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Ascorbic Acid Protects Against Anxiogenic-Like Effect Induced by Methylmercury in Zebrafish: Action on the Serotonergic System

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Abstract

To evaluate the protector effect of ascorbic acid (AA) against anxiogenic-like effect induced by methylmercury (MeHg) exposure, adult zebrafish were treated with AA (2 mg g^{-1}, intraperitoneal [i.p.]) before MeHg administration (1.0 μg g^{-1}, i.p.). Groups were tested for the light/dark preference as a behavioral model of anxiety, and the content of serotonin and its oxidized metabolite tryptamine-4,5-dione (T-4,5-D) in the brain was determined by high-performance liquid chromatography. MeHg has produced a marked anxiogenic profile in both tests, and this effect was accompanied by a decrease in the extracellular levels of serotonin, and an increase in the extracellular levels of T-4,5-D. Added to this, a marked increase in the formation of a marker of oxidative stress accompanied these parameters. Interestingly, the anxiogenic-like effect and biochemical alterations induced by MeHg were blocked by pretreatment with AA. These results for the first time demonstrated the potential protector action of AA in neurobehavioral and neurochemical alterations induced by methylmecury exposure demonstrating that zebrafish model could be used as an important tool for testing substances with neuroprotector actions.

Introduction

Methylmercury (MeHg) represents a potent environmental pollutant and several studies have shown that chronic and acute MeHg exposure evokes severe neurotoxicity effects in humans.[1–4] Recent studies have reported that populations living in the Amazon region are at risk from MeHg toxicity, this phenomenon is associated with intense consumption of contaminated fishes by riverside communities.[5–10] Previous works have already demonstrated that MeHg can elicit a range of neurological alterations.[11–14] In this way, the development of animal experimental models to evaluate mercurial neurotoxicity represents an important tool to understand the underlying mechanisms related to MeHg toxicity as well as to characterize the protector agents.

Several reports reveal that the utilization of experimental models is able to reproduce neurological alterations observed in humans as learning deficits, memory impairment, and anxiety-like behaviors.[15–17] The dark/light preference (scototaxis) protocol represents a behavioral model used to evaluate the anxiety-like behavior in rodents and fishes, including the zebrafish species.[18–20] In fact, the effects of pharmacological agents on the behavioral changes are commonly used to investigate the genetic, epigenetic, and biochemical bases of anxiety-related behavior.[21,22] Added to this, studies reveal that zebrafish has a marked preference for the dark compartment and an increase in the white compartment activity (duration and/or entries) should reflect an antianxiety behavior, whereas an increase in the dark compartment activity should reflect an anxiety-promoting behavior.[22] This model was previously submitted to behavioral and pharmacological validations with remarkable consistency in the analyzed parameters.[21–24] Different experimental procedures used for methodological validation included re-exposition (three sessions with a 15-min interval among them, and five sessions with a 24-h interval), effects of the forced exposition to a white chamber, lightness level (250 and 500 lux), effects of the chamber's proportion of dark/

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light environments, and the effects of several anxiogenic and anxiolytic drugs (chlordiazepoxide, clonazepam, diazepam, buspirone, moclobemide, alcohol, and caffeine).[22] All results indicate a consistent response, few influences of parametric manipulations, and sensitivity to drugs recommended for anxiety treatment.[21–24] In this context, research utilizing zebrafish has emerged as an important experimental model to evaluate the mechanism of toxicity induced by an environmental contaminant such as MeHg. In fact, our group has recently reported that MeHg produced a marked anxiogenic-like effect in zebrafish.[18] These behavior profiles were accompanied by a decrease in the extracellular content of serotonin and an increase in the extracellular levels of tryptamine-4,5-dione (T-4,5-D), a partially oxidized metabolite of serotonin.[18] We also observed a remarkable increase in the formation of malondialdehyde (MDA), a marker of oxidative stress. Our results suggested that MeHg induced oxidative stress, produced mitochondrial dysfunction, and originated (T-4,5-D), which could have further inhibited tryptophan hydroxylase; however, future studies are necessary for the detailed characterization of this phenomenon.[18]

The idea that oxidative stress is a mediator phenomenon of the MeHg neurotoxicity is well characterized,[16,25–27] however, few studies describe the effect of antioxidant treatment on the neurochemical and neurobehavioral alteration evoked by MeHg.[28–32] Natural antioxidants have been used in an attempt to reverse the damage caused by oxidative stress by retaking the cellular redox balance.[33–35] Ascorbic acid (AA), an important natural antioxidant, is present in 85% of fruits and vegetables consumed and several studies reveal that AA represents an important water-soluble antioxidant.[36–38] Some reports have already demonstrated that AA can act as a radical scavenger by eliminating free radicals and providing protection to the cell in the aqueous medium.[37,38] In the present study, we demonstrated a biochemical mechanism of protection mediated by AA against anxiety-like behavior induced by MeHg in zebrafish. Added to this, the present work introduces the zebrafish as an alternative experimental model for the studies with chemical compounds that exert protection against the injury induced by MeHg in the central nervous system.

Materials and Methods

Animals

Forty-five zebrafish (*Danio rerio*) of wild-type long-fin phenotype were obtained in a local fish store (Ananindeua-PA). They were acclimated in 40-L tanks, in appropriate conditions of temperature (28°C) and pH (7.0–8.0) for at least 1 week before the experiments. The animals were kept in 12-h light–12-h dark cycle. The animals (n = 9 per group) were divided into four groups: control, AA, MeHg, and AA + MeHg. They were tested in a light–dark preference and later euthanized and their brains analyzed by biochemical assays as described below.

Experimental drugs

MeHg chloride (MeHg; Sigma) stocks were diluted in Cortland's salt solution pH 7.4 (124 mM NaCl; 5.1 mM KCl; 2.9 mM Na_2PO_4; 24 mM $MgSO_4.7H_2O$; 1.4 mM $CaCl_2.H_2O$; 12 mM $NaHCO_3$; 1000 U heparin, and distilled water). All groups treated with MeHg, including AA + MeHg, were injected intraperitoneally (i.p.) with a dose of 1.0 μg/g of MeHg. The work solution of AA (Sigma) was diluted in distilled water (2 mg/kg), and i.p. injections in all groups were performed using 10-μL syringes (Hamilton USA), according to Kinkel *et al.*[39] Since MeHg was diluted in Cortland's salt solution and AA was diluted in distilled water, in the present study, we used as control group, the animals injected i.p. with Cortland's salt solution, as described previously by Maximino *et al.*[18] No behavioral differences were observed between animals not submitted to injections and animals injected with Cortland's salt solution (data not shown). Animals were cold anesthetized and transferred to the surgical table composed of a 20 mm soft sponge embedded in a 60-mm Petri dish.

Light–dark preference

The apparatus used in the present work was an acrylic tank (15 cm height × 10 cm depth × 45 cm length) divided equally into one-half white and one-half black compartments, with central sliding doors colored with the same color of the substratum defining a central compartment, as described by Maximino *et al.*[22] The walls and bottom are either black or white, so as to warrant uniform substrata for each compartment. The water column is kept at 10 cm yielding a final volume of 4.5 L, and the tank contains central sliding doors colored with the same color of the aquarium side, thereby defining an uncolored central compartment measuring 15 × 10 × 10 cm.

Pretreatment with AA was performed 24 h before MeHg poisoning. After 24 h of vehicle administration (Cortland's salt solution) and MeHg exposure, the control group (vehicle), MeHg group, AA group, and MeHg + AA group were independently transferred to the central compartment for 3 min. After acclimation period, the doors that delimited this compartment were removed and the animal was allowed to freely explore the apparatus for 15 min. The following variables were measured in the white compartment: (1) *time on the white compartment*: the time spent in the top third of the tank (percentage of the trial); squares crossed: the number of 10 cm^2 squares crossed by the animal in the white compartment; (2) *latency to white*: the amount of time the animal spends in the black compartment before its first entry in the white compartment (s); and (3) *entries in white compartment*: the number of entries the animal makes in the white compartment in the whole session. After each trial within one session, the tank was rotated by 180°, so as to eliminate spatial effects and the tank was illuminated by environmental light (60 W light bulb, located at 1.80 m above the tank top), which kept illumination uniform and constant between trials. All groups were tested in a unique day, and the experiments were repeated three times.

Subcellular fractionation

After behavioral testing, animals were sacrificed, their brains were quickly dissected and incubated for 30 min in 2 mL of 50 mM tris buffered saline (TBS), pH 7.4, containing 90 mM NaCl, 2.5 mM $CaCl_2$, 1 mM glutathione for 30 min at 4°C to extract the extracellular fluid (ECF). After that, the brains were then homogenized in 2 mL TBS, and half of the resulted homogenates (Ho) were centrifuged at 1000 g for 10 min at 4°C. The protein content in the brains was determined by the Bradford[40] method.

Analysis of indoleamines

We used the high-performance liquid chromatography (HPLC) to determinate the indoleamine content. After brain incubations, the homogenization buffer (TBS) was collected in assay tubes and separated for injecting into the chromatographic column. The different components in the sample pass through the column at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase (Colum). The presence of indoleamines in the column effluent was recorded by an electrochemical detector, as described above. The content of indoleamines in the sample was determinate utilizing the standard curve with known concentrations of commercial indoleamines.

Standard of serotonin and 5-hydroxyindolacetic acid (5-HIAA) were dissolved in 100 mL of eluting solution (50 mL Milli-Q water, 0.43 mL 70% $HClO_4$ [0.2 N], 10 mg EDTA, 9.5 mg sodium metabisulfite). The T-4,5-D was synthesized chemically according to Chen *et al.*,[41] an eightfold amount of Fremy's salt to a 5-HT·HCl solution (3.5 mg in 2 mL of 0.01 N HCl). The HPLC system consisted of a delivery pump (LC-20AT; Shimadzu), a 20 μL sample injector (Rheodyne), a degasser (DGU-20As; Shimadzu), and an analytical column (Shimadzu Shim-PackVP-ODS, 250×4.6 mm internal diameter). The integrating recorder was a CBM-20A (Shimadzu). An electrochemical detector (Model L-ECD-6A; Shimadzu) with glassy carbon was be used at a voltage setting of +0.83 V (5-HT and 5-HIAA) or +7.5 V (T-4,5-D), with a sensitivity set at 8 nA full deflection. The mobile phase consisted of a solution of 70 mM phosphate buffer (pH 2.9), 0.2 mM EDTA, 5% methanol, and 20% sodium metabisulfite as a conservative. The isocratic flow rate was 1.8 mL/min 0.5 mL of ECF, extracted as described above and was mixed with 0.5 mL of the eluting solution described above. The analyses of peaks were performed using the LC-solution software (Shimadzu), and concentrations of serotonin, tryptamine, and 5-HIAA-4,5-dione were analyzed by comparing the results with standard curves.

Lipid peroxidation assay

Lipid peroxidation was assessed by following the formation of thiobarbituric acid-reactive substances (TBARS).[36] Brains homogenates (100 μL) were added to the same volume of TBA reagent (30% TCA, 0.0375% TBA, and 25% 1 N HCl). The formation of TBAR produced a coloring that can be read with a spectrophotometer to 535 nm, and the sample absorbencies were compared with a standard curve of MDA. Raw data were corrected by the protein content and normalized as percentage of control values.

Statistical analyses

Data were analyzed with one-way analyses of variance (ANOVAs) followed by Tukey's tests, whenever $p < 0.05$.

Results

Anxiogenic-like behavior induced by MeHg is prevented by AA treatment

Our data have shown that treatment with AA has blocked the anxiogenic-like effect induced by MeHg in zebrafishes

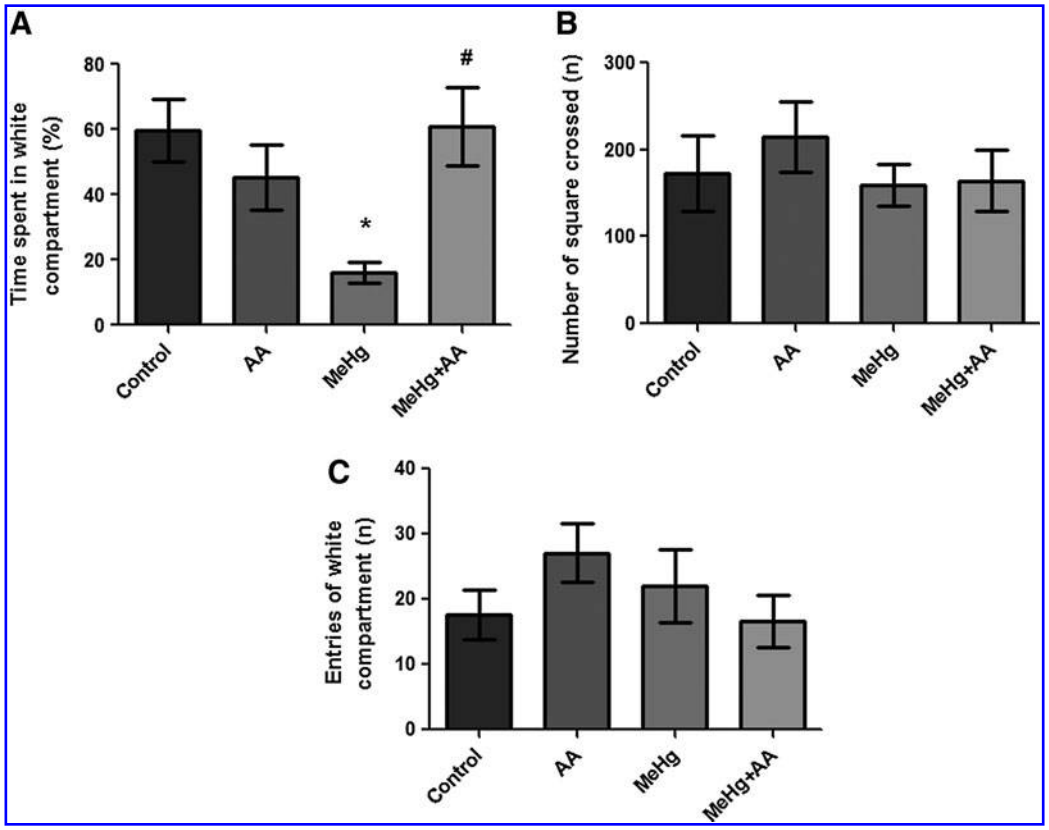

FIG. 1. Effects of ascorbic acid (AA) pretreatment in animals exposed to methylmercury. **(A)** Time spent in the white compartment, **(B)** number of squares crossed in the white compartment, **(C)** entries in the white compartment of the light/dark preference test. ANOVA Tukey's post-test. *$p < 0.01$ versus control; #$p < 0.01$ versus methylmercury (MeHg).

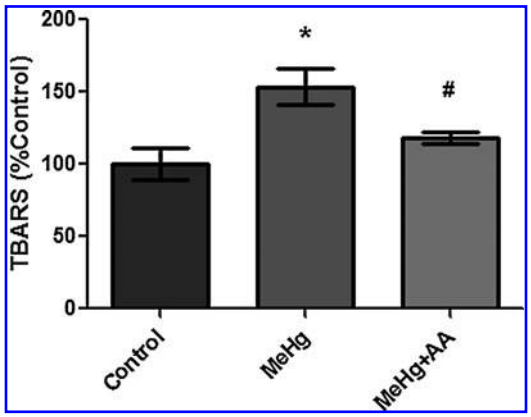

FIG. 2. Effects of AA pretretament on lipid peroxidation induced by MeHg in the zebrafish brain. $^{*}p < 0.01$ versus control; $^{\#}p < 0.05$ versus MeHg. Animals were injected with AA 24 h before MeHg poisoning and 48 h before brain dissection and homogenization. ANOVA Tukey's post-test $^{*}p < 0.01$ versus control; $^{\#}p < 0.01$ versus MeHg.

(F[3,20] = 5.0090, $p < 0.05$). As observed in Figure 1, MeHg treatment induces a significant decrease in the time spent in the white compartment (59.65% ± 9.48% control vs. 16.05% ± 3.12% MeHg) and this effects was avoided by AA treatment (16.05% ± 3.12% MeHg vs. 60.85% ± 11.95% AA + MeHg). Lack of alterations in the number of squares crossed in the white compartment and entries in the white compartment (F[3,24] = 0.48) (Fig. 1) suggest that a decrease in the time spent in the white compartment induced by MeHg exposure was not associated with motor alterations.

Oxidative stress induced by MeHg in zebrafish brains is prevented by AA treatment

To evaluate whether behavioral effect induced by MeHg treatment could be associated with oxidative stress, we performed a lipid peroxidation assay in the brain tissues, as described in the Materials and Methods section. Our data demonstrated that the MeHg group showed an increase in the TBARS levels compared with nontreated animals; however, in the animals treated with AA, a considerable decrease in the TBARS levels was observed in relation to the MeHg group (F[2,18] = 7.71; $p < 0.01$; 100% ± 5.5% control vs. 153.59% ± 6% MeHg and $p < 0.05$, 153.59% ± 6% MeHg vs. 117.86% ± 2.3% AA + MeHg) (Fig. 2).

Indoleamine levels

Our results have demonstrated that the ECF serotonin content was decreased in MeHg groups. This alteration seemed to be prevented by AA pretreatment (F[3,8] = 112.88; $p < 0.01$; 111.34% ± 4.14% control vs. 65.69% ± 2.44%% MeHg vs. 94.13% ± 3.5% AA + MeHg) (Fig. 3A). We also have showed that MeHg treatment induced significant increases in the extracellular levels of T-4,5-D (F[3,8] = 86.5682; $p < 0.0001$) compared to control ($p < 0.05$), and AA pretreatment prevented the tripamina-4,5-diona increase by the MeHg treatment (F[3,8] = 85.56; $p < 0.01$; 0.7% ± 0.92% control vs. 11.61% ± 1.58% MeHg vs. 2.06% ± 0.59% AA + MeHg) (Fig. 3B). No difference in the extracellular levels of 5-HIAA was observed (F[3.8] = 0.93) (Fig. 3C).

Discussion

In the present work, we used for the first time the zebrafish model to evaluate the protector effect of antioxidant

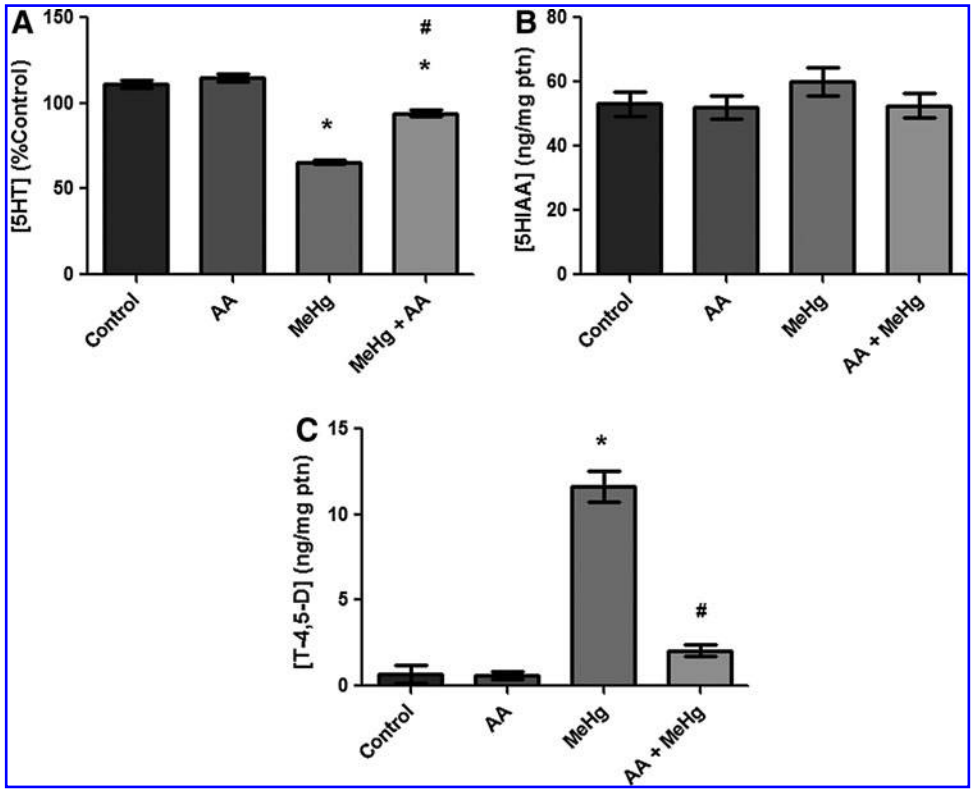

FIG. 3. Effects of MeHg on the extracellular contents of **(A)** serotonin (5-HT), **(B)** tryptamine-4,5-dione (T-4,5-D), and **(C)** 5-hydroxyindolacetic acid (5-HIAA). Animals were injected with AA pretreatment and 24 h before MeHg poisoning, brain dissection, and extracellular fluid extraction. ANOVA Tukey's post-test $^{*}p < 0.01$ versus control; $^{\#}p < 0.01$ versus MeHg.

against behavior alterations induced by MeHg in the central nervous system. Studies performed by our group have recently demonstrated that zebrafishes exposed to different MeHg levels showed an anxiogenic-like behavior, this phenomenon being associated with the decrease of extracellular serotonin levels and increase in the content of T-4,5-D a oxidized serotonin.[41]

The anxiogenic-like effect and biochemical alterations induced by MeHg was blocked by pretreatment with AA. These results support our hypothesis that oxidative stress represents the main molecular mechanism related to anxiogenic like induced by MeHg. Several studies have demonstrated that generation of reactive oxygen species is an important mechanism of MeHg toxicity in the central nervous system,[29] as well as that oxidative stress has been implicated in anxiety disorders and high anxiety levels.[41–46] In the present work, we demonstrated that AA treatment is able to exert protection against neurobehavior and neurochemical alterations induced by mercury exposure. These results suggest that the zebrafish model can be used for the evaluation and development of drugs with a protector activity against agents that evoke toxicity in the central nervous system.

Previous reports have demonstrated that several fruits of the Amazon Region have in its constitution, a high concentration of AA[37]; in this context, the characterization of AA as a protector agent can contribute to a future public health policy by inclusion of a diet with fruits with a high concentration of vitamin C for populations that live in areas contaminated with MeHg, although several posterior studies need to be performed for its characterization as an effective protector agent in humans.

Disclosure Statement

No competing financial interests exist.

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