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TAURINE PREVENTS ENHANCEMENT OF ACETYLCHOLINESTERASE ACTIVITY INDUCED BY ACUTE ETHANOL EXPOSURE AND DECREASES THE LEVEL OF MARKERS OF OXIDATIVE STRESS IN ZEBRAFISH BRAIN

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Abstract—Ethanol (EtOH) is a drug widely consumed throughout the world that promotes several neurochemical disorders. Its deleterious effects are generally associated with modifications in oxidative stress parameters, signaling transduction pathways, and neurotransmitter systems, leading to distinct behavioral changes. Taurine (2-aminoethanesulfonic acid) is a β-amino acid not incorporated into proteins found in mM range in the central nervous system (CNS). The actions of taurine as an inhibitory neurotransmitter, neuromodulator, and antioxidant make it attractive for studying a potential protective role against EtOH-mediated neurotoxicity. In this study, we investigated whether acute taurine cotreatment or pretreatment (1 h) prevent EtOH-induced changes in acetylcholinesterase (AChE) activity and in oxidative stress parameters in zebrafish brain. The results showed that EtOH exposure (1% in volume) during 1 h increased AChE activity, whereas the cotreatment with 400 mg·L^{-1} taurine prevented this enhancement. A similar protective effect of 150 and 400 mg·L^{-1} taurine was also observed when the animals were pretreated with this amino acid. Taurine treatments also prevented the alterations promoted in superoxide dismutase and catalase activities by EtOH, suggesting a modulatory role in enzymatic antioxidant defenses. The pretreatment with 150 and 400 mg·L^{-1} taurine significantly increased the sulfydryl levels as compared to control and EtOH groups. Moreover, 150 and 400 mg·L^{-1} taurine significantly decreased thiobarbituric acid reactive species (TBARS) levels, but the cotreatment with EtOH plus 400 mg·L^{-1} taurine did not prevent the EtOH-induced lipoperoxidation. In contrast, the pretreatment with 150 and 400 mg·L^{-1} taurine prevented the TBARS increase besides decreased the basal levels of lipid peroxides. Altogether, our data showed for the first time that EtOH induced oxidative stress in adult zebrafish brain and reinforce the idea that this vertebrate is an attractive alternative model to evaluate the beneficial effect of taurine against acute EtOH exposure.

Key words: taurine, ethanol, acetylcholinesterase, oxidative stress parameters, zebrafish, brain.

The zebrafish (*Danio rerio*) is emerging as a promising model organism for experimental studies in different biomedical areas. As a relatively simple vertebrate species, zebrafish is an ideal animal model for laboratory research because they are inexpensive, low-maintenance, and abundantly produce offspring (Gerlai et al., 2006; Egan et al., 2009). Because zebrafish genes are highly conserved sharing a 70–80% homology to those of humans (Barbazuk et al., 2000), it is a tempting vertebrate model for modeling behavioral and functional parameters related to human pathogenesis and for clinical treatments screening, including alcohol abuse and therapeutic strategies.

Ethanol (EtOH) is a drug widely consumed throughout the world. Alcoholic consumption is linked to the occurrence of several pathological conditions such as various forms of cancer, liver failure, brain damage, and fetal injuries (Quertemont et al., 2005; Dalitz et al., 2008). The cerebral effects of acute EtOH exposure in central nervous system (CNS) lead to an impairment of motor coordination, sensory perception and cognition, which can be correlated to oxidative stress and modifications of neurotransmitter systems and intricate signaling pathways (Hanchar et al., 2005; Belmeguenai et al., 2008).

Acetylcholine (ACh) is a neurotransmitter that elicits its effects through nicotinic and muscarinic receptors. In the extracellular space, acetylcholinesterase (EC 3.1.1.7;

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Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; CAT, catalase; CNS, central nervous system; DTNB, 5,5′-dithionitrobis 2-nitrobenzoic acid; EtOH, ethanol; GSH, reduced glutathione; ROS, reactive oxygen species; SOD, superoxide dismutase; TAR, total antioxidant reactivity; TBARS, thiobarbituric acid reactive species; TRAP, total antioxidant potential.

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AChE) is the enzyme responsible for terminating the cholinergic transmission by degradation of ACh into choline and acetate. Since the cholinergic system can also be involved in the modulation of behavioral and cognitive functions (Sarter and Bruno, 2004; Furey et al., 2008) the levels of ACh in extracellular milieu must be tightly regulated and the screening for molecules able to modulate directly and/or indirectly AChE activity could be a pharmacological strategy to lead the maintenance of brain homeostasis.

Taurine (2-aminoethanesulfonic acid) is a simple sulfur-containing β-amino acid, which is not incorporated into proteins and is found free in virtually all animal cells. In particular, high concentrations of taurine are detected in electrically excitable tissues such as brain, retina, heart, and skeletal muscles (Huxtable, 1992; Saransaari and Oja, 2000; Oja and Saransaari, 2007). In the CNS, taurine plays a critical role for brain function, being implicated in cell volume regulation and also in neuromodulation or inhibitory neurotransmission (Banerjee et al., 2008). In certain tissues such as brain, intracellular taurine concentrations can range up to 50 mM, whereas extracellular concentrations are in the micromolar range (Huxtable, 1992). The intracellular taurine accumulation results primarily from uptake by an efficient transport system (TauT protein) in the plasma membrane which utilizes transmembrane gradients of Na^+ and Cl^- as the driving force, as well from intracellular biosynthesis of taurine (Kozlowski et al., 2008; Kang, 2009). Several roles of taurine have been reported, including its trophic actions during the CNS development; antioxidant functions, the ability for modifying protein phosphorylation, maintenance of calcium homeostasis, and membrane integrity (Wu et al., 2005; Oliveira et al., 2010; Junyent et al., 2010). Although the mechanisms involved in taurine actions still remains poorly understood, it is conceivable that its extracellular effects are mediated by opening the chloride channels through the interaction with $GABA_A$ receptors, glycine receptors, or putative taurine receptors (Albrecht and Schousboe, 2005). Due to its biochemical properties, taurine interacts with other transmitter systems and acts as a neuroprotector against various types of injury, including alcohol abuse (Oja and Saransaari, 2007; Chen et al., 2009).

The teratogenic properties of EtOH have been previously established in zebrafish (Dlugos and Rabin, 2003; Reimers et al., 2006). It has been shown that EtOH modulates distinct behavioral parameters in this species such as swimming activity, aggression, group preference, and pigment response, possibly through alterations in neurotransmitter systems and in cell signaling cascades (Gerlai et al., 2000; Rico et al., 2007; Peng et al., 2009). Concerning the cholinergic signaling, zebrafish presents a unique situation among vertebrates because its genome does not encode a functional butyrylcholinesterase, being AChE the only ACh-hydrolyzing enzyme in this organism (Behra et al., 2004). It was reported that brain AChE activity in zebrafish is altered after acute EtOH exposure, suggesting an involvement of cholinergic parameters on EtOH-mediated responses (Rico et al., 2007). Moreover, previous study characterized the expression profile of TauT in zebrafish during embryogenesis, showing a high degree of homology to mammalian taurine transporter (Kozlowski et al., 2008). Although it has been previously reported that the EtOH-mediated toxicity in zebrafish embryos can be partially attenuated by antioxidants (Reimers et al., 2006), little is known about the effects of EtOH and taurine on the brain function of adult zebrafish. In this context, the influence of acute EtOH exposure on oxidative stress parameters and the beneficial actions of taurine in this species still remain obscure. Therefore, in the present study we focused for the first time our attention on the potential neuroprotective effects of taurine in zebrafish brain. First, we evaluated whether acute cotreatment or pretreatment with taurine prevent EtOH-induced enhancement of AChE activity. In addition, the effect of acute EtOH exposure on oxidative stress parameters and the influence of both taurine treatments on neurochemical redox profile were also studied.

EXPERIMENTAL PROCEDURES

Animals

Adult males and females of the "wild type" (short fin—SF) zebrafish (*Danio rerio*) strain (3–6 months-old, weighing 0.43±0.07 g) were obtained from a commercial supplier (Delphis, RS, Brazil) and acclimated for at least 2 weeks in 50-L thermostated aquarium before the experiments. All tanks were filled with unchlorinated water previously treated with 132 $\mu L.L^{-1}$ AquaSafe® (Tetra, USA) and kept under mechanical and chemical filtration at a targeted temperature of 26±2 °C and water pH and conductivity at 7.0–8.0 and 1,500–1,600 $\mu S.cm^{-1}$, respectively. Illumination was provided by ceiling-mounted fluorescent light tubes on a 12-h light-dark photoperiod (on 7:00 h; off 19:00 h). Fish were fed twice a day to satiety with commercial alcon BASIC® (Alcon, Brazil) as flake fish food. For the experiments, fish were euthanized by decapitation and the brains were quickly dissected. Each independent experiment was performed using biological preparations from a pool of three animals for AChE experiments and 10 animals for oxidative stress evaluation. All animals used were naive being healthy and free of any signs of disease and maintained in accordance to the National Institute of Health Guide for Care and Use of Laboratory Animals. The protocols were approved by the Ethical Committee of the Federal University of Rio Grande do Sul under the number 2007950.

Chemicals

Ethanol (C_2H_6O; CAS number 64-17-5) was purchased from Merck (Darmstadt, Germany). All other reagents used were purchased from Sigma (St. Louis, MO, USA).

Experimental design

For acute EtOH exposure, fish were placed in 3-L aquarium and kept in a solution of 1% EtOH in volume during 1 h. The same time of exposure and EtOH concentration have been successfully tested in adult zebrafish (Gerlai et al., 2000; Dlugos and Rabin, 2003; Rico et al., 2007) leading to alterations in behavioral and cholinergic signaling parameters of this species. In addition, the blood alcohol levels achieved with it are expected to be in the range seen in the human clinic after mild to moderate acute alcohol consumption (Gerlai et al., 2008). Acute taurine treatments were performed using distinct concentrations (42, 150, and 400 $mg{\cdot}L^{-1}$) during 1 h, the same time used for EtOH exposure. Taurine chosen concentrations correspond to a range previously

used in other studies related to taurine effects on several biochemical parameters, which vary from 0.33 to 3.2 mM (Wu et al., 2005; Kong et al., 2006; Rosemberg et al., 2010b). Two treatment protocols with taurine were performed: (i) concomitant treatment with EtOH; and (ii) a pretreatment during 1 h before EtOH exposure. Importantly, the acute treatments procedure (e.g., the origin and quality of the system water and the timing of taurine delivery, etc.) was identical for all fish. Different experimental groups were analyzed: (1) control (1 h into water); (2) EtOH-exposed during 1 h; (3) taurine-treated during 1 h; (4) concomitant taurine treatment with EtOH during 1 h (cotreatment); (5) 1 h into water before transference to another aquaria+1 h into water (pretreatment—control group unexposed to taurine); (6) 1 h into water before transference to another aquaria+EtOH exposure during 1 h (pretreatment—EtOH group unexposed to taurine); (7) taurine-treated during 1 h before transference to another aquaria+EtOH exposure during 1 h (pretreatment—experimental group); and (8) taurine-treated during 1 h before transference to another aquaria+1 h into water (pretreatment—control group treated with taurine).

Determination of AChE activity

The brains were homogenized on ice in 60 volumes (v/w) of Tris–citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) using a Potter–Elvehjen-type glass homogenizer. The rate of hydrolysis of acetylthiocholine iodide (0.88 mM) was determined in a final volume of 300 μL, with 33 μL of 100 mM phosphate buffer, pH 7.5 mixed to 2.0 mM 5,5′-dithionitrobis 2-nitrobenzoic acid (DTNB). Samples containing 5 μg protein and the reaction medium specified above were preincubated for 10 min at 25 °C. The hydrolysis of acetylthiocholine iodide was monitored in a microplate reader by the formation of thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s) (Ellman et al., 1961). Controls without the homogenate preparation were performed in order to determine the non-enzymatic hydrolysis of the substrate. AChE activity was expressed as μmol thiocholine (SCh). h^{-1}. mg $protein^{-1}$. All experiments were performed in quadruplicate.

Oxidative stress analyses

Zebrafish brains were dissected out in ice immediately after the fish were euthanized and homogenized in 1.0 mL phosphate buffer saline (PBS) pH 7.4, containing in mM: 137 NaCl, 10.1 Na_2HPO_4, and 1.76 KH_2PO_4. The homogenates were centrifuged (700×g, 5 min) to remove cellular debris. Supernatants were collected and used to all biochemical assays described herein.

Antioxidant enzyme activities determination

Superoxide dismutase (EC 1.15.1.1, SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation at 480 nm (Misra and Fridovich, 1972), and the results were expressed as Units SOD. mg $protein^{-1}$. Catalase (EC 1.11.1.6; CAT) activity was assessed by measuring the rate of decrease in H_2O_2 absorbance at 240 nm (Aebi, 1984), and the results were expressed as Units CAT. mg $protein^{-1}$. For SOD assay, protein amounts ranged from 15–60 μg, while CAT activity was determined using 30–50 μg protein. To better understand the effect promoted by the treatments upon these two oxidant-detoxifying enzymes that work in sequence converting superoxide anion to water, a ratio SOD/CAT activities was calculated. An imbalance between their activities is thought to facilitate oxidative-dependent alterations in the cellular environment, which could culminate in oxidative stress.

Non-enzymatic antioxidant defenses

The non-enzymatic antioxidant potential of zebrafish brains was estimated by the total antioxidant potential (TRAP) and total antioxidant reactivity (TAR) (Lissi et al., 1995). The reaction was initiated by adding luminol (5-Amino-2,3-dihydro-1,4-phthalazinedione, 4 mM)—an external probe to monitoring radical production—and AAPH (2,2′-azobis[2-methylpropionamidine]dihydrochloride, 10 mM)—a free radical source that produces peroxyl radical at a constant rate—in glycine buffer (0.1 M) pH 8.6 at room temperature, resulting in a steady luminescence emission (system counts). Chemiluminescence was read in a liquid scintillation counter (Wallace, 1409) as counts per minutes. Sample addition decreases the luminescence proportionately to its antioxidant potential. The luminescence emission was followed for 40 min after the addition of the sample (10 μg protein) in a TRAP protocol, and the area under the curve was quantified. In the TAR protocol, results were calculated as percentage of radical production (system counts considered as 100% of radical production).

Total reduced thiol content

Oxidative alterations in proteins can be evaluated by the level of protein thiol content in samples. Briefly, samples (40 μg protein) were mixed to 35 μL of 0.2 mM EDTA, 100 mM boric acid buffer, pH 8.5. DTNB (0.01 M dissolved in ethanol) was added and the intense yellow color developed was measured at 412 nm after 1 h (Ellman, 1959). Total reduced sulfydryl content was estimated in a final volume of 210 μL and the results were expressed as μmol SH. mg $protein^{-1}$.

Thiobarbituric acid reactive species (TBARS)

The formation of TBARS during an acid-heating reaction, which is widely performed for measurement of lipid redox state (Draper and Hadley, 1990), was used as an index of lipid peroxidation. Briefly, 300 μL of samples (80–100 μg protein) were mixed with 600 μL of 15% trichloroacetic acid (TCA) and centrifuged (10.000×g, 10 min). Supernatants (100 μL) were mixed with 100 μL of 0.67% thiobarbituric acid (TBA, 4,6-Dihydroxypyrimidine-2-thiol) and heated at 100 °C for 30 min. TBARS levels were determined by the absorbance at 532 nm using 1,1,3,3-tetramethoxypropane (TMP) as standard. Results were expressed as nmol TBARS. mg $protein^{-1}$.

Protein quantification

For AChE activity assays, the protein was measured by the Coomassie Blue method (Bradford, 1976). For oxidative stress analyses, the protein concentration was determined according to Peterson (1977). Bovine serum albumin was used as a protein standard.

Statistics

Data were expressed as mean ± standard error of the mean (SEM) and P-values were considered significant for $P \leq 0.05$. Differences within the experimental groups were determined by one or two-way analysis of variance (ANOVA). Comparison among means was carried out using Bonferroni's multiple comparison test as post hoc. All experiments were performed at least in triplicate.

RESULTS

Taurine prevents alterations in AChE activity promoted by acute EtOH exposure

As previously reported by our group (Rico et al., 2007), we demonstrate that acute EtOH exposure (1% in volume) increased AChE activity in zebrafish brain (37%, $n=6$) when compared to control group (Fig. 1). When the animals were treated concomitantly with taurine (42 and 150

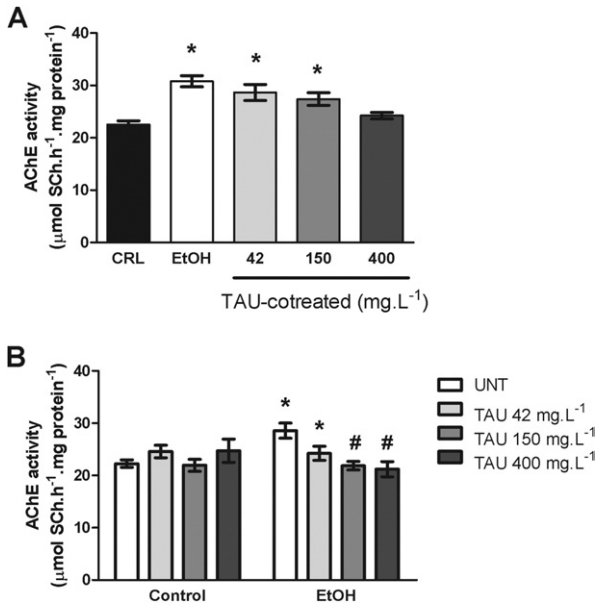

Fig. 1. Effect of taurine on the EtOH-induced enhancement of AChE activity after acute exposure. (A) Cotreatment with taurine and EtOH. The groups were represented as control (CRL), ethanol (EtOH), taurine-cotreated animals. Data represent mean±SEM of at least three independent experiments. * Significant difference compared to control (one-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$). (B) Pretreatment with taurine followed by water (control group) or EtOH exposure. The treatments were represented as taurine-untreated (UNT) and taurine-treated (TAU). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to UNT from control group. # Significant difference compared to UNT from EtOH group (two-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$).

mg·L^{-1}) and EtOH, the AChE activity did not change in comparison to EtOH group. However, taurine cotreatment at 400 mg·L^{-1} prevented zebrafish brain from EtOH-induced enhancement of AChE activity ($n=6$) (Fig. 1A).

Next, we evaluated the effect of taurine pretreatment on EtOH-induced AChE stimulation (Fig. 1B). A two-way ANOVA revealed a significant effect of taurine pretreatment×EtOH exposure interaction ($F(3,32)=3.63$, $P<0.05$). Post hoc analyses indicated that pretreatment with 150 and 400 mg·L^{-1} taurine prevented the effects promoted by EtOH in AChE activity.

To verify whether taurine alters AChE activity, the animals were treated with the same concentrations tested (42, 150, and 400 mg·L^{-1}). The results demonstrated that AChE activity remained similar to control group ($n=4$).

Taurine prevents changes in antioxidant enzyme activities induced by EtOH

SOD and CAT play a key role in the control of reactive oxygen species (ROS) levels and in the prevention of oxidative damage. Acute exposure to EtOH significantly decreased SOD activity (49%, $n=3$) (Fig. 2) and concomitantly increased CAT activity (89%, $n=4$) (Fig. 3). The cotreatment with 400 mg·L^{-1} taurine prevented the EtOH-induced changes in SOD (Fig. 2A) and CAT activities (Fig. 3A) ($n=4$).

The influence of taurine pretreatment on the effects of EtOH in SOD (Fig. 2B) and CAT activities (Fig. 3B) was also evaluated. A two-way ANOVA revealed a significant effect of taurine pretreatment ($F(2,28)=6.15$, $P<0.05$) and EtOH exposure ($F(1,28)=4.5$, $P<0.05$). Post hoc analyses indicated that pretreatment with 150 and 400 mg·L^{-1} taurine prevented the EtOH-induced decrease in SOD activity. In relation to CAT activity, a two-way ANOVA revealed a significant effect of taurine pretreatment×EtOH exposure interaction ($F(2,19)=10.37$, $P<0.05$) and EtOH exposure ($F(1,19)=8.79$, $P<0.05$). Post hoc analyses indicated that pretreatment with 150 and 400 mg·L^{-1} taurine also prevented the EtOH-induced increase in CAT activity. Both taurine concentrations *per se* did not significantly alter SOD and CAT activities in zebrafish brain.

As depicted in Fig. 4, EtOH induced an imbalance in the SOD/CAT ratio ($n=4$; $P<0.05$). Both cotreatment (400 mg·L^{-1}) (Fig. 4A) and pretreatment (150 and 400 mg·L^{-1}) (Fig. 4B) with taurine reversed SOD/CAT ratio to the basal level ($n=5$).

Non-enzymatic antioxidant defenses are altered neither by EtOH exposure nor taurine treatments

The effects promoted by EtOH, taurine and both cotreatment and pretreatment on non-enzymatic antioxidant

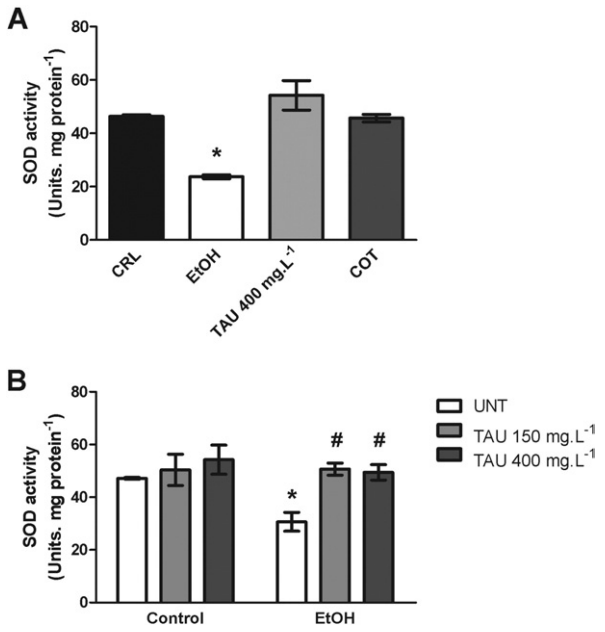

Fig. 2. Effect of EtOH and taurine treatments in SOD activity. (A) Cotreatment with taurine and EtOH. The groups were represented as control (CRL), ethanol (EtOH), taurine-treated (TAU) and taurine-cotreated animals (COT). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to control (one-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$). (B) Pretreatment with taurine followed by water (control group) or EtOH exposure. The treatments were represented as taurine-untreated (UNT) and taurine-treated (TAU). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to UNT from control group. # Significant difference compared to UNT from EtOH group (two-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$).

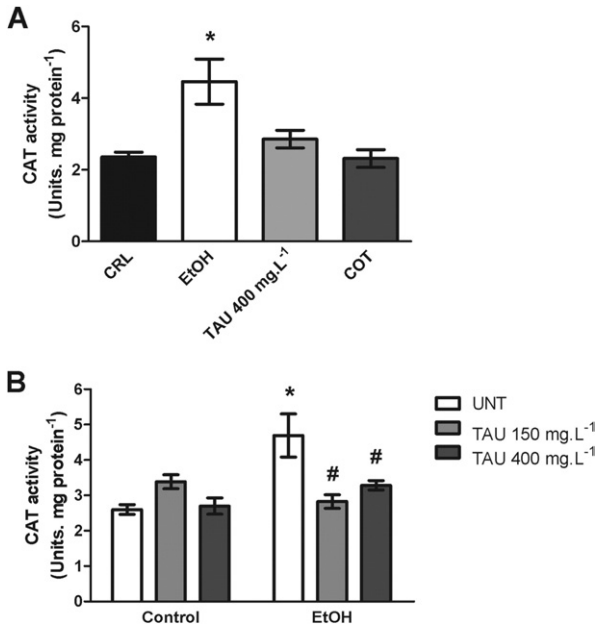

Fig. 3. Effect of EtOH and taurine treatments in CAT activity. (A) Cotreatment with taurine and EtOH. The groups were represented as control (CRL), ethanol (EtOH), taurine-treated (TAU) and taurine-cotreated animals (COT). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to control (one-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$). (B) Pretreatment with taurine followed by water (control group) or EtOH exposure. The treatments were represented as taurine-untreated (UNT) and taurine-treated (TAU). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to UNT from control group. # Significant difference compared to UNT from EtOH group (two-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$).

defenses were also evaluated. Neither EtOH exposure nor taurine treatments significantly changed TRAP (according to decreased radical production during TRAP experiment) and TAR in zebrafish brain ($n=3$) (data not shown).

Effect of taurine treatments on total reduced thiol content

EtOH, at the concentration and time of exposure tested, did not induce any significant changes in the total reduced thiol content of zebrafish brain (Fig. 5). Similarly, 150 and 400 mg·L^{-1} taurine did not alter total thiol content. Furthermore, the cotreatment with 400 mg·L^{-1} taurine did not significantly change the basal total reduced thiol content as compared to control and EtOH-treated groups ($n=4$) (Fig. 5A). However, a two-way ANOVA revealed a significant effect of EtOH exposure ($F(1,20)=12.47$, $P<0.05$) and taurine pretreatment ($F(2,20)=5.27$, $P<0.05$). Post hoc analyses indicated that pretreatment with 150 and 400 mg·L^{-1} taurine followed by EtOH exposure induced a significant increase in total reduced sulfydryl content (52% and 64%, respectively, $n=4$) as compared to control and EtOH-treated groups ($n=5$) (Fig. 5B).

Protective effect of taurine against EtOH-induced lipid peroxidation

To evaluate whether EtOH exposure alters lipid peroxidation in zebrafish brain, we assessed the formation of TBARS during an acid-heating reaction. Acute EtOH exposure significantly increased by 40% ($n=3$) the lipid peroxidation (Fig. 6). When the animals were treated with 150 and 400 mg·L^{-1} taurine, there was a significant decrease of TBARS levels (30% and 50%, respectively) as compared to the control ($n=3$). Interestingly, the cotreatment with taurine (400 mg·L^{-1}) did not prevent the increase of TBARS levels induced by EtOH ($n=5$) (Fig. 6A).

Regarding the taurine pretreatment experiment (Fig. 6B), a two-way ANOVA revealed significant effects of taurine pretreatment ($F(2,23)=74.45$, $P<0.05$), taurine pretreatment×EtOH exposure interaction ($F(2,23)=6.27$, $P<0.05$) and EtOH exposure ($F(1,23)=4.75$, $P<0.05$). Post hoc analyses indicated that pretreatment with 150 and 400 mg·L^{-1} taurine prevented EtOH-induced enhancement of TBARS formation and the lipid peroxidation levels were statistically lower than that determined in control group (34% and 41%, respectively, $n=3$).

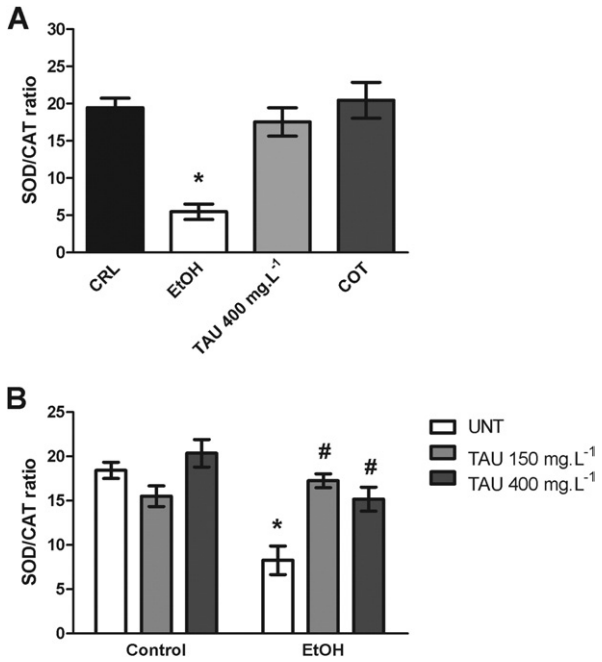

Fig. 4. Protective effect of taurine in SOD/CAT ratio. (A) Cotreatment with taurine and EtOH. The groups were represented as control (CRL), ethanol (EtOH), taurine-treated (TAU) and taurine-cotreated animals (COT). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to control (one-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$). (B) Pretreatment with taurine followed by water (control group) or EtOH exposure. The treatments were represented as taurine-untreated (UNT) and taurine-treated (TAU). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to UNT from control group. # Significant difference compared to UNT from EtOH group (two-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$).

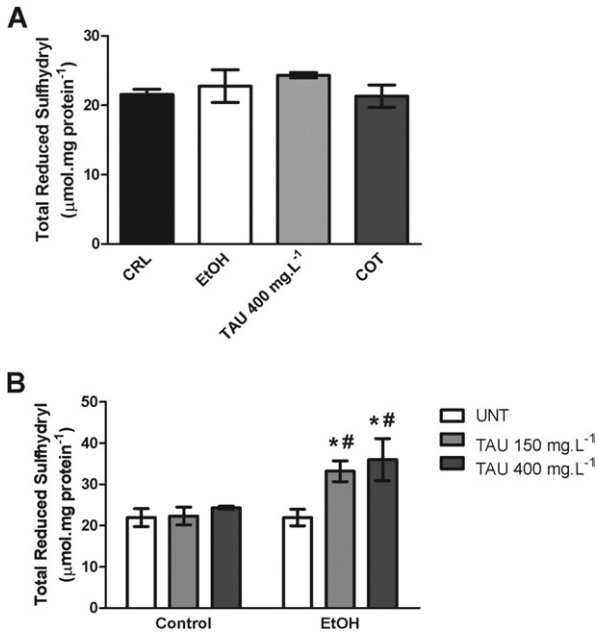

Fig. 5. Effect of EtOH and taurine on total thiol content in zebrafish brain. (A) Cotreatment with taurine and EtOH. The groups were represented as control (CRL), ethanol (EtOH), taurine-treated (TAU) and taurine-cotreated animals (COT). Data represent mean±SEM of at least three independent experiments. (B) Pretreatment with taurine followed by water (control group) or EtOH exposure. The treatments were represented as taurine-untreated (UNT) and taurine-treated (TAU). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to UNT from control group. # Significant difference compared to UNT from EtOH group (two-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$).

DISCUSSION

Studies have demonstrated that the behavioral changes induced by acute EtOH exposure in mammalian models are generally associated to its influence on several neurotransmitter systems (Hanchar et al., 2005; Belmeguenai et al., 2008). Due to the susceptibility of zebrafish to environmental and pharmacological manipulations and the characterization of distinct neurotransmitter parameters in this species, it is rapidly becoming a popular vertebrate model in behavioral and neuroscience research (Egan et al., 2009; Rico et al., 2010; Rosemberg et al., 2010a). It has been shown that acute EtOH exposure during 1 h in the tank water induced significant alterations in behavioral tasks of zebrafish and also increased the levels of dopamine, serotonin, and its metabolite 5-HIAA in whole brain extracts (Chatterjee and Gerlai, 2009). Zebrafish may be especially suitable for modeling the effects of EtOH because of the simplicity of alcohol delivery (Gerlai et al., 2000). The EtOH mixed in their environment is absorbed by the blood vessels of the gill and the skin of the fish so that blood alcohol levels reach equilibrium with the external alcohol concentration quickly (Gerlai et al., 2000; Dlugos and Rabin, 2003; Chatterjee and Gerlai, 2009). Despite to the relative differences in comparison to mammalian metabolism of EtOH ingestion, the alcohol mixed to the fish tank is rapidly diffused through systemic circulation and reaches distinct tissues. There is evidence suggesting that hepatic alcohol dehydrogenase and aldehyde dehydrogenase play a role in alcohol metabolism in fish, since 32 h of continuous 2% EtOH exposure caused characteristics signs of acute alcoholic liver disease in zebrafish larvae, including hepatomegaly, steatosis and changes in hepatic gene expression (Passeri et al., 2009). Furthermore, it has been shown that significant brain alcohol levels can be detected 15 min after exposure to 0.5% (v/v) EtOH in adult zebrafish brain, reaching a steady-state level that is maintained for at least 24 h without significant difference among distinct strains (Dlugos and Rabin, 2003). These data are in accordance with studies involving other fish species (Ryback et al., 1969; Galizio et al., 1985) in that, following a few hours of exposure, an equilibrium between the level of alcohol in the tank and EtOH content in brain was reached. Previous report demonstrated that the brain alcohol level of the zebrafish was achieved when brain alcohol levels were approximately 90% of the tank alcohol level (Dlugos and Rabin, 2003; Gerlai et al., 2006). Thus, it is comprehensive that the physiological responses promoted by acute EtOH exposure might involve alterations in neurotransmitter systems and also in oxidative stress parameters of zebrafish, suggesting that studies which comprise both approaches are tempting for the

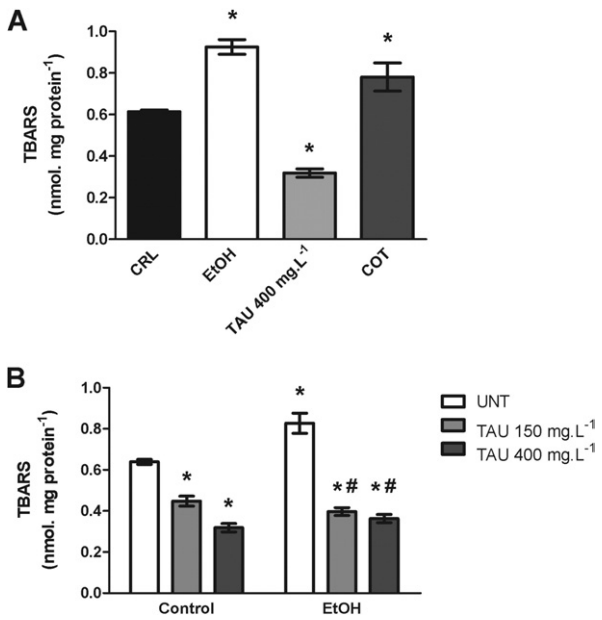

Fig. 6. Effect of EtOH and taurine on lipid peroxidation measured as TBARS formation in zebrafish brain. (A) Cotreatment with taurine and EtOH. The groups were represented as control (CRL), ethanol (EtOH), taurine-treated (TAU) and taurine-cotreated animals (COT). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to control (one-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$). (B) Pretreatment with taurine followed by water (control group) or EtOH exposure. The treatments were represented as taurine-untreated (UNT) and taurine-treated (TAU). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to UNT from control group. # Significant difference compared to UNT from EtOH group (two-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$).

assessment of potential beneficial effects against EtOH-mediated neurotoxicity.

In order to determine whether taurine could prevent the enhancement of AChE activity in zebrafish brain, we performed a cotreatment and pretreatment using different taurine concentrations during 1 h. The results demonstrated that only 400 $mg{\cdot}L^{-1}$ taurine prevented the increase of AChE activity when cotreated with EtOH. However, we further verified that the EtOH-induced enhancement of AChE activity was prevented by the pretreatment with 150 and 400 $mg{\cdot}L^{-1}$ taurine. Probably, the increase in AChE activity promoted by EtOH and the protective effect of taurine are not due to a direct mechanism, since both compounds did not alter the enzyme activity *in vitro* (data not shown). In this sense, the investigations related to the effects of taurine against EtOH-induced modifications in oxidative stress parameters were performed using the amino acid concentrations which prevented the enhancement of AChE activity for each treatment.

Previous studies have shown that ROS are products of normal cellular metabolism that act as important signaling molecules at low/moderate concentrations (Avshalumov et al., 2007; Chiarugi and Fiaschi, 2007). In this context, SOD and CAT activities normally have a synergism under physiological situations playing a key role in the fine-tuning regulation of superoxide anion (O_2^-) and H_2O_2 levels. Our results demonstrated that even though EtOH did not induce significant changes in TRAP and TAR, the acute EtOH exposure significantly decreased SOD activity and increased CAT activity from zebrafish brain. Although these data seem contradictory, there are some possible explanations for these effects and even potential consequences. It is important to emphasize that EtOH is an exogenous drug that impairs distinct neurotransmitter systems and also promotes oxidative stress in several organisms. Hence, we suggest that an imbalance between oxidants and enzymatic/non-enzymatic antioxidant defenses might contribute to an increase of ROS and its harmful effects on living cells. Previous studies demonstrated that i.p. injections of EtOH led to an inhibition of SOD activity in rat brain (Ledig et al., 1981) and also that 400 $mg.dL^{-1}$ EtOH reduced SOD activity in cerebellar granule cells (Siler-Marsiglio et al., 2004). Therefore, the significant decrease in SOD activity could be due to a direct effect of EtOH or an indirect effect mediated by its metabolite acetaldehyde. As a consequence, it is possible to suggest an increase of O_2^- after EtOH exposure which could mediate toxic effects by itself or via another ROS such as nitric oxide (NO), peroxynitrite ($ONOO^-$) and the radical OH (Halliwell and Gutteridge, 1984; Radi et al., 2002). Concerning CAT activity, it has been demonstrated that EtOH increased this enzyme activity in cerebellar granule cells (Siler-Marsiglio et al., 2004). In the same way, another study showed that EtOH orally administered in rats (2, 4, and 6 $g.kg^{-1}$) also promoted a significant increase of CAT activity in rat brain subcellular fractions (Reddy et al., 1999). There is evidence that CAT is the major enzyme responsible for oxidizing brain EtOH to acetaldehyde after alcohol consumption (Cohen et al., 1980; Swift, 2003). This metabolite has been implicated to mediate central effects that follow EtOH exposure and also plays a key role in the mediation of psychopharmacological effects of EtOH (Pastor et al., 2002). Moreover, acetaldehyde is highly reactive and its neurotoxicity has been associated to the ability to form protein–acetaldehyde adducts or to induce structural chromosomal changes (Nakamura et al., 2003; Kayani and Parry, 2010). Besides the EtOH-induced increase in CAT activity initially appears to be protective, this alcohol also serves as a substrate of CAT, forming the toxic metabolite, acetaldehyde. Thus, CAT activity in the presence of EtOH could be a double-edged sword, ridding the cell of H_2O_2 while producing acetaldehyde. Finally, we demonstrated that SOD/CAT ratio was altered after acute EtOH exposure which suggests that EtOH induces oxidative damage in zebrafish brain. In contrast, both taurine cotreatment and pretreatment maintained SOD/CAT ratio at control levels, suggesting that this amino acid might exert a neuroprotective role by modulating enzymatic antioxidant defenses, which could be important for controlling the levels of O_2^-, H_2O_2, and acetaldehyde in the CNS.

Lipid peroxidation is one important cause of neuronal damage and the major consequence of enhanced lipid peroxidation is attributed to the oxidative deterioration of cellular membranes (Kaizer et al., 2005). Our data showed that EtOH enhanced lipid peroxides, as measured by TBARS formation. Taurine, at 150 and 400 $mg{\cdot}L^{-1}$, promoted a significant decrease in TBARS levels in a concentration-dependent fashion. When cotreated with EtOH, 400 $mg{\cdot}L^{-1}$ taurine did not reduce the EtOH-induced lipid peroxidation, whereas the pretreatment with 150 and 400 $mg{\cdot}L^{-1}$ taurine prevented the increase of TBARS maintaining the levels of lipid peroxidation significantly lower than the control group. Considering these data, it is possible to suggest that the decrease of lipid peroxidation could be correlated to the increase in total reduced thiol content detected after taurine pretreatment and alcohol exposure. Studies reported that taurine is known to attenuate tissue lipid peroxidation either by scavenging or quenching oxygen-derived free radicals, H_2O_2 or hypochlorous acid directly or by binding free metal ion species like Fe^{2+} or Cu^{2+} by its sulfonic acid group (Franconi et al., 2004; Hagar, 2004). In addition, it has been demonstrated that taurine treatment has been found to increase reduced glutathione (GSH) levels by directing cysteine into the GSH synthesis pathway (Schaffer et al., 2003; Hagar, 2004), which could result in the alterations observed in the reduced sulfydryl content after the pretreatment with taurine and EtOH exposure.

Because AChE is anchored to the plasma membrane, there is evidence that lipid peroxidation can alter its activity (Flora et al., 2003; Kaizer et al., 2005). Previous study demonstrated that AChE activation could be correlated with an enhancement of lipid peroxidation (Kaizer et al., 2005). Similarly, our results showed that acute EtOH exposure increased AChE activity and lipid peroxides in zebrafish brain. However, lipid peroxidation is probably not the only explanation for the modulation of AChE activity, considering that the cotreatment with EtOH prevented the

enzyme activation but did not reduce TBARS levels. Our recent study showed that taurine increases AMP hydrolysis and concomitantly decreases adenosine deaminase activity in zebrafish brain membranes, suggesting a potential role of adenosine in taurine-mediated effects (Rosemberg et al., 2010b). Furthermore, it is known that taurine also modulates Ca^{2+} signaling pathways (Wu et al., 2005; Junyent et al., 2010), which are potential targets of ROS (Turrens, 2003). Although speculating about the physiological relevance of our data and also about the putative mechanisms involved in the potential neuroprotective of taurine in zebrafish are tempting, it must be acknowledged that both EtOH and taurine act through numerous neurotransmitters, second messenger systems and other molecular targets. Therefore, our speculations are only correlative, and conclusions regarding the precise molecular mechanism cannot be drawn at this moment. Briefly, the manipulation and/or the systemic assay of all possible neurotransmitter systems and also the exploration of other molecular changes, perhaps at gene expression level and/or transduction signaling pathways, could be interesting strategies to better understand the mechanistic details of the actions promoted by alcohol and taurine in zebrafish brain.

CONCLUSION

In conclusion, this is the first study demonstrating that acute EtOH exposure induced oxidative damage in adult zebrafish brain, which could be correlated to the EtOH-induced modifications in cholinergic signaling parameters and in behavioral tasks in this species. The impairment of the physiological synergism of SOD and CAT activities associated to an increase of lipid peroxides suggests an imbalance between $O_2^{\cdot -}$ and H_2O_2 levels and that acetaldehyde could also play a role in mediating EtOH effects in zebrafish CNS. Moreover, our data report the first evidence that two different acute taurine treatments (cotreatment and pretreatment) prevented AChE activation, maintained SOD/CAT ratio at basal levels and differently changed total reduced sulfydryl content and TBARS formation. Therefore, we suggest that free radicals could in part be involved in the effects of alcohol on zebrafish brain function. There is certainly value in further examining the neural basis of the effects promoted by EtOH and taurine in this species—not only in terms of its face validity (in producing behavioral symptoms related to alcohol consumption) but also its construct validity in relation to modeling the underlying mechanisms related to the alcohol-mediated responses and also to the potential neuroprotective effect of taurine in adult zebrafish brain. In this sense, it is important to reinforce the idea that our data also provide implications for future studies. First, they open the possibility of analyzing the effects EtOH and taurine in oxidative stress parameters of distinct zebrafish strains. Furthermore, one would need to assess a potential functional role of taurine treatments against the neurochemical changes promoted by alcohol exposure in zebrafish by performing additional pharmacokinetic assays and testing whether this amino acid can prevent/attenuate or even reverse EtOH-induced changes in different behavioral paradigms of this vertebrate. Nevertheless, our zebrafish assay may therefore complement existing approaches, bringing new insights related to black box screening and rational experimental design. The current paper also demonstrates that researchers now have yet another available tool which may help in solving the puzzle related to EtOH and taurine effects in zebrafish brain and allows that other questions might be addressed.

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