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ORIGINAL ARTICLE

Cholinergic System and Oxidative Stress Changes in the Brain of a Zebrafish Model Chronically Exposed to Ethanol

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Abstract Ethanol is a widely used drug, and excess or even moderate consumption of ethanol is associated with changes in several neurotransmitter systems, including the cholinergic system. The incidence of alcoholic dementia and its insults are well supported by multiple studies, although the mechanisms of neurotoxicity are still poorly understood. Considering that zebrafish have a complete central nervous system (CNS) and that several signaling systems have already been identified in zebrafish, this neurotoxicological model has become useful. In the present study, we investigated the long-term effects of ethanol consumption on the cholinergic system, on oxidative stress, and on inflammatory parameters in the zebrafish brain. Animals were exposed to 0.5% (*v*/*v*) ethanol for 7, 14, and 28 days. Ethanol inhibited choline acetyltransferase activity after 7 and 14 days but not after 28 days. Acetylcholinesterase activity did not change after any of the exposure periods. When compared to the control group, thiobarbituric acid reactive species and dichlorodihydrofluorescein levels were increased after chronic ethanol exposure. Antioxidant activity promoted by the CAT/SOD ratio was altered after chronic ethanol exposure, suggesting that EtOH can induce oxidative damage in the zebrafish brain. In contrast, nitrate and nitrite levels and sulfhydryl content were not altered. Ethanol did not modify gene expression of the inflammatory cytokines *il-1b*, *il-10*, or *tnf-α* in the zebrafish brain. Therefore, the cholinergic system and the oxidative balance were targeted by chronic ethanol toxicity. This neurochemical regulatory mechanism may play an important role in understanding the effects of long-term ethanol consumption and tolerance in zebrafish model studies.

Keywords Ethanol · Cholinergic system · Oxidative stress · Inflammation · Zebrafish · Brain

Introduction

Ethanol is a psychoactive substance that is obtained through fermentation and that has been used for centuries in ritual and festive practices in many cultures (McGovern 2009). The consumption pattern has changed over the years, and its harmful use is a risk factor for increasing morbidity, mortality, and disability. Chronic ethanol consumption describes alcoholism, whose symptoms are a strong desire to drink, difficulty in controlling ingesting, increased tolerance, and sometimes a physical abstinence state. In addition, alcoholism is related to pathogenic factors in several diseases such as congestive heart failure (Ronksley et al. 2011), diabetes (Munukutla et al., 2016), and cancer (Cao and Giovannucci 2016). Chronic ethanol consumption can cause toxic effects on the central nervous system (CNS), impacting behavioral and social skills.

There is increasing evidence that ethanol can affect the CNS by interfering with amino acid neurotransmitter systems,

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especially the excitatory amino acid neurotransmitters (aspartate and glutamate), which activate post-synaptic cells, and the inhibitory amino acids (γ-aminobutyric acid [GABA] and taurine), which depress post-synaptic cellular activity (De Witte 2004). Studies have shown that ethanol is a GABA agonist (De Witte 2004; Quertemont et al. 2005), thus favoring post-synaptic inhibitory activity. During chronic ethanol exposure, glutamatergic N-methyl-D-aspartate (NMDA) receptors are stimulated in a physiological response to the depressant effects of ethanol in order to restore the balance of inhibitory/excitatory neurotransmission (Gonzalez and Jaworski 1997; Rao and Sari 2012). In addition to this mechanism, the opioid (Harshberger et al. 2016), purinergic (Rico et al. 2011), and dopaminergic systems (Esel 2006) demonstrate neurochemical alterations due to chronic ethanol exposure. Another neurotransmitter system influenced by ethanol consumption is the cholinergic system. Rodents chronically treated with ethanol demonstrated a decrease in cholinergic neuron expression in the cerebral cortex, hippocampus, and amygdala (Arendt et al. 1988; Miller and Rieck 1993).

Ethanol has effects on some cholinergic neurotransmitter mechanisms, such as a reduction in acetylcholine (ACh) levels and a decrease in activity of the cholinergic transmission enzymes, choline acetyltransferase (ChAT; EC 2.3.1.6) and acetylcholinesterase (AChE; EC 3.1.1.7) (Arendt et al. 1988; Miller e Rieck 1993; Floyd et al. 1997). The cholinergic system has been suggested as an important neuroimmune interaction mediator, and this system mechanism has been elucidated (Nizri et al., 2006). Moreover, deregulation of the cholinergic system by oxidative stress is linked to cognitive and memory impairment with ethanol consumption (Tiwari et al. 2012; Tiwari and Chopra 2013).

The zebrafish is a small, freshwater fish commonly used as an experimental model in several scientific fields. In neuroscience, use of both adult and larval zebrafish has grown significantly in the last few decades because this vertebrate species has high physiological and genetic homology to humans, it is easy to perform genetic manipulation, and this species has similar CNS morphology to that of humans (Stern and Zon 2003; Kalueff et al. 2014).

This species absorbs components directly from the water by its gills, storing them in different tissues, including the CNS (Froehlicher et al. 2000; Yang et al. 2009). Several neurotransmission systems widely studied in mammals have been identified and described in zebrafish, such as the dopaminergic (Boehmler et al. 2004), GABAergic (Kim et al. 2004), glutamatergic (Edwards and Michel 2002), serotoninergic (Rink and Guo 2004), and purinergic (Rico et al. 2003; Senger et al. 2004; Rosemberg et al. 2010; Vuaden et al. 2016) systems. Regarding the cholinergic system, studies have already sequenced and identified these system parameters in the zebrafish brain (Arenzana et al. 2005), and AChE and ChAT enzymes have been evaluated in this animal model (Bertrand et al. 2001; Mueller et al. 2004). Zebrafish mutants for the AChE gene (*ache*^{sb55}/+) have been used to investigate mechanisms related to amphetamine addiction (Ninkovic et al. 2006).

In our lab, we determined that ethanol can modify AChE activity and expression patterns in the zebrafish brain (Rico et al. 2007). In addition to an experimental model for neurobiological processes, zebrafish also represent an important system for modeling human inflammatory diseases. In relation to the neuroinflammatory mechanisms, the zebrafish brain has a microglial cellular network formed by 25–30 cells participating in this process (Sierger and Peri 2013). Some studies have already identified cytokines and transcription factors known to humans in the zebrafish CNS (Zhang et al. 2005; Fénero et al. 2016). Moreover, several studies have shown that zebrafish have antioxidant defenses similar to those of mammals, suggesting similar cellular responses to oxidative stress among species (Nunes et al. 2016; Mohanty et al. 2017).

In this study, we assessed the cholinergic system by analyzing ChAT and AChE activity in the zebrafish brain after long-term ethanol exposure, and we investigated the parameters related to oxidative stress and the related gene expression patterns of cytokines. Considering that (i) prolonged ethanol consumption promoted neurological dysfunction, (ii) the cholinergic system, oxidative balance, and neuroinflammation are associated with neurological disorders promoted by ethanol abuse, and that (iii) the zebrafish has become a prominent vertebrate to study neurological disorders related to human diseases, we aimed to test the cholinergic system through ChAT and AChE activity in the zebrafish brain after long-term ethanol exposure and to investigate the parameters related to oxidative stress and the related gene expression patterns of cytokines.

Materials and Methods

Reagents

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

Animals

Adult zebrafish were obtained from the Federal University of Rio Grande do Sul (UFRGS). All fish were acclimated to their new environment for at least 2 weeks in a 50-L aquarium conditioned at 25 ± 2 °C under a natural light-dark photoperiod. The fish were used according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and were healthy and free of any signs of disease. The Ethics Committee of the University

of Southern Santa Catarina (UNESC) approved the protocol under the number 051/2017-1.

Ethanol Exposure and Sample Preparation

For chronic treatment, fish were introduced to the test aquariums (10 L each) containing an ethanol solution at 0.5% (*v*/*v*) and were maintained in the test aquarium for 7, 14, and 28 days. To ensure the aquarium ethanol concentration, the solution was replaced every 2 days, according to the infrared ethanol analysis conducted by Rico et al. (2011). Immediately after the exposure, the fish were euthanized, and the brains were removed, cleaned, and maintained at − 80 °C until the analyses were performed. Six independent experiments ($n = 6$) were performed using biological preparations from a pool of five animals for each analysis (30 animals per group per procedure). Considering that three different techniques (biochemical, molecular, and immunolabeling) were employed, 90 animals were used in this study.

Cholinergic System Evaluation

Determination of ChAT Activity

ChAT activity was determined according to Chao and Wolfgram (1973). The homogenate fraction was preincubated for 10 min at 37 °C in the reaction solution containing 0.5 M sodium phosphate buffer (pH 7.2), 6.2 mM acetyl-CoA, 1 M choline chloride, 0.76 mM neostigmine sulfate, 76 mM sodium chloride, 3 M sodium chloride, and 1.1 mM ethylenediaminetetraacetic acid (EDTA). Next, 1 mM 4,4′-dithiodipyridine (4-PDS) was added, and the absorbance was measured at 324 nm for 20 min. Activity was measured by the formation of the 4-thiopyridone (4-TP) conjugate, a product resulting from the binding of CoA to 4-PDS. The results were calculated using the molar extinction coefficient of 4-TP, $1.98 \times 10^4\ M^{-1}\ cm^{-1}$, and are expressed as nanomoles per minute per milligram of protein.

Determination of AChE Activity

The brains were homogenized on ice in 500 μL of Tris-citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA [pH 7.4], with citric acid) in a motor-driven Teflon-glass homogenizer. The rate of hydrolysis of acetylthiocholine (AcSCh, 0.8 mM) in 2-mL assay solutions with 100 mM phosphate buffer (pH 7.5) and 1.0 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) was determined as described previously (Ellman et al. 1961). Before the addition of substrate, samples containing protein (10 μg) and the reaction medium mentioned above were preincubated for 10 min at 25 °C. Hydrolysis of the substrate was monitored by the formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s). Control reactions without the homogenate preparation were performed to determine the non-enzymatic hydrolysis of AcSCh. The linearity of absorbance over time and protein concentration was previously determined. All reactions were performed in duplicate, and AChE activity is expressed as micromoles of thiocholine (SCh) released per hour per milligram of protein.

Western Blot Analysis

To perform the immunoblot experiments, the samples were first homogenized in buffer (1% Triton-X 100, 100 mM Tris [pH 7.4], 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF, and 0.1 mg aprotinin/mL). Equal amounts of protein (25 μg/well) were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. The protein loading and electroblotting efficiency were verified with Ponceau S staining. The membranes were blocked in Tween Tris-buffered saline (TTBS: 100 mM Tris-HCl [pH 7.5], 0.9% NaCl, and 0.1% Tween-20) containing 5% skim milk. The membranes were incubated overnight at 4 °C with an antibody against ChAT (AS-55681, AnaSpec, Fremont, CA) at a 1:1000 dilution. The primary antibody was then removed, and the membranes were washed four times for 15 min. After washing, an anti-rabbit peroxidase-linked secondary antibody was incubated with the membranes for 2 h (1:2500 dilution) and the membranes were washed again. Finally, the immunoreactivity was detected using an enhanced chemiluminescence (ECL Plus kit, Amersham Life Science). After exposure, the membranes were stripped and incubated with a mouse monoclonal antibody to β-actin (Sigma, A2228) in the presence of 5% milk. An anti-mouse IgG peroxidase-linked secondary antibody was incubated with the membranes for 1 h (1:2500 dilution), and the membranes were washed again. Densitometry was performed using ImageJ v.1.34 software. Amersham ECL Full-Range Rainbow Molecular Weight Marker (GE Healthcare Life Sciences, UK) was used as a molecular weight marker to make sure that the correct bands were analyzed for ChAT and β-actin.

Acetylcholine Levels

Acetylcholine levels were determined using an Acetylcholine Assay Kit (Cell Biolabs, STA-603) according to the specifications of the manufacturer. Data are expressed as micromolars per milligram of protein.

Evaluation of Oxidative Stress Parameters

Tissue Preparation

Tissues were homogenized in 1 mL of 20 mM sodium phosphate buffer (pH 7.4) containing 140 mM KCl. Homogenates

were centrifuged at 750×*g* for 10 min at 4 °C to discard nuclei and cell debris (Evelson et al. 2001). The pellet was discarded, and the supernatant was collected and used for the evaluation of oxidative stress parameters.

Sulfhydryl (Thiol) Group Oxidation

This assay is based on the reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm. The protein-bound sulfhydryl content is inversely correlated to the oxidative damage of proteins. The results are reported as nanomoles of per milligram of protein (Aksenov and Markesbery 2001).

TBA-RS Levels

Thiobarbituric acid-reactive species (TBA-RS) levels, a parameter of lipid peroxidation, were determined according to Esterbauer and Cheeseman (1990). A calibration curve was established using 1,1,3,3-tetramethoxypropane, and the standard used to create each curve point was subjected to the same treatment as the supernatants. TBA-RS values are presented as nanomoles of TBA-RS per milligram of protein.

DCFH Oxidation

Reactive species production was assessed according to Lebel et al. (1992), using 2′,7′-dihydrodichlorofluorescein diacetate. The dichlorofluorescein (DCF) fluorescence intensity parallels the amount of reactive species formed. A calibration curve was generated with standard DCF (0.25–10 μM), and the levels of reactive species were calculated as picomoles of DCF formed per milligram of protein.

Nitrate and Nitrite Determination

Nitrate and nitrite levels were determined according to Miranda et al. (2001), using Griess reagent (2% sulfanilamide in 5% HCl and 0.1% N-1-[naphthyl]ethylenediamine in H_2O). A calibration curve was established using sodium nitrate, and the standard used to create each curve point was subjected to the same treatment as the supernatants. The concentrations were calculated as micromoles per milligram of protein.

Catalase Activity

The catalase (EC 1.11.1.6) activity assay was performed according to Aebi (1984), by measuring the decrease in absorbance at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, and 10 mM potassium phosphate buffer (pH 7.0), and the supernatants contained 0.1–0.3 mg of protein mL^{-1}. The specific activity is expressed as nanomoles per minute per milligram of protein.

SOD Activity

Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined according to Bannister and Calabrese (1987) using a spectrophotometric assay based on the superoxide-dependent oxidation of epinephrine to adrenochrome at 32 °C. Absorbance was measured at 480 nm. The reaction medium consisted of 50 mM glycine buffer (pH 10.2), 0.1 mM catalase, and 1 mM epinephrine. SOD specific activity is represented as nanomoles per minute per milligram of protein.

Quantitative Real-Time PCR

Total RNA was extracted from zebrafish brains using the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instructions. Two micrograms of RNA was reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, USA). Gene expression was measured using TaqMan assays (Life Technologies, USA) for genes involved in inflammation (Table 1). Differences in gene expression were calculated using ef-1α as the internal control (Baldo et al. 2011).

Protein Determination

Total protein quantification in the samples was performed using the method by Lowry et al. (1951) for oxidative stress parameters, and the other analysis was performed using the method by Bradford (1976), with bovine serum albumin used as the standard for both methods.

Results

In this study, we assessed the effects of chronic ethanol exposure on cholinergic neurotransmission, oxidative stress, and inflammatory parameters in the zebrafish brain. First, we evaluated the alcohol in vivo effect on ACh levels in animals that were exposed to ethanol at a concentration of 0.5% (*v*/*v*) for 7, 14, and 28 days (Fig. 1). The brains were dissected, and ACh was quantified. There was a significant decrease in ACh levels at 7 days (46%; $p < 0.001$) and 14 days (34%; $p < 0.05$) of

Table 1 TaqMan (Life Technologies, USA) ID assays for genes analyzed in this study

Gene symbol	Assay ID
tnf-α	dr03126848
il-1b	dr03114368
il-10	dr03103209
ef-1α	dr03432748

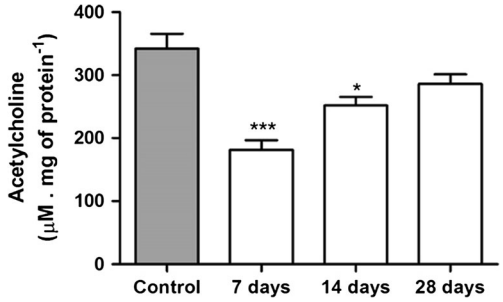

Fig. 1 Effects of chronic ethanol exposure on acetylcholine levels in the zebrafish brain. The results represent the mean ± SD (n = 6), each in duplicate. Values are expressed as micromolars per milligram of protein. *p < 0.05; ***p < 0.001 compared to the control group (one-way ANOVA followed by Tukey's post hoc test)

ethanol exposure. ACh levels did not present significant changes after the 28-day exposure.

To verify whether ChAT and AChE were altered when zebrafish were chronically exposed to ethanol, we performed enzymatic assays after treatment (7, 14, and 28 days). ChAT activity was significantly decreased in the groups exposed to ethanol for 7 days (34%; $p < 0.001$) and 14 days (54%; $p < 0.05$) when compared to the control group, whereas the 28-day exposure group did not change when compared to the control group (Fig. 2a). There was no significant difference in AChE activity between the groups (Fig. 2b).

The ChAT activity decrease promoted by ethanol exposure could be a consequence of transcriptional control and/or post-translational regulation. The brain immunocontent for this protein was evaluated in zebrafish chronically exposed to ethanol (Fig. 3). Our results show that different periods of chronic ethanol exposure did not alter ChAT immunocontent.

Ethanol metabolism to acetaldehyde and then to acetate is associated with reactive oxygen species production that accentuates the cellular oxidative state. Therefore, we assessed the oxidative stress involvement in chronic ethanol exposure in the zebrafish brain. The sulfhydryl group content (a marker of protein oxidative damage) was evaluated. The results showed that there was no significant difference between the groups (Fig. 4a). Levels of TBA-RS, a marker of lipid peroxidation, were evaluated, and a significant increase was observed after 7 days (301%; $p < 0.001$) and 14 days (245%; $p < 0.001$; Fig. 4b).

To identify a cause of the observed lipid peroxidation, we studied the effect of chronic ethanol exposure on reactive species production in zebrafish. Nitrate and nitrite levels were quantified (Fig. 5a), and DCFH oxidation was evaluated (Fig. 5b). Only in the 7-day ethanol exposure group (137%; $p < 0.05$) was there an observed increase in DCFH oxidation when compared to the control group. On the other hand, there was no significant difference in nitrate or nitrite levels after the different chronic ethanol exposure periods.

SOD and CAT play important roles in regulating reactive oxygen species levels and preventing oxidative damage. First, chronic ethanol exposure presented a significant decrease in SOD activity (30, 18, and 13% for 7, 14, and 28 days, respectively; $p < 0.001$; Fig. 6a). The effect of ethanol on CAT activity was also evaluated, and no difference was observed between groups (Fig. 6b). As described in Fig. 6c, an ethanol-induced increase was observed in the CAT/SOD ratio for 7 days (433%; $p < 0.05$), 14 days (501%; $p < 0.05$), and 28 days (802%; $p < 0.001$) of ethanol exposure.

Considering the presence of oxidative stress activity, we verified whether ethanol could alter the gene expression of inflammatory cytokines in the zebrafish brain when chronically exposed to ethanol. No differences were found between the ethanol-exposed groups and the control group (Fig. 7).

Discussion

Alcohol abuse is an important public health problem, especially due to the severe damage caused by chronic exposure

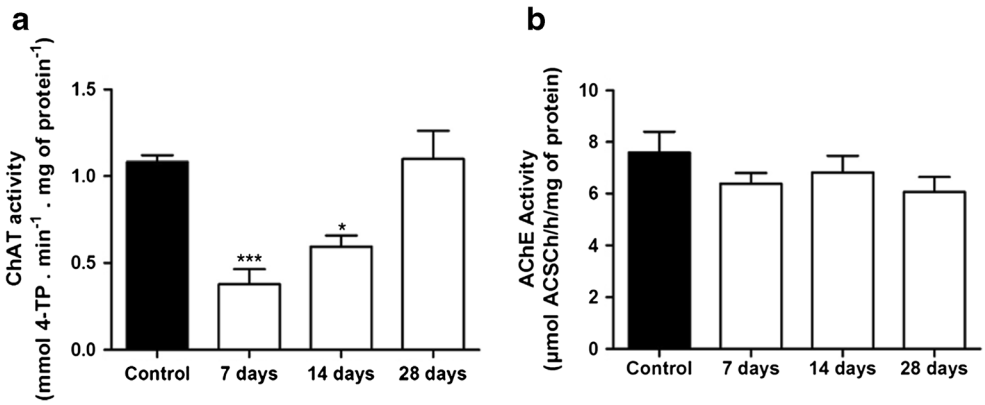

Fig. 2 Effect of chronic ethanol exposure on choline acetyltransferase (ChAT) activity (**a**) and acetylcholinesterase (AChE) activity (**b**) in the zebrafish brain. The results represent the mean ± SD (n = 6), each in duplicate. The enzymatic activities values are expressed in nanomoles of 4-TP per minute per milligram of protein and micromoles of ACSC per hour per milligram of protein, respectively. *p < 0.05; ***p < 0.001 compared to the control group (one-way ANOVA followed by Tukey's post hoc test)

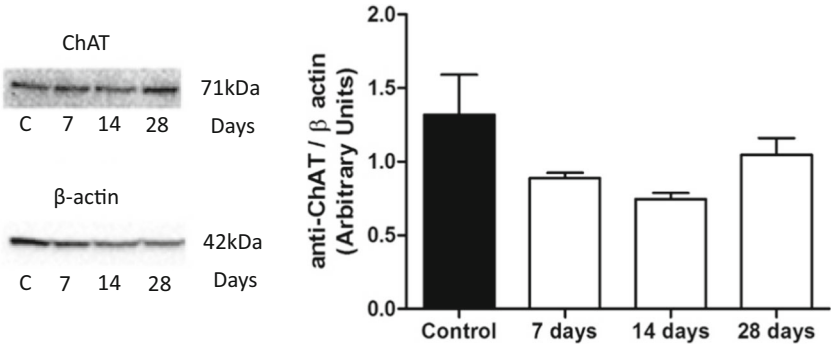

Fig. 3 Effect of chronic ethanol exposure on choline acetyltransferase (ChAT) immunocontent in the zebrafish brain. The results represent the mean ± SD (n = 6), each in duplicate. The values are expressed in an arbitrary unit/*β actin*. There was no significant difference when compared to the control group ($p > 0.05$; one-way ANOVA followed by Tukey's post hoc test)

that affects many physiological and human behavioral processes, such as memory, motor function, and cognitive abilities. Many of these consequences are related to alcohol metabolism and its oxidation in the brain leading to neurochemical modifications, which can induce neurotoxicity and neurodegeneration. There is evidence for the effects of ethanol on the CNS, including not only the GABAergic and glutamatergic receptors but also other receptors and neurotransmitters, such as the cholinergic system (Banerjee 2014). The zebrafish is an important model not only in neuroscience but also in chronic alcohol consumption-related studies. The levels of dopamine, serotonin, glutamate, GABA, aspartate, glycine, taurine, and purine metabolism can be altered in the zebrafish brain after chronic exposure to ethanol (Chatterjee et al. 2014; Rico et al. 2011). This is the first study showing the effects of chronic alcohol exposure on parameters related to the cholinergic system in a long-term alcohol exposure model using this species.

The cholinergic system is involved in cognition, emotion, and brain electrical activity modulation (Giovannini et al. 2015; Graef et al. 2011). Homeostasis variations in this system can induce responses such as dementia and cognitive and behavioral changes as well as chemical dependence (Talesa 2001; Gawel et al. 2016). Studies have shown that chronic ethanol consumption can lead to cognitive impairments, such as learning and memory problems, and alterations in the cholinergic system can be associated with these damages (Tiwari and Chopra 2013). In the present study, we evaluated ACh levels in zebrafish brain after long-term ethanol exposure. A decrease in ACh levels was observed after the 7- and 14-day exposure when compared to non-exposed animals, but ethanol did not alter ACh levels after the 28-day exposure. Studies have related ethanol neurotoxicity to the loss of cholinergic neurons, leading to cholinergic system damages, including enzymatic functions (Boutros et al. 2014; Vetreno et al. 2014). Based on the evidence that this chronic ethanol consumption period altered ACh levels in the zebrafish brain, two important markers, ChAT and AChE, were evaluated to better understand the alterations in the cholinergic system. ChAT is an enzyme responsible for the ACh synthesis in presynaptic neurons (Dobransky and Rylett 2005; Kumar et al. 2016), whereas AChE is an enzyme responsible for the ACh synthesis, inactivation, and regulation in the synaptic cleft (Soreq and Seidman 2001). Similar to ACh levels, ChAT activity decreased after the 7- and 14-day ethanol exposure but not after 28 days. Our results suggest that the decrease in ACh neurotransmitter levels in zebrafish chronically exposed to ethanol may be associated with the decrease in ChAT enzyme activity, which is responsible for ACh synthesis from acetyl-coenzyme A and choline in presynaptic neurons. Furthermore,

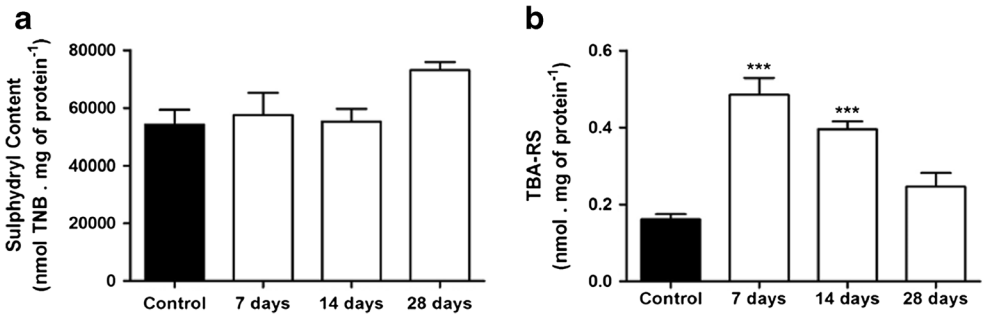

Fig. 4 Effect of chronic ethanol exposure on the content of sulfhydryl groups (**a**) and thiobarbituric acid (TBA-RS) levels (**b**) on the zebrafish brain. The results represent the mean ± SD (n = 6), each in duplicate. The values are expressed in nanomoles of TNB per milligram of protein and nanomoles per milligram of protein, respectively. ***$p < 0.001$ compared to the control group (one-way ANOVA followed by Tukey's post hoc test)

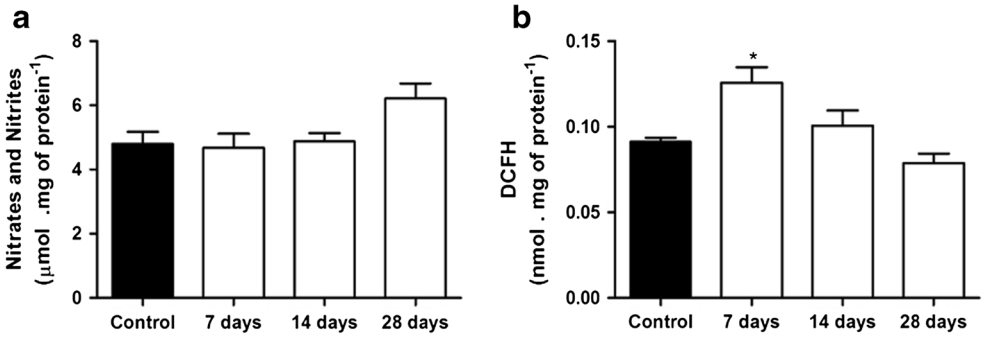

Fig. 5 Effect of chronic ethanol exposure on DCFH oxidation (**a**) and nitrate and nitrite levels (**b**) in the zebrafish brain. The results represent the mean ± SD (n = 6), each in duplicate. The values are expressed in nanomoles per milligram of protein and micromoles per milligram of protein, respectively. *p < 0.05 compared to the control group (one-way ANOVA followed by Tukey's post hoc test)

Floyd et al. (1997) demonstrated a decrease in ACh levels and ChAT activity in several brain structures in animals chronically treated with ethanol.

Ethanol and acetaldehyde play an important role by mediating the behavioral, neuropharmacological, neurotoxic, and other CNS effects of ethanol, either directly or by altering biogenic aldehyde metabolism (Quertemont et al. 2005). Acetaldehyde is a highly reactive molecule that is responsible for some deleterious effects of ethanol, such as perturbation in neurotransmission, including the cholinergic system (Jamal et al. 2007). Acetaldehyde showed remarkable reductions of neuroactive amino acid content and ChAT activity in cerebral cortical neurons in primary cultures (Kuriyama et al. 1987), suggesting that changes in acetylcholine synthesis promoted by ethanol could be due not only to its direct action but also to its indirect action via acetaldehyde.

To verify the inhibition of ChAT activity in zebrafish, western blotting assessed ChAT immunocontent. The results demonstrated that ChAT expression was not susceptible to the chronic ethanol effect, suggesting that the observed increase in ChAT activity is not directly related to ChAT expression. Neuronal alcohol responses can involve the activation of several signal transduction pathways mediated by hormones and neurotransmitters, which in the short and long term may influence post-translational protein events (Mailliard and Diamond 2004; Krishna et al. 2006). Post-translational modifications at protein kinase phosphorylation sites may influence ChAT activity, as already demonstrated (Dobransky and Rylett 2005). The observed decrease in ChAT activity after chronic ethanol exposure can also be associated with possible changes in phosphorylation sites as consequences of post-translational changes.

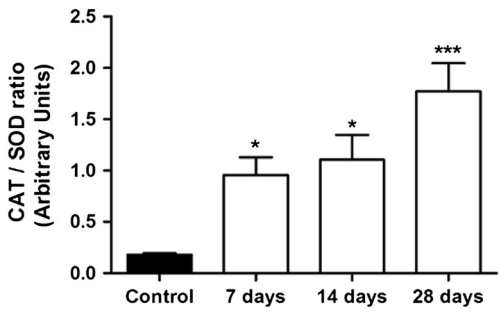

Fig. 6 Effect of chronic ethanol exposure on the ratio of CAT to SOD activities in the zebrafish brain. The results represent the mean ± SD (n = 6), each in duplicate. *p < 0.05; ***p < 0.001 compared to the control group (one-way ANOVA followed by Tukey's post hoc test)

Another cholinergic activity indicator, AChE, which is responsible for ACh inactivation and regulation in the synaptic cleft, was assessed in zebrafish chronically exposed to ethanol. The results showed that long-term ethanol exposure did not modify AChE activity in the zebrafish brain. A previous study demonstrated a significant increase in AChE activity in different brain regions of rats chronically treated with ethanol (Tiwari and Chopra 2013). In our group, we showed that short-term ethanol treatment increased AChE activity in the zebrafish brain (Rico et al. 2007). Considering that the cholinergic system was influenced by long-term ethanol exposure, it is possible that the mechanism regulating acetylcholine synthesis was susceptible to ethanol in the zebrafish brain.

Among the neurochemical and pharmacological effects promoted by consumption of alcohol and by its metabolites, other products have a role in ethanol cytotoxicity, inducing neuronal degeneration (Takeuchi and Saito 2005). Oxidative stress, which is caused by excessive reactive oxygen species production, has been proposed as a potential mechanism for ethanol-induced neuronal damage (Antonio and Druse 2008; Heaton et al. 2006). Here, we demonstrated the inhibitory effect of ethanol on ChAT activity in the zebrafish brain after 7 and 14 days but not 28 days. In addition, oxidation of DCF and TBA-RS content showed a similar profile in which the zebrafish with 28 days of ethanol exposure were more comparable to non-exposed animals. These results suggest that the profile of ethanol effects on ChAT activity could be related to the formation of reactive oxygen species.

The sensitivity of chronic ethanol administration leads to adaptive changes in the CNS that manifest as tolerance and

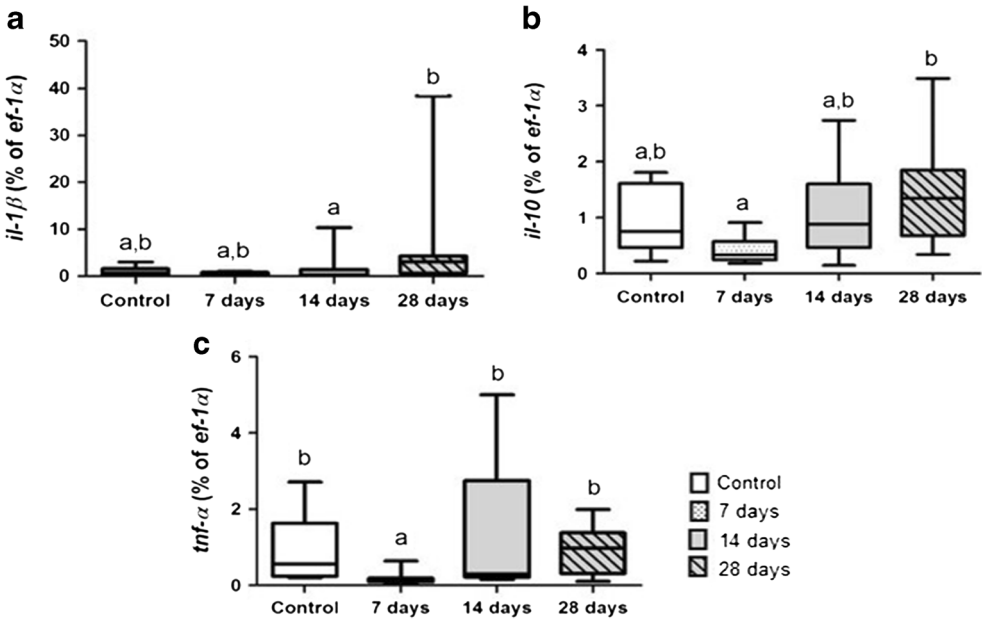

Fig. 7 Effect of chronic ethanol on gene expression of the inflammatory cytokines *il-1b* (**a**), *il-10* (**b**), and *tnf-α* (**c**) in the zebrafish brain. Variables were analyzed using the Kruskal-Wallis test followed by Dunn's post hoc test and are expressed in percentage of *ef-1α*

physical dependence. Recently, studies have suggested that the ethanol-induced changes in the cholinergic system are transient and that ethanol exposure chronicity may lead to neuroadaptations (Ehrlich et al. 2012; Pereira et al. 2014). Studies have shown that long-term ethanol exposure leads to behavioral adaptations in the zebrafish model (Gerlai et al. 2006; Damodarn et al. 2006; Dlugos et al. 2011). Ethanol-induced adaptations to chronic treatment have also been demonstrated at the neurochemical level, including the dopaminergic, serotoninergic (Gerlai et al. 2009), and purinergic systems (Rico et al. 2011). These alterations in ACh synthesis and in radical formation could be important for explaining the functional actions of ethanol and its tolerance over time on cholinergic neurotransmission and reactive oxygen species regulation in zebrafish brain.

Studies have shown that alcohol administration induces SOD activity inhibition in the rat brain. Intraperitoneal ethanol injections led to a progressively decreased activity, reaching a plateau after a 6-week treatment (Ledig et al. 1981), and the direct exposure to ethanol reduced SOD activity in cerebellar granule cells (Siler-Marsiglio et al., 2007). Antioxidant enzymes play an important role in cellular ROS elimination. In this study, we found that the CAT/SOD ratio was altered after chronic ethanol exposure, suggesting that ethanol can induce oxidative damage in the zebrafish brain, and the resulting increase in ROS is observed as an increase in DCF levels. This reduction in antioxidant defense promoted by SOD could be associated with reactive oxygen species production and possibly with the toxic effects of ethanol. Another explanation would be that the observed SOD decrease could be associated with pro-oxidant ethanol effects.

Oxidative stress and chronic alcohol consumption may be associated with activation of the inflammatory response (Qin et al. 2008; Cross et al. 2000). Tiwari and collaborators (2009) observed a significant increase in TNFα and IL-1β levels in the cerebral cortex and hippocampus of ethanol-treated rats, indicating important neuroinflammation in the two main brain regions involved in memory and learning. In our study, we did not observe a significant difference in the gene expression pattern of inflammatory cytokines between ethanol-treated and control groups. This finding suggests that ethanol is not directly influencing the transcriptional machinery of these genes in brain tissue. However, we cannot exclude the possibility that other inflammatory events are related to long-term ethanol consumption. Thus, further studies are still needed to elucidate other events such as microglia or systemic inflammatory processes.

The present study confirms our hypothesis that chronic ethanol exposure can promote neural cholinergic system dysfunction in the short term when compared to long-term exposure in zebrafish. This simple vertebrate presents high enough psychopharmacological similarities to rodents and humans and allows a comparative approach to identify evolutionarily conserved mechanisms (Gerlai et al. 2000; Kily et al. 2008; Rico et al. 2011b). These results may contribute to understanding the pathological, neurochemical, and cognitive impairments observed in chronic ethanol consumption, including sensitization and tolerance.

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Authors' Contributions Rico had full access to all study data and takes responsibility for the data integrity and analysis accuracy.

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Data analysis and interpretation: Agostini, Cruz, Longo, Machado, and Rico.

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Compliance with Ethical Standards

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

Ethics Statement The Ethics Committee of the University of Southern Santa Catarina (UNESC) has approved the present study.

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