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Time does matter! Acute copper exposure abolishes rhythmicity of clock gene in *Danio rerio*

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ABSTRACT

The circadian clock is a key cellular timing system that coordinates physiology and behavior. Light is a key regulator of the clock mechanism via its activation of Per and Cry clock gene expression. Evidence points to a key role of reactive oxygen species (ROS) in resetting this process. In this context, the aim of the present study was to explore copper as a ROS generator, using an innovative approach investigating its effects on circadian timing. Liver and brain from *Danio rerio* specimens exposed to 0, 5, 25 and 45 μg/L copper concentrations were obtained. Daily oscillations of superoxide dismutase (SOD) and catalase (CAT) enzymatic activity and their correlations both with clock genes (per1, per2, and cry1a) and with organism energy cost were determined. CAT expression correlates with per2 and cry1a and, thus, provides data to support the hypothesis of hydrogen peroxide production by a phototransducing flavin-containing oxidase. Higher SOD activity is correlated with higher intracellular ATP levels. Copper disturbed the daily oscillation of antioxidant enzymes and clock genes, with disturbed per1 rhythmicity in both the brain and liver, while cry1a rhythmicity was abolished in the liver at 25 μg/L copper. Coordination between the SOD and the CAT enzymes was lost when copper concentrations exceeded the limits established by international laws. These results indicate that organism synchronization with the environment may be impaired due to acute copper exposure.

1. Introduction

Organisms, from unicellular species to mammals and plants, display rhythmic oscillations, within the 24-h period, named circadian rhythms. These are highly conserved endogenous temporal organizations that allow the anticipation of environmental cycles and provide temporal coordination for physiological and behavioral activities (Kulczykowska et al., 2010; Zelinski et al., 2014; Moore and Whitmore, 2014). Molecularly, the vertebrate circadian clock is regulated by self-oscillating transcription translation feedback loops, composed of activator and repressor clock proteins (Nader et al., 2010; Mracek et al., 2012).

The major environmental timing cue for the entrainment of the circadian clock is light. In this way, organisms have evolved dedicated photoreceptors and signaling pathways at all levels of organization, from cells to tissues and organs, that rely on this lighting information (Pando and Sassone-Corsi, 2002; Mracek et al., 2012). Fish, especially *Danio rerio*, present peripheral clock oscillators directly entrained by light (Whitmore et al., 2000; Vallone et al., 2004). Molecularly, light can promptly activate the transcription of per2 and cr1a genes, that lead to the entrainment of the circadian clock and consequent organism synchronization with the environment within (Ziv et al., 2005; Tamai et al., 2007; Hirayama et al., 2007).

Additionally, it has been demonstrated that ROS (reactive oxygen species), more specifically H_2O_2, via the MAPK signaling pathway, can activate the transcription of those light-regulated genes and, with a delay, also the catalase enzyme. The activation of the catalase will decompose the hydrogen peroxide and downregulate the cycle (Hirayama et al., 2007). Likewise, because circadian rhythms in daily activities, such as feeding, locomotion, brain activity and sleep/wake

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cycles, modulate the intensity of metabolic processes during the day (Hardeland et al., 2003; Patel et al., 2014), the close connection between the circadian clock and redox signaling have been already pointed out by several studies (Rutter et al., 2002; Hirayama et al., 2007; Krishnan et al., 2008; Jiménez-Ortega et al., 2011; O'Neill and Feeney, 2014; Wu and Reddy, 2014).

However, when ROS production rates rise, oxidative stress, a disturbance in organism homeostasis resulting from the actions of intrinsic or extrinsic stressors, can occur (Wendelaar Bonga, 1997; Livingstone, 2001; Patel et al., 2014). Since, it is known that ROS play important physiological roles in intracellular signaling pathways (Nordberg and Arnér, 2001; Dröge, 2002; Wang et al., 2010; Tseng et al., 2012), oxidative stress may disrupt cellular homeostasis. Therefore, to protect the organism against excessive ROS formation, a symmetric and compensatory induction of the antioxidant defense occurs (Hardeland et al., 2003; Patel et al., 2014). Furthermore, since the ROS detoxification chain is based on a sequence in which the products of one reaction serve as substrates for the next, the expression and activity of various components of the antioxidant defense system must be coordinated (Hardeland et al., 2003; Volpato and Trajano, 2005; Patel et al., 2014). Two important components are the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), which convert superoxide anions into H_2O_2 and H_2O and O_2, respectively (Craig et al., 2007).

Copper (Cu) is the oldest metal manipulated by man and exposure to it is inevitable (Navarro, 2006). Natural Cu levels range from 2 to 30 μg/L but, in areas with a prevalence of agricultural and mining activities, industries and municipal effluents, Cu may be present in concentrations ranging from 50 to 560 μg/L (Hem, 1985; Robins et al., 1997). Furthermore, copper sulfate ($CuSO_4$) is widely used in aquaculture as a therapeutic agent for the control of protozoa, fungi, bacteria and cyanobacteria in fish (Varo et al., 2007; Straus and Tucker, 1993), being frequent in concentrations that reach seven to one hundred times greater than those found in the environment (Macfarlane et al., 1986; Paquin et al., 2002). Therefore, although Cu is an essential trace element in organisms, present in a large number of enzymes involved in a variety of biological processes, in high concentrations it can become toxic (Simonato et al., 2015).

Cu ions shows high reactivity to H_2O_2 and the potential to undergo the Fenton reaction, a process that can directly produce ROS, responsible for exerting the toxic function of excess Cu through oxidative stress. (Valko et al., 2005; Helsel and Franz, 2015). Farther, Cu and consequently ROS production, has been demonstrated to activate several signaling pathways but, mainly, the MAPK (Ostrakhovitch et al., 2002; Mattie et al., 2008; Song et al., 2009; McElwee et al., 2009; Wang et al., 2010; Tseng et al., 2012; Turski et al., 2012). This demonstrates that Cu not only can activate antioxidant defenses and interfere with cellular survival, growth and proliferation and apoptosis control, but also it can potentially affect the control of circadian rhythms by exerting oxidative stress.

Currently, an emerging issue is how the circadian apparatus is adjusted to maintain coordination between physiology and the ever-changing environment (Jiménez-Ortega et al., 2011). In this context, As Cu activates the MAPK pathway by producing ROS, and the fact that CAT may also be involved on the control of circadian rhythmicity, the hypothesis or the present work is that exposure to Cu may not only disturb antioxidant defenses, but also deregulate the circadian timing and proper synchronization between the organism and the environment. Consequently, the present study aimed to evaluate and establish a correlation between the expression and activity of the antioxidant enzymes SOD and CAT with the expression of per1, per2 and cry1a genes, responsible for the control of circadian rhythms, in liver and brain of *Danio rerio* between 48 and 72 h after Cu exposure to three different concentrations of copper (9 μg/L, 45 μg/L and 90 μg/L).

2. Material and methods

2.1. Animals and experimental design

In total, approximately 2500, 1:1 males/females between 4 and 5 months old weighting 275.32 ± 46.29 mg, wild-type zebrafish were obtained from an ornamental fish farm (Lindóia; Muriaé-MG) and were acclimated in 30 L or 20 L glass-aquaria for two weeks prior to the beginning of the experiment. The animals were maintained under controlled temperature (26.5 ± 1.5 °C), pH (7 ± 0.2) and lighting conditions of 12:12 light/dark cycles (LD). Fish were fed with ALCON Shrimp twice a day and half of the water in the aquaria was renewed every 72 h.

Four experimental groups were evaluated: one negative control group (CTRL), with no Cu added to the water, and three groups exposed to two ecologically relevant waterborne Cu concentrations of 5 and 25 μg/L and one concentration expected only in areas influenced by anthropogenic sources of 45 μg/L. A fresh 1 mg/L of Cu stock solution was prepared from copper sulfate (Sigma; ≥ 99.99% trace metals basis) and deionized water before animal exposure. Cu was added to the aquaria at 8:00 a.m., when the lights went on. Fish sampling began after 72 h of exposure and at each sampling time, 24 fish were randomly collected from the aquaria. The sampling occurred every three hours, starting at time zero (T0) at 8:00 a.m. and ending at 5:00 a.m. the next morning (T21), totaling eight collection points during twenty-four hours. The experiments were performed in independent triplicates. For each replica and for each experimental group consisted in 4 glass-aquaria with stocking density of 2–3 fish/liter, as recommended by the European zebrafish community and the Federation for Laboratory Animal Science Associations (FELASA).

After anesthetization with MS222 (1 g/L) liver and brain of all fish were obtained and immediately frozen in liquid N_2 for further analysis of enzymatic activity and ATP quantification by HPLC. For the qRT-PCR assay, tissues were embedded in RNAlater® and frozen in liquid N_2 until RNA extraction. Each time point evaluated for qRT-PCR or enzyme activity had n = 15 animals, except the HPLC measurement that had n = 5. At the first (T0) and last (T21) samplings, a 500 ml water sample was taken from each aquarium and added to acidified polyethylene plastic bottles with ultrapure MERCK nitric acid. The bottles were protected from light with aluminum foil and stored at − 4 °C until Cu concentration analyses.

This project complies with national and international ethical standards. All the animal procedures were approved by the Ethics Committee on the Use of Animals of the Biological Sciences Sector of the Federal University of Paraná (CEUA/BIO - UFPR). Certificate no. 962; Process no. 23075.118616/2016-33.

2.2. qRT-PCR

Pools of three livers and three brains was used for RNA extraction, based on the TRIzol® method (Chomczynski, 1993). RNA integrity was checked with 1% agarose gel with ethidium bromide at 0.5 μg/ml. The electrophoretic run was performed in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 7.5). RNA was dosed on a NanoVue (GE Healthcare, UK) and only samples with a 260/280 nm ratio above 0.8 were used for the Quantitative real-time PCR analysis (qRT-PCR). The primers for per1, per2, cry1a, cu/zn sod, cat and βactin (Table 1) were ordered from EXXTEND (Campinas, São Paulo, Brazil) and purified by the RP-OPC technique, which excludes truncated sequence oligos. The specificity of each primer pair was verified by normal PCR and real-time PCR.

The Power SYBR® Green RNA-to-CT 1-Step Kit Kit (Applied Biosystems) was used to evaluate the expression of the genes presented in Table 1. Standardization for 10 μl qRT-PCR reactions, for both liver and brain, comprised 5 ng of total RNA and 800 nM for all the primers. All samples were run in triplicate and submitted to 45 amplification