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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.