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Acute restraint stress induces an imbalance in the oxidative status of the zebrafish brain

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HIGHLIGHTS

- Acute restraint stress (ARS) induces oxidative stress in zebrafish brain.
- ARS increased SOD/CAT activity ratio and lipid peroxides.
- ARS increased non-protein thiol levels without altering total reduced thiols.
- Zebrafish is a suitable organism for studying acute stress protocols.

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ABSTRACT

The zebrafish (*Danio rerio*) has become an emergent model organism for translational approaches focused on the neurobiology of stress due to its genetic, neuroanatomical, and histological similarities with mammalian systems. However, despite the increasing number of studies using zebrafish, reports examining the impact of stress on relevant neurochemical parameters are still elementary when compared to studies using rodents. Additionally, it is important to further validate this model organism by comparing its stress response with those described in other species. Here, we evaluated the effects of an acute restraint stress (ARS) protocol on oxidative stress-related parameters in the zebrafish brain. Our data revealed that ARS significantly decreased catalase activity without altering the activity of superoxide dismutase. Oxidative stress was also indicated by increased levels of lipid peroxides. ARS significantly increased the levels of non-protein thiols, although significant changes in total reduced sulfhydryl content were not detected. These results suggest that ARS is an interesting strategy for evaluating the mechanisms underlying the neurochemical basis of the oxidative profile triggered by acute stressors in the zebrafish brain. Furthermore, this protocol may be suitable for screening new compounds with protective properties against oxidative stress, which plays an increasingly important role in many psychiatric disorders.

1. Introduction

In many vertebrate organisms, stressful stimuli have been shown to evoke a coordinated pattern of physiological and behavioral changes [1]. When a threatening stimulus is presented, the acute response is "fight or flight", which consists of a set of physiological reactions that culminate in a fast adaptation of cardiac, muscular, and visual functions [2]. The mechanisms underlying these adaptive responses are the activation of the hypothalamus-pituitary-adrenal (HPA) axis and the sympathoadrenal system. Both autonomic and neuroendocrine responses are part of the physiological component of behavioral responses required for the maintenance of homeostasis during stressful events [3].

Zebrafish (*Danio rerio*) is a suitable model organism for studying the deleterious effects promoted by stressors and examining

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the behavioral and neurochemical outcomes [4–8]. This species has a low cost, small size, and high fecundity rate, which are valuable characteristics for high-throughput analyses [9,10]. The high degree of homology between zebrafish and human genomes [11], the conserved function of brain areas [12] and neurotransmitter systems [13], and the presence of an HPA axis homolog (the hypothalamic-pituitary-interrenal axis) [14,15] make zebrafish an attractive model organism for studying the neurobiology of stress and complementing the current rodent approaches. Moreover, similar to humans, the main glucocorticoid involved in the stress response in zebrafish is cortisol, the level of which increases significantly after exposure to a wide range of stressors [16–18].

Reactive oxygen species (ROS) and free radicals are produced by the mitochondria, which is the major site of their intracellular production, during normal aerobic metabolism [19]. The superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) produced by the respiratory chain may generate the reactive hydroxyl radical ($^{\bullet}OH$) via the Fenton reaction [20]. Overproduction of $^{\bullet}OH$ is related to lipid, protein, and DNA oxidation that may trigger several events, culminating in cell death [21]. However, endogenous antioxidant systems act as scavengers by counteracting these deleterious effects [22]. Enzymatic antioxidant protection is provided by superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), while non-enzymatic defense is provided by reduced glutathione (GSH) but also by vitamins from the diet. Oxidative stress occurs when the antioxidant systems are overcome by ROS production [22]. Nerve cells are particularly susceptible to oxidative stress due to elevated polyunsaturated fatty acid content, high oxygen utilization, and the presence of redox-active metals [23]. Indeed, ROS overproduction and oxidative stress have been implicated in the pathophysiology of several neurodegenerative and psychiatric diseases [24–26]. It is conceivable that stressful conditions stimulate intricate cell signal transduction pathways that lead to an increase in ROS formation. In this regard, restraint stress can affect central nervous system (CNS) homeostasis by disrupting neurochemical and endocrine parameters, inducing an imbalance of antioxidant status [27–29]. Because zebrafish is considered a promising model organism for translational neuroscience research, the evaluation of oxidative status in the CNS after application of an acute restraint stress protocol (ARS) is a fundamental step for the validation of the model at the construct level. Thus, the goal of the current report was to investigate the effects of ARS on oxidative stress parameters in the zebrafish brain.

2. Materials and methods

2.1. Animals

A total of 100 male and female wild-type short-fin strain adult zebrafish (4–6 months old, 50:50 male:female ratio) were obtained from the heterogeneous breeding stock of Universidade de Passo Fundo. The fish were kept in 50-L aquariums (80–100 fish per tank) for 2 weeks prior to the experiments to acclimate them to laboratory conditions. All experiments were performed in tanks filled with non-chlorinated water that was mechanically and chemically filtered and maintained at $26 \pm 2\,^{\circ}C$ with a light/dark cycle of 14/10 h (lights on at 7:00 AM). The fish were fed twice a day with a commercial flake fish food (Alcon BASIC®, Alcon, Brazil). All protocols were approved by the Ethics Committee of Unochapecó (#003/2012).

2.2. Reagents

Epinephrine, 4,6-dihydroxypyrimidine-2-thiol (TBA), 1,1,3,3-tetramethoxypropane (TMP), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

2.3. Acute restraint stress protocol (ARS)

In this protocol, which has been previously reported by our group, 50 animals can be tested concomitantly, and only a small space is required [6,17]. The protocol involved enclosing each animal in 2-mL plastic microcentrifuge tubes containing openings at both ends (one at the cap and other at the bottom end of the tube) that were placed in a 20-L tank for 90 min. The openings were manually shaped and were large enough (approximately 5 mm in diameter) to allow adequate water circulation inside the tube for 90 min. Importantly, the time period chosen was previously utilized for zebrafish; within this time period, the animals showed significant alterations in CRF levels and increased whole-body cortisol [18]. The control group (non-stressed) remained in the same room. Aeration (8 ppm) and temperature ($26 \pm 2\,^{\circ}C$) were controlled during the test, and pilot experiments showed that the oxygen levels did not significantly differ in either the tank water or the water collected inside the plastic tube. After ARS, the fish were cryoanaesthetized and euthanized by decapitation to remove the brain.

2.4. Oxidative stress analyses

Each of the samples used for biochemical assays consisted of a pool of ten whole brains, which were gently homogenized in 1 mL of phosphate-buffered saline solution (PBS), pH 7.4, containing 137 mM NaCl, 10.1 mM Na_2HPO_4, and 1.76 mM KH_2PO_4. The samples were further centrifuged at $700 \times g$ for 5 min at $4\,^{\circ}C$. The resultant pellets were discarded, and the supernatants were collected for the experiments described herein, which were performed similarly to experiments reported previously for zebrafish [7].

2.5. Superoxide dismutase (SOD) and catalase (CAT) activities

Superoxide dismutase (EC 1.15.1.1, SOD) activity was quantified according to Misra and Fridovich [30] by spectrophotometrically determining the inhibition of auto-oxidation of epinephrine to adrenochrome at an alkaline pH at 480 nm. Catalase (EC 1.11.1.6; CAT) activity was assessed by measuring the rate of decrease in H_2O_2 absorbance at 240 nm [31]. For the SOD assay, 20–90 μg of protein was used, whereas CAT activity was determined using 50–80 μg protein. All results were calculated and expressed as U per mg protein.

2.6. Lipid peroxidation

The determination of the lipid redox state was measured based on the formation of thiobarbituric acid reactive substances (TBARS) [32]. Briefly, 300 μL of sample was mixed with 700 μL of 15% trichloroacetic acid (TCA) and centrifuged at $10{,}000 \times g$ for 10 min. The supernatants were mixed with 0.67% TBA (at a 1:1 proportion) and heated at $100\,^{\circ}C$ for 30 min. The TBARS levels were determined in triplicate based on the absorbance at 532 nm using TMP as a standard. The results were expressed as nmol TBARS per mg protein.

2.7. Total content of reduced thiol and non-protein thiol groups

To quantify the total reduced thiol content, 200 μL of homogenate was incubated in the presence of 0.2 mM EDTA and 100 mM boric acid buffer (pH 8.5). Subsequently, DTNB (0.01 M dissolved in ethanol) was added, and after 1 h, the product was measured at 412 nm [33]. The non-protein sulfhydryl groups were similarly assessed, except that the samples were centrifuged at