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N-Acetylcysteine Reverses Anxiety and Oxidative Damage Induced by Unpredictable Chronic Stress in Zebrafish

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Abstract
There is accumulating evidence on the use of *N*-acetylcysteine (NAC) in the treatment of patients with neuropsychiatric disorders. As a multi-target drug and a glutathione precursor, NAC is a promising molecule in the management of stress-related disorders, for which there is an expanding field of research investigating novel therapies targeting oxidative pathways. The deleterious effects of chronic stress in the central nervous system are a result of glutamatergic hyperactivation, glutathione (GSH) depletion, oxidative stress, and increased inflammatory response, among others. The aim of this study was to investigate the effects of NAC in zebrafish submitted to unpredictable chronic stress (UCS). Animals were initially stressed or not for 7 days, followed by treatment with NAC (1 mg/L, 10 min) or vehicle for 7 days. UCS decreased the number of entries and time spent in the top area in the novel tank test, which indicate increased anxiety levels. It also increased reactive oxygen species (ROS) levels and lipid peroxidation (TBARS) while decreased non-protein thiols (NPSH) and superoxide dismutase (SOD) activity. NAC reversed the anxiety-like behavior and oxidative damage observed in stressed animals. Additional studies are needed to investigate the effects of this agent on glutamatergic modulation and inflammatory markers related to stress. Nevertheless, our study adds to the existing body of evidence supporting the clinical evaluation of NAC in mood disorders, anxiety, post-traumatic stress disorder, and other conditions associated with stress.

Keywords *N*-acetylcysteine · Unpredictable chronic stress · Behavior · Oxidative status · Zebrafish

Introduction

Clinical studies have suggested that oral administration of *N*-acetylcysteine (NAC) may improve several outcomes in patients with neuropsychiatric disorders such as anxiety and depression [1–5]. The positive effects observed in these conditions are probably related to actions on oxidative status and the glutamatergic system [6], since NAC is a glutathione (GSH) precursor and modulates glutamatergic transmission [7]. In addition to clinical studies, preclinical findings have also demonstrated beneficial effects of NAC in different animal models, as evidenced by modulation of behavioral, biochemical, and molecular parameters involved in psychiatric and neurological conditions (e.g., anxiety, depression, Parkinson's and Alzheimer's disease, schizophrenia, alcoholism, drug addiction, gambling) [3, 6, 8–14].

Our group has previously reported that NAC induces anxiolytic-like effects in both mice and zebrafish in different protocols; specifically regarding stress-induced outcomes, NAC was able to prevent the deleterious effects observed in zebrafish submitted to an acute stress protocol [9, 10, 13]. Other studies have shown beneficial and

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protective effects of NAC against the imbalance on inflammatory (interleukin-1beta (IL-1b), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-a)) and oxidative stress markers (lipid peroxidation (TBARS), superoxide dismutase (SOD), catalase (CAT), and gluthatione peroxidase (GPx) activities), besides its neurotrophic actions (increase of brain-derived neurotrophic factor (BDNF)) [5, 11, 15, 16].

Chronic stress is a powerful trigger to the development of neuropsychiatric disorders and is particularly relevant in the context of modern life [17]. Its neurobiology is heterogeneous and includes dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and neurotransmitter systems (glutamatergic, noradrenergic, dopaminergic, serotoninergic, and GABAergic), decrease in GSH levels, imbalance in oxidative status parameters, activation of neuroinflammatory and apoptosis pathways, and behavioral changes in response to adverse situations [17–25].

As demonstrated by our laboratory and others, unpredictable chronic stress (UCS) protocols increased anxiety and reactive oxygen species (ROS) levels in zebrafish, besides increasing inflammatory cytokines (IL-1b, IL-6, and IL-10) and neurotrophic factor (BDNF) [26, 27]. Considering the multi-target effects of NAC in many systems related to stress and the deleterious effects of UCS, the aim of this study was to evaluate the potential of this drug in reversing the effects of UCS on anxiety-like behavior and oxidative markers in zebrafish.

Materials and Methods

Animals

A total of 44 adult (4–6-month-old) zebrafish (*Danio rerio*) of short-fin wild-type (WT) phenotype of both sexes (50:50 male:female ratio) was obtained from a local specialized commercial supplier (Delphis, RS, Brazil). Fish were housed in a maximum density of two fish per liter and acclimatized for 2 weeks prior to testing in 16-L tanks (40 × 20 × 24 cm) filled with non-chlorinated water kept under constant mechanical, biological, and chemical filtration at 26 ± 2 °C. Fish were kept on a 14–10-h day/night cycle (lights on at 07:00 a.m.) and fed three times a day with brine shrimp (*Artemia salina*) and commercial flake fish food (Alcon BASIC®, Brazil). More detailed information on housing conditions is described in our previous study [8]. All protocols were approved by the Ethics Committee of Federal University of Rio Grande do Sul (process number #30914).

Drug

N-acetylcysteine (NAC, CAS number 616-91-1) was acquired from Sigma-Aldrich (St Louis, Missouri, USA). NAC concentration (1.0 mg/L) was based on a previous study published by our group [9].

Experimental Design and Procedures

Initially, fish were divided into control (non-stressed group, S−) and UCS (stressed group, S+). After 7 days, the experimental groups were subdivided again into control and NAC (1.0 mg/L). The animals were daily transferred at 08:00 a.m. to 5-L tanks containing fresh water (control groups) or NAC (treated groups) for 10 min and were gently returned to the housing tanks, resulting in 14 days of UCS and 7 days of treatment. The experimental design is summarized in Fig. 1.

The UCS protocol followed our previous studies [28–30]. Stressors were presented randomly twice a day for a total of 14 days to avoid habituation. The stressors included chasing with a net (8 min); low water level on housing tanks until dorsal body wall was exposed (2 min); crowding in a 250-mL beaker (50 min); cooling tank water to 23 °C (30 min); heating tank water up to 33 °C (30 min); tank change, three consecutive times with 30 min interval. All stressors were applied between 08:30 a.m. and 17:00 p.m. The non-stressed group was left undisturbed throughout the experiments. Aeration and temperature were controlled during each stressor presentation (except during heating and cooling stress). A white frosted cardboard (30 × 60 cm) was placed in between tanks to prevent visual contact of fish from different tanks in the same horizontal plane. Two identical tanks were ran in parallel for each experimental group; no tank effects were observed in the analysis of results, so data from tanks of the same experimental group were pooled together.

Behavioral Analyses

The behavioral test was performed on the 15th day. The animals were individually transferred to the novel tank test (NTT) and video recorded for 6 min. The videos were later analyzed by the ANY-Maze™ software (Stoelting Co., USA). Tests were performed between 08:00 a.m. and 01:00 p.m. The NTT apparatus and the parameters studied are described in detail in our previous studies [8, 9, 28].

Tissue Preparation

For the biochemical analyses, a proportion of two brains to 300 μL of phosphate-buffered saline (PBS, pH = 7.4, Sigma-Aldrich®) was used. Immediately after the behavioral test, fish were anesthetized by rapid cooling (immersion in water at 2–4 °C until unable to swim and cessation of opercular

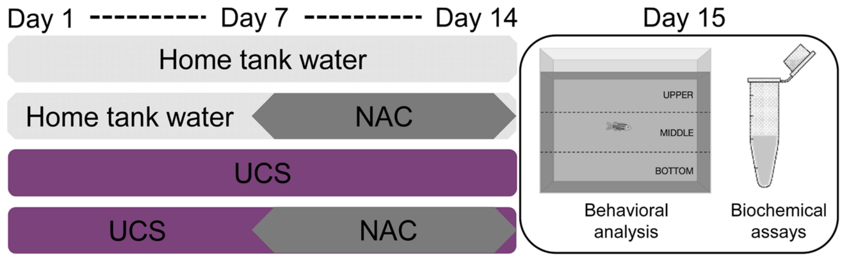

Fig. 1 Outline of the experimental design. Unpredictable chronic stress (UCS); *N*-acetylcysteine (NAC)

movement). Each animal was then euthanized by decapitation for brain tissue sampling. The brains were dissected and gently homogenized. The homogenate was centrifuged at 10,000×*g* for 10 min at 4 °C in a cooling centrifuge, and the supernatant packed in microtubes was used for assays. We quantified the following parameters: reactive oxygen species (ROS) levels (dichlorofluorescein—DCF), lipid peroxidation (thiobarbituric acid reactive species—TBARS), non-protein thiols (NPSH), and antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)). All biochemical measures were performed in duplicate. Details of each procedure are described in previous studies [8, 31].

Statistics

Data were analyzed after confirmation of normality and homogeneity of variances using D'Agostino-Person and Levene tests, respectively. Two-way ANOVAs were carried out to identify the main effects of stress and treatment, as well as their interactions. Bonferroni post hoc test was performed when significant interactions were obtained. Data are expressed as a mean + standard error of the mean (S.E.M). The level of significance was set at $p < 0.05$.

Results

Figure 2 shows the influence of NAC on behavioral parameters in zebrafish submitted to UCS. As expected, UCS increased the time spent in the bottom area (Fig. 2d) and decreased the entries and time in the top area (Fig. 2e, f, respectively). Two-way ANOVA revealed a main effect of treatment ($F_{1, 41} = 9.85$, $p = 0.0031$) and an interaction effect ($F_{1, 41} = 15.65$, $p = 0.0003$) for time in bottom area (Fig. 2d), as well as interaction effects for entries in top area (Fig. 2e, $F_{1, 41} = 6.01$, $p = 0.0185$) and time in the top area (Fig. 2f, $F_{1, 41} = 10.66$, $p = 0.0022$). NAC reversed the UCS-induced decrease in top swimming and the complementary increase in bottom dwelling. The distance traveled (Fig. 2a), the number of crossings (Fig. 2b) and entries to the bottom area (Fig. 2c) was not affected by any intervention.

The effects of the UCS on oxidative status are presented in Figs. 3 and 4. As shown in Fig. 3a, b, respectively, UCS promoted an increase in ROS levels and lipid peroxidation. Two-way ANOVA revealed an interaction between the factors ($F_{1, 18} = 21.49$, $p = 0.0002$) for ROS levels (DCF assay), and main effects of stress ($F_{1, 18} = 15.32$, $p = 0.0010$), treatment ($F_{1, 18} = 5.33$, $p = 0.0330$), and interaction ($F_{1, 18} = 12.71$, $p = 0.0022$) for lipid peroxidation (TBARS assay). NAC was able to reverse the increase on ROS levels and lipid peroxidation in zebrafish submitted to UCS, while it did not alter such parameters in non-stressed animals.

Regarding SOD activity (Fig. 4a), two-way ANOVA revealed an interaction between treatment and stress ($F_{1, 18} = 7.95$, $p = 0.0113$); UCS decreased SOD activity, while NAC blocked this effect in stressed fish, without affecting non-stressed animals. For NPSH (Fig. 4c), the two-way ANOVA revealed main effects of stress ($F_{1, 18} = 5.19$, $p = 0.0351$), treatment ($F_{1, 18} = 8.94$, $p = 0.0078$), and interaction ($F_{1, 18} = 7.45$, $p = 0.0137$); UCS decreased NPSH levels, and NAC again protected against this UCS-induced reduction in antioxidant defenses, without effects in non-stressed animals. No effects of stress or treatment were observed for CAT activity (Fig. 4b).

Discussion

Here, we provide the first evidence that *N*-acetylcysteine (NAC) reverses the behavioral alterations and oxidative stress induced by an unpredictable chronic stress (UCS) protocol in zebrafish. Fish submitted to 14 days of stress presented behavioral changes in the number of entries and time spent in the top area in the novel tank test (NTT); moreover, the protocol increased ROS levels (DCF) and lipid peroxidation (TBARS), while decreased non-protein thiols (NPSH) and superoxide dismutase (SOD) activity, without affecting catalase (CAT). All these effects were reversed by treatment with NAC in the last 7 days of UCS. This suggests that the behavioral changes induced by the UCS protocol are accompanied by oxidative damage in the zebrafish brain. Although a causal link cannot be established, it is possible that oxidative stress is somehow involved in the behavioral alterations induced by stress, since treatment with the antioxidant NAC reversed such changes.

We opted to submit zebrafish to UCS for 7 days before starting the treatment with NAC to recapitulate a more realistic scenario considering the potential use of NAC in patients with

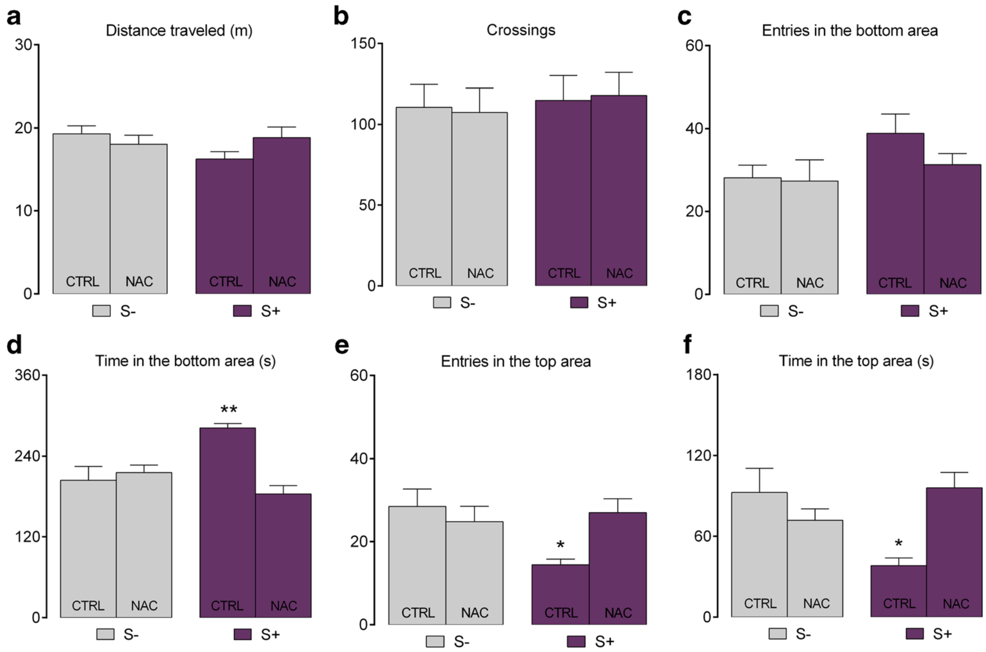

Fig. 2 Effects of NAC treatment against UCS-induced changes on behavioral parameters in zebrafish. **a** Distance traveled. **b** Crossings. **c** Entries in the bottom area. **d** Time in the bottom area. **e** Entries in the top area. **f** Time in the top area. Non-stressed group (S−); stressed group (S+). Data are expressed as mean + S.E.M. Two-way ANOVA followed by Bonferroni's test. $n = 10–12$. *$p < 0.05$ vs. control (S−)

mental disorders related to stress. In the NTT, total distance traveled and crossings were used as an indicator of locomotor activity. The increase in time spent in the bottom area and decrease in time spent in the top area is used as a proxy for anxiety behavior [32], which corresponds in rodents to thigmotaxis in the open-field test [33, 34]. As expected, our protocol increased anxiety-like behavior, as evidenced by the increased time spent in the bottom area and decreased entries and time in the upper zone of the tank, in agreement with previous works from our group [27, 28]. NAC did not affect locomotor activity and was devoid of effect in non-stressed animals. Similar effects in the novel tank test were observed for different anxiolytics such as bromazepam, diazepam, buspirone, and fluoxetine [9, 28, 35].

As all animals were daily transferred to control or NAC-containing tanks during the treatment exposure period, all groups were habituated to handling and to the presence of a net. On the testing day, the handling was performed carefully in the same manner as previous days. This circumvented the possibility of the UCS group being less stressed on the testing day if compared to a never handled control group. Furthermore, stressors were applied twice a day in a randomized unpredictable fashion, precisely to avoid habituation in stressed groups.

Our group has recently shown that UCS for 7 days is able to increase the production of ROS in zebrafish brain [27]. Here, we show for the first time the effects of UCS for 14 days on oxidative status parameters in zebrafish. We observed that ROS levels and TBARS significantly increased after UCS, an indication of lipid peroxidation that corroborates studies in other animal models [36, 37]. We also detected a decrease in NPSH levels, probably as a result of reduction in the amount of GSH [38]. NAC, as a GSH precursor, blocked the decrease in NPSH levels induced by UCS. Our results are in agreement

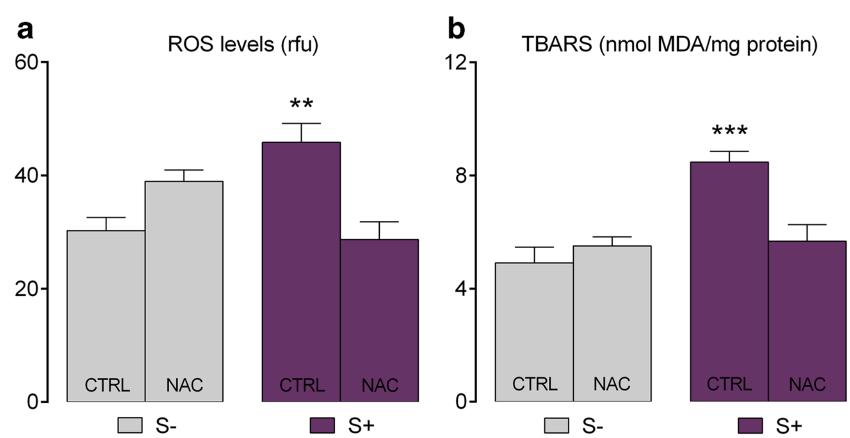

Fig. 3 Effects of NAC treatment against UCS-induced changes in levels of reactive oxygen species (**a**) and lipid peroxidation (**b**) in whole-brain zebrafish. Non-stressed group (S−); stressed group (S+). Data are expressed as mean + S.E.M. Two-way ANOVA followed by Bonferroni's test. $n = 5–6$. **$p < 0.01$; ***$p < 0.001$ vs. control (S −)

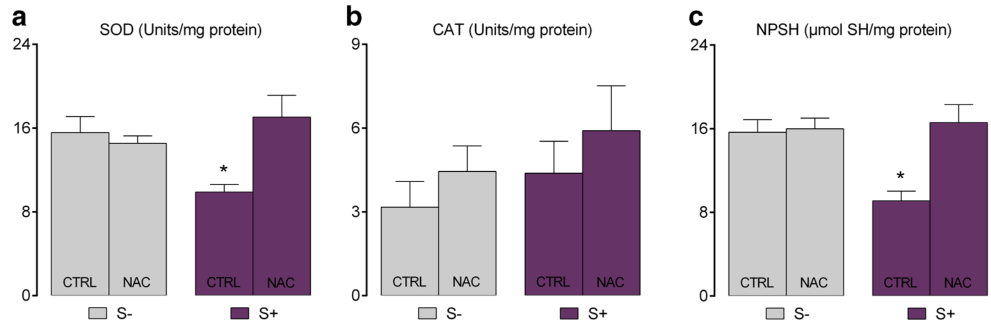

Fig. 4 Effects of NAC treatment against UCS-induced changes on oxidative status in zebrafish whole-brain. **a** SOD activity. **b** CAT activity. **c** Non-protein thiols. Non-stressed group (S−); stressed group (S+). Data are expressed as mean + S.E.M. Two-way ANOVA followed by Bonferroni's test. $n = 5–6$. $^{*}p < 0.05$ vs. control (S−)

with rodent studies, in which chronic stress impaired antioxidant defenses and resulted in lipid peroxidation and damage to the brain [39–41].

One of the defense lines against the excessive production of ROS and consequent brain damage is the increased activity of antioxidant enzymes, for instance, SOD and CAT [42]. SOD catalyzes the reaction of superoxide anion radical ($O_2^{\bullet -}$) dismutation into hydrogen (H_2) and hydrogen peroxide (H_2O_2), while the reduction of H_2O_2 to O_2 and H_2O is catalyzed by CAT or glutathione peroxidase (GPx) [21, 43]. UCS significantly decreased SOD, but not CAT activity in zebrafish. This decrease in enzymatic antioxidant defense induced by UCS indicates a possible maladaptive response to the long-term effects of stressful conditions, that is, the organism was not able to properly cope with the increased ROS levels and prevent oxidative damage [44]. Nevertheless, the oxidative damage induced by UCS was reversed by NAC, which blocked SOD activity decrease in stressed fish (Fig. 5).

Manganese superoxide dismutase (MnSOD) is the most important specific SOD enzyme present in the inner membrane and matrix of mitochondria [45, 46]. Chronic stress increases peroxynitrite levels, thus causing inhibition of MnSOD enzyme through nitration of its tyrosine residues [47–50]. Overexpression of MnSOD, however, is able to avoid stress-induced lipid peroxidation and neuronal death in rats [51]. NAC is an indirect antioxidant and increases SOD activity by increasing MnSOD expression [52]. The increased levels of MnSOD associated with NAC may reflect augmented de novo synthesis or decreased elimination rates due to lower oxidation levels [53]. Studies report that MnSOD mRNA is regulated at different transcriptional levels, and while NAC may potentially modulate its expression, the exact molecular mechanisms involved are still unknown [54–57]. We should also consider the possibility that peroxynitrite scavenging by NAC may underlie its ability to preserve SOD activity in stressed animals.

A limitation of our study is that we cannot assign the benefits of NAC exclusively to its antioxidant properties since it also modulates glutamatergic transmission and other relevant pathways [3, 5]. Chronic stress causes glutamate release and leads to maladaptive synaptic changes, including reduced extracellular glutamate clearance by glial cells, increased activation of extrasynaptic *N*-methyl-D-aspartate receptors and excitotoxicity, which may result in neuronal death [23]. Recent studies evidence the potential therapeutic usefulness of mGlu2/3 ligands and mGlu5 receptor antagonists in stress-related disorders [58]. NAC regulates intra and extracellular glutamate [59] by increasing the activity of the cystine-glutamate antiporter expressed by astrocytes, which inhibits glutamate release and excitotoxicity through activation of mGluR2/3 receptors [1, 3, 5].

Considering our observations of the effects of NAC in reversing the deleterious effects caused by UCS, we reinforce the idea that NAC would be a beneficial and promising agent

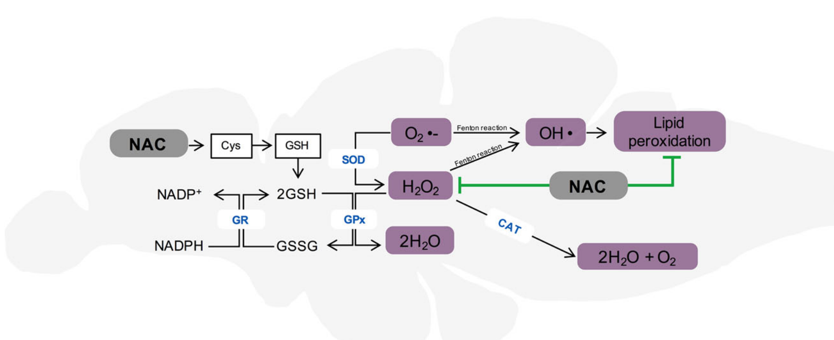

Fig. 5 Schematic representation of the mechanism of NAC on UCS-induced oxidative damage in the zebrafish brain. The illustration depicts an involvement of NPSH and SOD in mediating UCS responses and the effects of NAC on TBARS and SOD. NAC is a precursor of GSH, which is the main non-protein thiol. *N*-acetylcysteine (NAC); lipid peroxidation (TBARS); non-protein thiols (NPSH); superoxide dismutase (SOD); catalase (CAT); cysteine (Cys); glutathione (GSH); glutathione peroxidase (GPx); glutathione reductase (GR)

for the treatment of stress and associated psychiatric disorders. Additional studies are therefore needed, but the data available thus far are encouraging for the clinical trials needed to further assess the efficacy of NAC in neuropsychiatric conditions.

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Compliance with Ethical Standards

All protocols were approved by the Ethics Committee of Federal University of Rio Grande do Sul (process number #30914).

Conflict of Interest The authors declare that they have no conflict of interest.

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