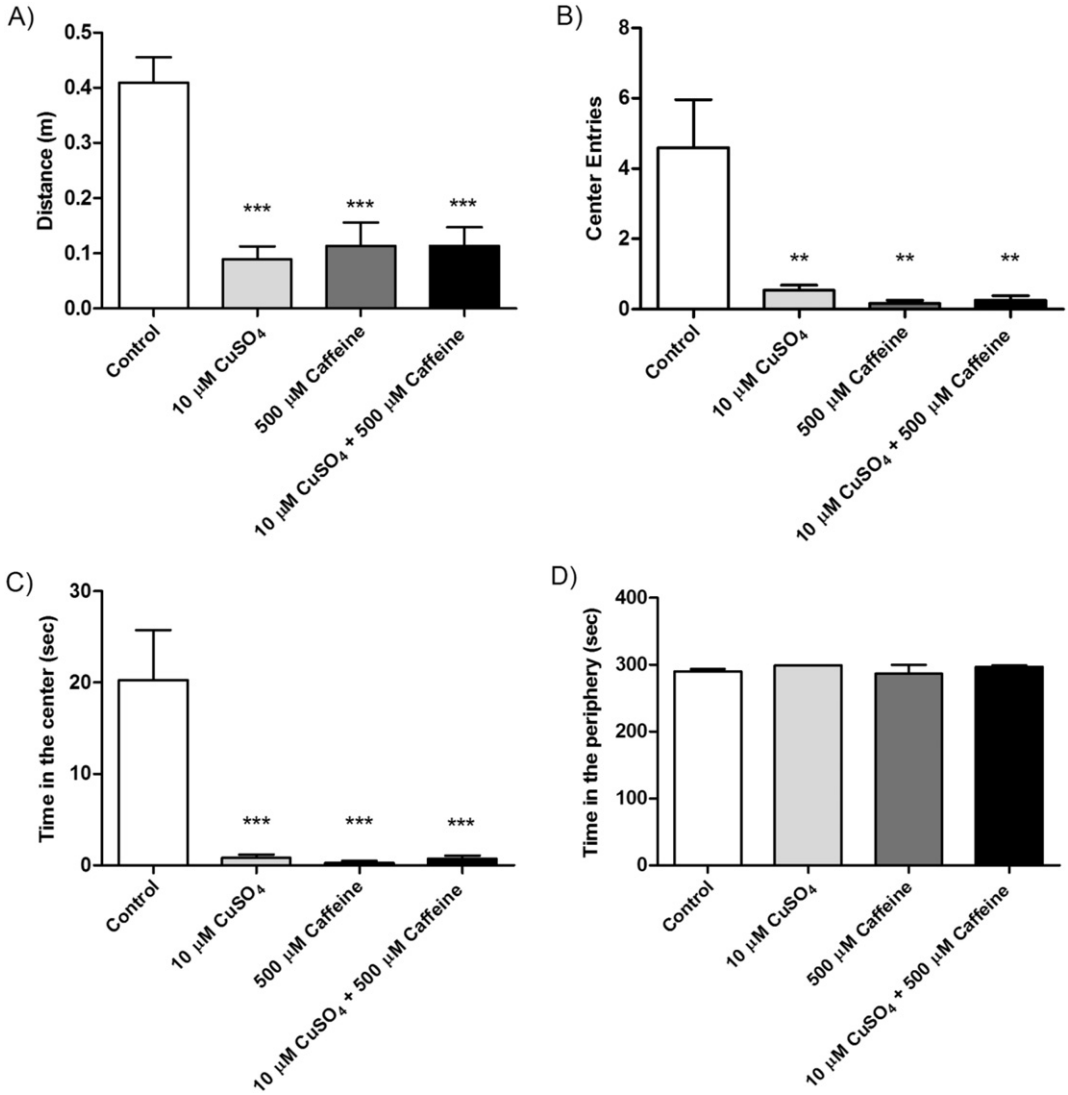

Fig. 3. Larvae behavior after 24 h exposure: Effect of 10 μM $CuSO_4$ and 500 μM caffeine in larvae behavior after 24 h treatment. The parameters analyzed were travelled distance (A), center entries (B), time spent in the center (C) and time spent in the periphery of the well (D). Each bar represents the mean of at least 12 larvae per group. Values are expressed as mean ± SEM. Mean values significantly different from control group $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Statistical analysis was performed by one-way ANOVA followed by Tukey's test.

COX-2 expression in the groups treated with 10 μM $CuSO_4$ (1.2 ± 0.23), and a decrease in the groups treated with 500 μM caffeine (0.55 ± 0.02), or 10 μM $CuSO_4$ associated to 500 μM caffeine (0.52 ± 0.03), in comparison to control. In relation to the group treated with 500 μM caffeine, 10 μM $CuSO_4$ had a significant increase of *COX-2* expression (Fig. 5A). After 24 h treatment, there was also an increase in *COX-2* expression in the groups treated with 10 μM $CuSO_4$ (1.25 ± 0.11), 500 μM caffeine (1.22 ± 0.39) or 10 μM $CuSO_4$ plus 500 μM caffeine (1.07 ± 0.15), when compared to the control group (Fig. 5A). After 4 h of exposure, TNF gene expression was also increased when the animals were treated with 10 μM $CuSO_4$ (3.03 ± 0.19), and showed an increase after the combined treatment of 10 μM $CuSO_4$ plus 500 μM caffeine (4.69 ± 0.25), when compared to control. In comparison with the group treated with 500 μM caffeine, there was a significant increase in *TNF* expression in larvae exposed to the combined treatment of copper and caffeine (Fig. 5B). At 24 h, *TNF* expression was increased after the treatment with 10 μM $CuSO_4$ and 500 μM caffeine (4.76 ± 0.81), when compared to control and to the group treated only with caffeine (Fig. 5B). No significant changes were observed in *IL-6* and *IL-10* gene expression at 4 h. Alternatively, after 24 h of exposure, there was an increase of *IL-6* expression in the 500 μM caffeine group (1.33 ± 0.12) (Fig. 5C), and a decrease of *IL-10* expression in the groups treated with 10 μM $CuSO_4$ (1.06 ± 0.03) or 10 μM $CuSO_4$ plus 500 μM caffeine (1.08 ± 0.06), when compared to the control. In comparison to 500 μM caffeine, *IL-10* gene expression showed higher expression in the group treated only with copper (Fig. 5D). Next, the gene expression of P1 receptors subtypes identified in zebrafish, A_1, A_{2A1}, A_{2A2} and A_{2B}, was evaluated after 4 and 24 h of exposure to copper and/or caffeine. After 4 h of exposure, there was an increase in the gene expression of A_1 receptor in the groups treated with 10 μM $CuSO_4$ (8.66 ± 0.27), 500 μM caffeine (8.09 ± 0.67), or 10 μM $CuSO_4$ plus 500 μM caffeine (9.73 ± 0.86) (Fig. 6A). Treatments with 10 μM $CuSO_4$ or 500 μM caffeine caused a significant increase in the expression of A_{2A1} receptors (7.46 ± 0.52; 7.14 ± 0.93) (Fig. 6B). A_{2A2} gene expression also presented an increase in the groups treated with 10 μM $CuSO_4$, 500 μM caffeine or 10 μM $CuSO_4$ plus 500 μM caffeine (1.30 ± 0.02; 1.44 ± 0.09 and 1.52 ± 0.07, respectively) (Fig. 6C). The treatment with 10 μM $CuSO_4$ (4.61 ± 0.39) or 10 μM $CuSO_4$ plus 500 μM caffeine (4.95 ± 0.46) significantly increased A_{2B} receptor gene expression (Fig. 6D). Furthermore, 24 h treatment caused an increase of A_1, A_{2A2} and A_{2B} genes expression only in the group treated with 10 μM $CuSO_4$ combined with 500 μM caffeine (8.39 ± 2.2; 1.51 ± 0.12 and 4.86 ± 0.78, respectively) (Fig. 6A, C and D).

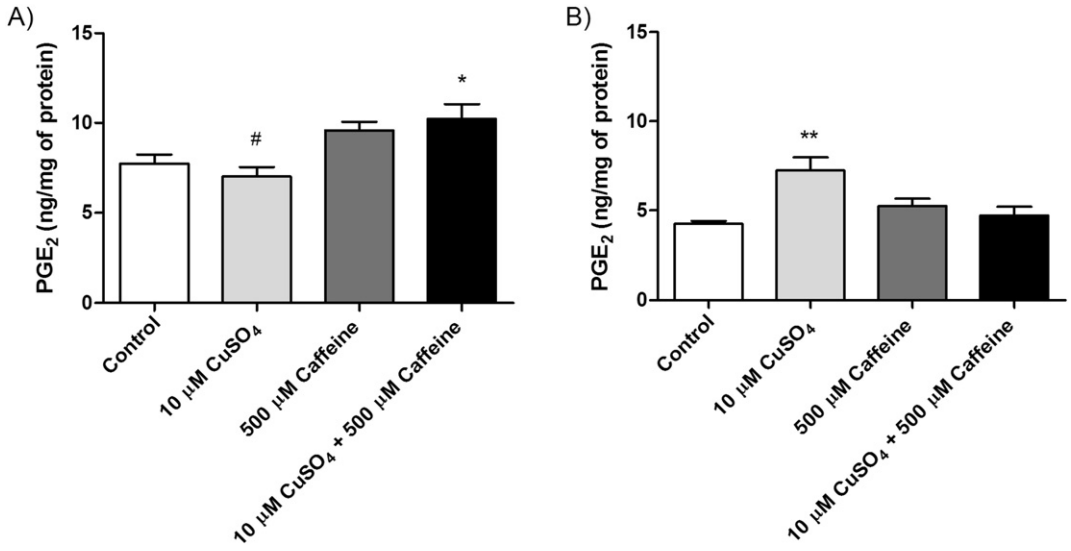

Fig. 4. Determination of PGE_2 levels: Profile of PGE_2 release after 4 and 24 h of exposure to 10 μM $CuSO_4$ and 500 μM caffeine. It was required 35 larvae per group ($n = 6$). The levels of PGE_2 were significantly higher than control at 4 and 24 h of exposure in copper plus caffeine and copper groups, respectively. Values are expressed as mean ± SEM. Mean values significantly different from control group $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{\#}P < 0.05$ in comparison to 500 μM caffeine group. Statistical analysis was performed by one-way ANOVA followed by Tukey's test.

4. Discussion

It has been already described that $CuSO_4$ can be used as an agent for the study of inflammatory processes in zebrafish larvae (Olivari et al., 2008; d'Alençon et al., 2010). Leite et al. (2012) demonstrated that copper, when tested at the concentration of 10 μM, did not cause a significant decrease of the zebrafish larvae survival, but it was able to induce oxidative stress, and a marked increase of inflammatory markers (Leite et al., 2013). Corroborating with these findings, our results show that, depending on the time of exposure, copper can induce the increase of PGE_2 and COX-2 and TNF genes expression, demonstrating its pro-inflammatory potential. Caffeine has several physiological targets, but adenosine receptors are the most relevant, especially when this nucleoside binds to A_{2A} receptor, which can increase the intensity of acute inflammation (Ohta et al., 2007; Ohta and Sitkovsky, 2009). In order to verify the inflammatory effect of caffeine, and choose treatments times and concentrations for the subsequent experiments, larvae were treated with copper combined to caffeine. Interestingly, treatments only with $CuSO_4$ or with any of the tested concentrations of caffeine alone did not show decreased in larvae survival, while the groups that were treated with copper plus caffeine (500 μM or 1 mM) had their survival rates significantly decreased. $CuSO_4$ and/or caffeine treatments between 8 and 24 h of treatments did not demonstrate any changes in larvae survival (data not shown). This result suggests that caffeine may be potentiating the inflammatory effect of copper, culminating in a significant death of the animals after 24 h of exposure. Compelling evidence demonstrated the relation of inflammation with neuropsychiatric disorders. It has been reported that during inflammation, pro-inflammatory cytokines can access the central nervous system and influence brain functions related to behavior, such as some pathways that control mood regulation, motor activity and anxiety (Capuron and Miller, 2011). In our study, after 4 h of exposure, caffeine or copper plus caffeine groups had the distance, number of entries and time spent in the center lower than larvae treated only with copper. Furthermore, time spend in the periphery was higher in groups treated with caffeine or cooper plus caffeine when compared to the control group. These set of results indicate that the behavioral changes may be induced by caffeine and not by copper. Of note, there was a reduction in the distance, the number of entries in the center and the time in the center of the well in all experimental groups, after 24 h of exposure. In fact, in this timepoint, copper showed an anxiogenic effect similar to caffeine, demonstrating that the inflammation induced by copper can alter the animal behavior and, although the larvae treated with copper or caffeine presented an anxiety-like behavior, the combination of these treatments did not show an additive effect. These results suggest that prolonged times of exposure to the inflammatory agent may be as anxiogenic as the treatment with caffeine alone. Interestingly, caffeine in the combined treatment did not potentiate the changes on the behavior of copper-treated animals. It has been previously reported that, depending on the dose used, caffeine can be anxiogenic to humans and animal models (Khor et al., 2013). Corroborating to our data, another study showed that caffeine induces an anxiety-like behavior in adult zebrafish, suggesting that this behavior is linked to the blockade of adenosine A_1 receptor subtype (Maximino et al., 2011). Herein, we demonstrated that the treatment with caffeine increased the gene expression of A_1 receptor subtype after 4 h of exposure, which could be a result of blocking the receptor with the antagonist. However, Maximino et al. (2011) reported an increase of locomotor activity of the animals, which was not observed in our study, since caffeine has decreased the distance traveled by the larvae in the times and concentrations evaluated. Chen et al. (2008) described that early exposure to caffeine in zebrafish caused muscle malformation, by disorganization of the muscle fibers alignment, which might influence the pattern of larvae locomotion. To evaluate the effect of the chosen caffeine concentration in larvae inflammatory profile, we decided to determine PGE_2 levels and COX-2, TNF, IL-6 (pro-inflammatory markers) and IL-10 (anti-inflammatory marker) gene expression. The PGE_2 levels were elevated only in the combined treatment of 10 μM $CuSO_4$ and 500 μM caffeine after 4 h. Therefore, it is reasonable to propose that caffeine can potentiate the effect of copper on the release of this inflammatory marker. At 24 h, only larvae treated with 10 μM $CuSO_4$ showed an increase of PGE_2 levels, suggesting that other pathways may be involved in the influence of caffeine in PGE_2 release after prolonged exposure to copper. In agreement with our data, Leite et al. (2013) demonstrated that 10 μM $CuSO_4$ treatment increased PGE_2 release in zebrafish larvae after 24 h of exposure. Regarding the gene expression of inflammatory markers, caffeine had different effects according to time of exposure. Alone or in combination with copper, caffeine treatment decreased *COX-2* expression at 4 h, and increased at 24 h. The fact that it is not possible to relate PGE_2 levels to the increase of *COX-2* expression may be due to a possible modulation of caffeine in the enzymes that metabolize PGE_2, such as 15-hydroxyprostaglandin dehydrogenase (15-PDGH), or it can have an effect on the enzyme activity, and not only in the gene expression. There was an increase of *TNF* expression at 4 h of exposure to 10 μM $CuSO_4$,

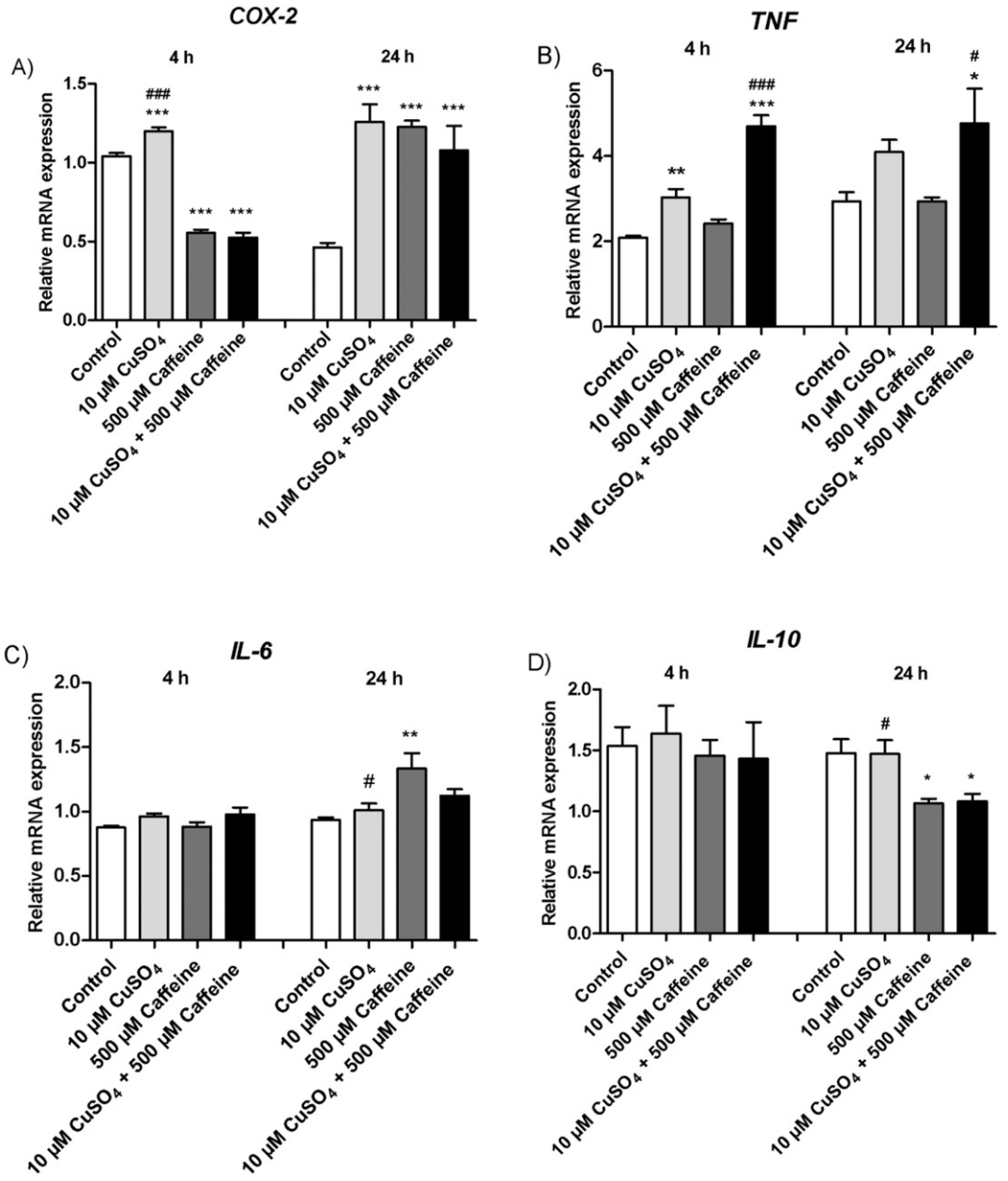

Fig. 5. Inflammatory markers gene expression: Effect of copper and caffeine in COX-2 (A), TNF (B), IL-6 (C) and IL-10 (D) gene expression, after 4 and 24 h of exposure. It was used a pool of 20 larvae per group ($n = 5$). Values are expressed as mean ± SEM. Mean values significantly different from control group $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.01$ in comparison to 500 μM caffeine group. Statistical analysis was performed by one-way ANOVA followed by Tukey's test.

and a further increase when caffeine was added to copper treatment, which indicates that there is an additive effect of the combination of treatments in *TNF* expression. In 24 h, only the treatment with 10 μM $CuSO_4$ plus 500 μM caffeine increased *TNF* expression, suggesting that caffeine may be prolonging the inflammatory effects mediated by copper. At 24 h, only caffeine at 500 μM was able to increase IL-6 gene expression, and *IL-10* expression was reduced after 10 μM $CuSO_4$ plus 500 μM caffeine reinforcing the notion of an inflammatory effect by caffeine. Adenosine metabolism is mediated by adenosine deaminase (ADA) and adenosine kinase (AK) enzymes, and the equilibrative nucleoside transporter (ENT) controls the adenosine diffusion between extracellular and intracellular medium (Ramakers et al., 2011). We believe that caffeine is possibly binding to adenosine receptors, promoting a free adenosine accumulation, which may result in adenosine metabolism to inosine by ADA. Moreover, the study of Leite et al. (2013) showed that zebrafish larvae treated with copper, presented a decrease of ADA activity that can occur probably with the intention of maintaining the increased adenosine levels and thus exert their anti-inflammatory action. Together, these results show that the adenosinergic system influences in copper-induced inflammation and caffeine may interfere in adenosine signaling. Further studies involving enzymes activities and using ENT inhibitors, such as dipyridamole, would be needed to better study the role of caffeine in these processes. Our results demonstrate an increased expression of adenosine receptors, depending on the time of exposure to treatments, which may be a result of the effect of caffeine as a non-selective P1 receptor antagonist. In general, the A_1 and A_{2B} receptors subtypes presents pro- and anti-inflammatory actions, while A_{2A} has anti-inflammatory effects (Antonioli et al., 2014). For a better understanding of each receptor effect on the release of each inflammatory marker and in larvae behavior, studies using specific receptor antagonists are needed. In the case of zebrafish genes for A_1 and A_{2B} receptors, and two genes for the A_{2A} receptor, named A_{2A1} and A_{2A2} have been described. However, their functional differences are not well established, and so far there is no evidence of the existence

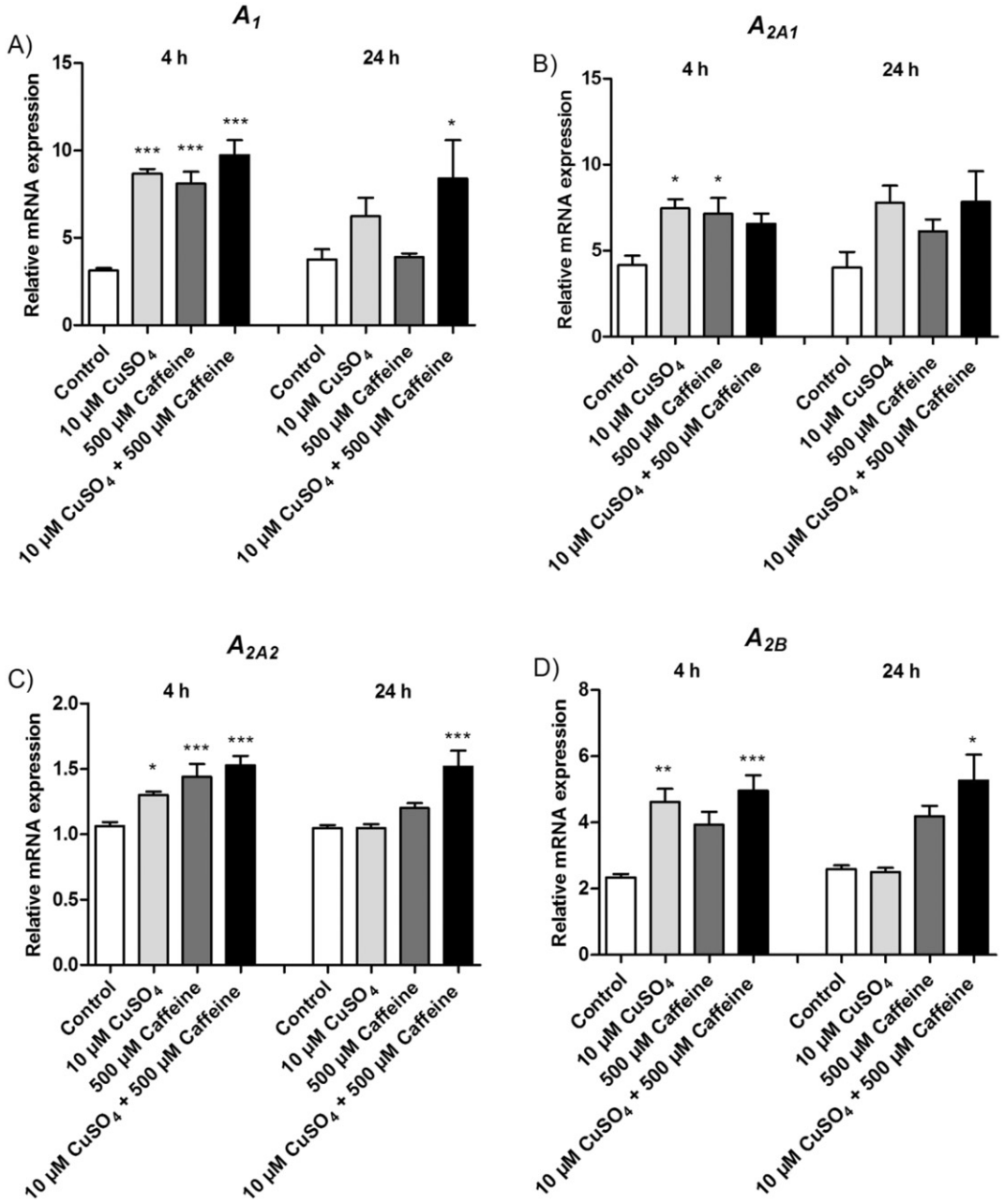

Fig. 6. Adenosine receptors gene expression: Effect of copper and caffeine in adenosine receptors subtypes A_1 (A), A_{2A1} (B), A_{2A2} (C) and A_{2B} (D) gene expression, after 4 and 24 h of exposure. It was used a pool of 20 larvae per group ($n = 5$). Values are expressed as mean ± SEM. Mean values significantly different from control group $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.01$ in comparison to 500 μM caffeine group. Statistical analysis was performed by one-way ANOVA followed by Tukey's test.

of the A_3 subtype (Boehmler et al., 2009). In fact, Ohta et al. (2007) disclose that caffeine can exacerbate tissue damage caused by inflammation via A_{2A} receptor, and that the caffeine concentrations in coffee consumers' blood can be high enough to block A_{2A} receptors (Ohta and Sitkovsky, 2009). Varani et al. (2011) demonstrated an increased gene expression of A_{2A} and A_3 receptors in lymphocytes from patients with rheumatoid arthritis, and also described that A_{2A} receptor activation inhibits the release of TNF, IL-1β and IL-6. Another study that supports the hypothesis of pro-inflammatory effects of caffeine is from Montesinos et al. (2000), which reported that caffeine reverses the anti-inflammatory effects of methotrexate in rheumatoid arthritis model in rodents by A_{2A} receptor pathway. In conclusion, copper is a heavy metal that can induce inflammation in a zebrafish model (Olivari et al., 2008; d'Alençon et al., 2010; Leite et al., 2012, 2013). Considering the concentrations and times of exposure tested, our data indicate that caffeine potentiate the inflammatory effects induced by copper in zebrafish larvae, showing the impact of adenosine receptors blockage. Moreover, the high intake of caffeine throughout the world makes this study very relevant in order to understand its effects in inflammatory conditions and diseases. Our study also suggests that those effects induced by copper and caffeine involve the modulation of P1 adenosine receptors expression.

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