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Low doses of methylmercury intoxication solely or associated to ethanol binge drinking induce psychiatric-like disorders in adolescent female rats

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ABSTRACT

Methylmercury (MeHg) is an environmental contaminant that provokes damage to developing brain. Simultaneously, the consumption of ethanol among adolescents has increased. Evidence concerning the effects of MeHg low doses *per se* or associated with ethanol during adolescence are scarce. Thus, we investigate behavioral disorders resulted from exposure to MeHg low doses and co-intoxicated with ethanol in adolescent rats. Wistar rats received chronic exposure to low doses of MeHg (40 μg/kg/day for 5 weeks) and/or ethanol binge drinking (3 g/kg/day at 3 days per week for 5 weeks). Animals were submitted to behavioral assays to assess emotionality and cognitive function. Total mercury content was evaluated in the brain and hair. Oxidative parameters were analyzed in blood samples. MeHg at low doses or associated to ethanol binge drinking produced psychiatric-like disorders and cognitive impairment. Peripherally, MeHg altered oxidative parameters when associated to ethanol. Ethanol administration reduced brain mercury deposit. We proposed that ethanol reduces the necessity of mercury tissue levels to display psychiatric-like disorders/cognitive impairment.

1. Introduction

Methylmercury (MeHg) is an environmental pollutant produced by industrial and mining activity, as well as by deforestation and is recognized as a neurotoxic compound for both the immature and the mature central nervous system (CNS) (Grandjean, 2007; Xu et al., 2012). MeHg consists of the most toxic form of mercury, which can accumulate in food-chain marine organisms that undergo bioaccumulation and biomagnification (Kraepiel et al., 2003). The oral pathway is the principal form of MeHg exposure (Ceccatelli et al., 2010; Višnjevec et al., 2014). For this reason, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) recommended a provisional tolerable weekly intake (PTWI) of 1.6 μgMeHg/kg body weight per week for adults (JECFA, 2006). In European countries, the maximum levels of total mercury in foodstuffs range from 0.5 to 1 mg/kg (Commission Regulation-EC No. 1881/2006). However, the intake over the PTWI concentrations of MeHg in the diet by fish-dependent populations appears to be common, with values found in hair samples greater than 40 ppm (Kong et al., 2013), including Tapajós river inhabitants (in Amazonia), who have blood- and hair-mercury levels around 60 mg/L and 18 mg/g, respectively (Passos et al., 2008). Several research studies have linked chronic exposure to low doses (CELDS) of MeHg in children with neurobehavioral deficits and higher prevalence of psychiatric disorders, including behavioral disturbances, mood alterations, and impaired intelligence in adulthood (Debes et al., 2006; Grandjean et al.,2014; Yorifuji et al., 2011). Besides, oxidative stress is one of the main toxic mechanism displayed by MeHg intoxication (Farina et al., 2011). Therefore, the impact of CELD of MeHg on adolescence is unclear, since it seems this group presents higher vulnerability compared to adults (WHO, 2008). In addition to MeHg neurotoxic effects, the occurrence of alcohol consumption in mercury contaminated areas (*i.e.*, mining regions) has been considerably high (Corbett et al., 2007), but the consequences of this comorbidity are unknown.

Ethanol acts on the CNS, which can cause dependence and brain

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disorders. In 2012, ethanol intake was responsible for 3.3 million deaths (5.9% of annual deaths) (WHO, 2014). The pattern of ethanol intake in a single episode, also known as binge drinking, leads to acute poisoning and has been associated with increased individual risk (Berridge et al., 2009; Silveira et al., 2008). According to the Second National Alcohol and Drugs Survey, binge drinking has increased among females in Brazil, especially adolescents (INPAD,2013). Alcohol abuse during adolescence plays an important role as a risk factor in psychosocial development (Skala and Walter, 2013). Moreover, the adolescent period presents a high risk for modifications in cerebral structures and neurodegeneration induced by alcohol misuse (Crews and Vetreno, 2014).

Actually, the two toxicants cause neurodegeneration by similar mechanisms, by enhancing lipid peroxidation and inducing phospholipase A2 (Farina et al., 2011; Moon et al., 2014; Tajuddin et al., 2014); glutamate-mediated excitotoxicity (Cippitelli et al., 2010; Deng et al., 2014); as well as mitochondrial disruption (Sokolowski et al., 2011; Glaser et al., 2014; Jung, 2014). Considering that MeHg and ethanol consist of neurotoxic compounds with well-established neurodegeneration effects (Maia et al., 2009; Maia et al., 2010a,b), we wondered whether CELDS of MeHg *per se* displays neurobehavioral disorders, related to psychiatric or neurological disturbance in adolescent female rats. Indeed, we hypothesize that MeHg intoxication associated to ethanol intake may intensify MeHg toxicological effects.

2. Material and methods

2.1. Ethical standards

All procedures followed the guidelines recommended by the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and were approved by the Ethics Committee on Experimental Animals of the Federal University of Pará under license number BIO 209-14.

2.2. Animals

Wistar (*Rattus novergicus*) adolescent female rats 40 days old (n = 52) were obtained from the animal facility of the Biological Science Institute/Federal University of Pará (UFPA) and kept in collective cages (five animals/cage) under controlled conditions with a light-dark cycle of 12 h (7 a.m. lights on), temperature, water and food *ad libitum*. Animals were divided into five experimental groups: basal (no treatment or behavioral tests, n = 5), control (water + water, n = 10), MeHg (MeHg 40 μg/kg/day + water, n = 12), ethanol (water + ethanol 3 g/kg/day, n = 12) and MeHg + ethanol (MeHg 40 μg/kg/day + ethanol 3 g/kg/day, n = 13) by oral route. Considering that the adolescence period starts from around 6 weeks old and the young adult period occurs at approximately at 63 days old (Andreollo et al., 2012; Sengupta, 2013), we elected the beginning of adolescence to initiate the toxicological protocol. The basal group served as baseline for Wistar rats for mercury total contents and oxidative stress assays. Twenty-four hours after last day of MeHg and/or ethanol treatment, the animals were submitted to behavioral assays.

2.3. Treatment

2.3.1. Methylmercury treatment

Animals received MeHg (Sigma-Aldrich, Germany) by oral gavage, at a dose of 40 μg/kg/day for 5 weeks (Kong et al., 2013). According to the study of Day et al. (2005), this sub-millimolar dose may not cause detectable neurological impairment. Thus, we used the present protocol to mimic the effects known as CELDs of MeHg in the adolescence period. Therefore, the dose employed reflects human exposure. Control animals received distilled water by gavage at the same volume as the MeHg dose.

2.3.2. Ethanol treatment

Animals received ethanol solution (20% w/v, NUCLEAR, Brazil) by oral gavage, at a dose of 3 g/kg/day, 3 days per week, with five weeks of treatment (5 cycles of binge drinking). This dose was based on data from the National Institute of Science and Technology for Public Policy on Alcohol and Other Drugs (INPAD, 2013) and NIAAA et al., 2004, which demonstrated an increase of alcohol intake (females), and the pattern of ethanol consumption prevalent among adolescents (binge drinking which consists of 4 doses/2 h, frequency of three consecutive days per week), respectively. This ethanol design employed by our group reached a blood alcohol concentration of BAC of 197.4 ± 19.46 (Fagundes et al., 2016). Control animals received distilled water at the same volume and frequency as ethanol administration.

2.4. Behavioral assays

2.4.1. Open field test

Locomotor activity and anxiety-like behavior were measuring with the open field test (OF), according to Walsh and Cummins (1976) and Pandolfo et al. (2007) protocols. The OF consists of a wooden apparatus (100 × 100 X 40 cm), divided into 25 quadrants and two zones (central *versus* peripheral), in which it is possible to evaluate locomotor activity as well as anxiety-like behavior. Briefly, animals were placed at the center of the apparatus and exploratory behavior was permitted for 5 min. The test was videotaped and analyzed by Any-Maze software (Stoelting Co., USA). The parameters measured were total number of crossed lines, number of entries in the central area and latency to start movement.

2.4.2. Elevated plus maze

Elevated plus-maze (EPM) is an anxiety test based on unconditional answers of rodents (Handley and Mithani, 1984). The wooden apparatus consists of two open plus two enclosed arms. Animals were placed at the center of the apparatus facing the enclosed arm and locomotion was permitted for 5 min. The test was videotaped and analyzed by Any-Maze software (Stoelting Co., USA). The parameters measured were frequency of open arm entries (OAE) and frequency of enclosed arm entries (EAE). The %OAE was calculated according to the formula open/totalx100 entries. Anxiety-like behavior was considered as reduction of %OAE (Hogg, 1996; Pellow and File, 1986). The risk assessment parameter was measured as attempts to enter into open or enclosed arms, with avoidance behavior (stretched attend posture and head-dipping) during the test session (Griebel et al., 1997).

2.4.3. Splash test

The splash test (ST) consists of an anhedonia-like behavior model, which reflects a motivational behavior considered parallel to some depression symptoms such as apathy (Willner, 2005). Animals were placed on the plexiglass individual box (9 × 7 × 11 cm) and sucrose solution (10% w/v) was applied on the dorsal region hair (Freitas et al., 2013; Isingrini et al., 2010). The time spent in grooming behavior over 5 min was recorded.

2.4.4. Forced swim test

The forced swim test (FST) is a depression-like behavior model for rodents (Porsolt et al., 1978). The apparatus consists of a glass cylinder (50 cm high × 30 cm diameter) containing water at 23 ± 1 °C. Animals were dropped into the apparatus with no escape possibility for 5 min. The immobility time was recorded in the last three minutes of the test. Reduction of immobility time suggests depressant-like behavior (Cryan et al., 2002; Porsolt et al.,1977).

2.4.5. Step-down inhibitory avoidance test

Animals were submitted to the inhibitory avoidance apparatus (EP 104R, Insight, Brazil), which consists of an acrylic box (20 × 7.5 × 25 cm) with a floor composed of parallel stainless-steel

bars (1 mm in diameter) separated by 1 cm from each other. In addition, a safe platform (7 × 2.5 cm) was localized against the left wall of the box. In summary, on the first day (habituation stage) each animal was placed individually on the safe platform of the apparatus and was allowed to explore the environment for 180 s. On the next day (training session), animals were placed on the secure platform and the rat step-down was measured on the grid with four paws (baseline 1-BL1) and a scrambled foot shock was applied (0.4 mA, for 1 s). Thereafter, animals were immediately removed from the apparatus and re-introduced after 1.5 h to the safe platform (test session) and step-down latency was measured (cut-off 180 s), baseline 2 (BL2), to evaluate short-term memory (STM)(Maia et al., 2009).

After behavioral tests, animal brain or blood samples were collected (N = 5 animals per group), including the basal group (N = 5). Animals were sacrificed by cervical dislocation and blood samples were collected by intraventricular puncture and stored in tubes containing ethylenediaminetetraacetic acid (EDTA). Biochemical parameters analyzed were superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) in total blood; Sulfhydryl group levels and nitrite/nitrate (NOx) were measured in plasma.

2.5. Total mercury dosage

Total mercury concentration from hair and CNS tissues (prefrontal cortex, motor cortex, and hippocampus; n = 5 per group) was determined according to Voegborlo and Akagi (2007). The procedure involved the digestion of the hair or tissue sample (10 mg) using a mixture of water (1 mL), a solution (2 mL) of nitric acid and perchloric acid (1:1), plus 5 mL of sulphuric acid added in turn and heated at a temperature of 200 °C. Distilled water was then added to the reaction product until reaching double the primary volume. The sample was analyzed by cold vapor atomic absorption spectrophotometry using an automatic mercury analyzer (HG-201, Sano Seisakusho Co. Ltd, Tokyo, Japan). The precision and accuracy of this method for hair have been verified by analysis of standard reference materials (IAEA 085). Results were expressed as part *per* billion (ppb) for hair and CNS tissues.

2.6. Oxidative stress assays

2.6.1. Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was measured according to McCord and Fridovich (1969), modified by Schalcher and collaborators (2014). The protocol is based on the conversion of O2- to hydrogen peroxide (H2O2) and O2˚ by SOD. Briefly, hemolyzed samples were added to a solution consisting of phosphate buffered saline (PBS), EDTA, cytochrome C, and xanthine oxidase enzyme. The principle of the assay relies on the prevention of cytochrome C reduction by SOD, which may be measured at a wavelength of 550 nm by ultraviolet spectrophotometry at time 0 (before adding xanthine oxidase) and 5 min after adding xanthine oxidase. Values were calculated in nmol/mL and results were expressed as percentage of the basal group.

2.6.2. Catalase (CAT) activity

Aebi (1984), modified by Bukowska and Kowalska (2004), developed the catalase (CAT) activity assay performed in the present study. The presence of CAT in the hemolyzed samples converts H2O2 to H2O and O2. Briefly, 50 µL of H2O2 30% (w/w) plus 2.5 mL of Tris(hydroxymethyl)aminomethane solution was added to blood samples. The rate of decay of H2O2 by CAT activity was measured by ultraviolet spectrophotometry at 240 nm for 60 s. Values were calculated as units per gram of total protein per minute (k/gProt/min) that was normalized by the total protein concentration, using a commercial kit (Doles, Brazil). Results were expressed as percentage of basal.

2.6.3. Total protein content

Total protein content was measured by the technique of modified biuret (Weichselbaum, 1946) using a commercial kit for total protein (Doles, Brazil). The analysis consisted of 2.5 mL of biuret reagent (trisodium citrate 0.114 M, sodium carbonate 0.21 M and copper sulfate 0.01 M) + 50 µL of sample or protein solution (4 g/dL). Two drops of sodium hydroxide 6 M were then added to the sample followed by spectrophotometer analysis (Model Spectra MAX 250, Molecular Devices, Union City, CA, USA) at a wavelength of 550 nm. The result was used for normalizing the results of CAT that was expressed as grams of protein (gProt).

2.6.4. Sulfhydryl group levels

Determination of sulfhydryl group levels was based on the ability of thiol to reduce 5,5-dithiobis-2-nitrobenzoic acid (DTNB) to nitrobenzoic acid (TNB), according to the methodology of Ellman (1959), modified by Schalcher et al. (2014). Briefly, hemolyzed samples (20 µL) were solubilized with distilled water plus PBS/EDTA (4 mL) and mixed by vortexing. Finally, the samples (3 mL) were analyzed with a spectrophotometer at time 0, followed by additional DTNB solution (100 µL) and read again at time 3 (after 3 min of addition). Sulfhydryl concentrations were expressed in µg/mL and as percentage of basal.

2.6.5. Determination of nitrate/nitrite (NOx)

Determination of NOx was done using the Griess method (Green et al., 1982). Briefly, samples were deproteinized by zinc sulfate (15 g/L) and centrifuged at 3000 rpm for 15 min. A sample of the supernatant was then collected (100 µL) and incubated with 100 µL of the reagent (50 µL of sulfanilamide solution 1% plus 50 µL of *N*-naphthyl-ethylenediamine solution 0.1% in H3PO4 2.5%) at room temperature for 10 min. Thereafter, the samples were measured on a spectrophotometer by absorbance at 570 nm. NOx concentration was determined using a standard curve generated using sodium nitrate (NaNO2). Results were expressed as µmol/liter (Schalcher et al., 2014).

2.6.6. Determination of lipid peroxidation

Oxidative damage can promote injury to cells, increasing the biomarker malondialdehyde (MDA), which is a product of cell death (Liu et al., 2014; Yang et al., 2015). Such method is based on a complex formation between MDA and thiobarbituric acid (TBA-MDA), according to Percario (1994). Firstly, TBA solution was prepared (Sigma Aldrich, Germany). In sequence, 1 mL of TBA solution was added to 500 µL of sample. TBA-MDA complexes were measured with a spectrophotometer at a wavelength of 535 nm. Lipid peroxidation was expressed as ng/mL of MDA.

2.7. Statistical analysis

All values are expressed as means + S.E.M. (n = 10–13 animals per group for behavioural tests and n = 4–5 per group for toxicological and biochemical analysis). Statistical comparison of body weight gain was performed using one-way analysis of variance (ANOVA) with repeated measures (days). Results from behavioral, toxicological and biochemical tests were evaluated with two-way ANOVA followed by Tukey's test as multiple post-hoc comparisons. The accepted level of significance was $P < 0.05$. Statistical analyses and graphical design were performed using the GraphPad Prism software version 6.01 (San Diego, CA, USA).

3. Results

3.1. Mercury final concentration was reduced by ethanol binge drinking in samples

The highest mercury levels were detected in hair, confirming this sample as an excellent peripheral marker of methylmercury exposure (Fig. 1). In the CNS areas, the levels of mercury concentration were dependent of brain region. In fact, mercury levels were higher in the

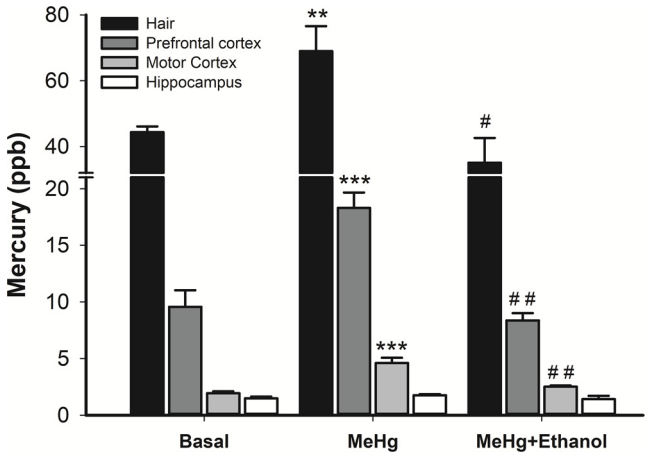

Fig. 1. Effect of chronic exposure to low doses of methylmercury (MeHg40 µg/kg/day for 5 weeks) and/or ethanol binge drinking (3 g/day at 3 days per week for 5 weeks) on the mercury concentration in the hair, pre-frontal cortex, motor cortex and hippocampus of rats. Basal group received distilled water (1 mL/kg/day). Results expressed as mean ± S.E.M. (n = 4–5). **p < 0.01*versus* Basal group; ***p < 0.001*versus* Basal group; #p < 0.05*versus*MeHg group; ##p < 0.01*versus*MeHg group. Two-way ANOVA followed by Tukey's *post hoc* test.

prefrontal and motor cortex than the hippocampus, which showed basal levels of mercury content (Fig. 1). The dosage of total mercury in hair samples showed that the ethanol administration (*e.g.* MeHg plus ethanol group) presented a reduction (approximately 42%) of mercury content compared to MeHg *per se* ($p < 0.05$; Fig. 1). Surprisingly, this result suggests an interference of ethanol in Hg accumulation, observed by a reduction of the metal deposit in the brain tissue and hair followed by ethanol administration.

3.2. Low dose of MeHg and/or ethanol binge drinking promote behavioral alterations

Body weight gain was monitored during the treatment period. Our results demonstrated that none of the groups presented a difference in this parameter through the five weeks (data in the Supplementary material). In the spontaneous motor behavior, all the intoxicated groups reduced equally the total crossed lines on the OF [Ethanol: $F_{(1,43)} = 14.29$, $P < 0.001$; Methylmercury: $F_{(1,43)} = 9.335$, $P < 0.01$; Interaction: $F_{(1,43)} = 9.431$, $P < 0.01$; Fig. 2a]. In order to quantify the spontaneous locomotor activity related to emotionality, the number of central crossed lines was measured. In this parameter, all treated groups showed a similar reduction of central ambulation [Ethanol: $F_{(1, 52)} = 6.11$, $P < 0.05$; Methylmercury: $F_{(1, 52)} = 8.596$, $P < 0.01$; Interaction: $F_{(1, 52)} = 10.25$, $P < 0.01$; Fig. 2b], which may be related to an anxiogenic-like behavior. The latency to start movement was increased among the groups that received ethanol [Ethanol: $F_{(1, 52)} = 10.81$, $P < 0.01$; Fig. 2c], which suggests motor skills damage.

Corroborating the previous results of anxiogenic-like behavior assessed on OF, the groups that received ethanol and MeHg reduced % OAE in the EPM test with no interaction [Ethanol: $F_{(1, 42)} = 10.91$, $P < 0.01$; Methylmercury: $F_{(1, 42)} = 6.057$, $P < 0.05$; Fig. 3a], which corresponds to anxiety-like behavior, with no motor effects (Fig. 3, panel b). The risk assessment parameter was reduced among animals that received MeHg *per se* independent of ethanol treatment [Methylmercury: $F_{(1, 40)} = 26.99$, $P < 0.001$; Fig. 3c], which suggests an impairment in planning skills.

In the depressive-like behavior, animals intoxicated with either ethanol or MeHg *per se*, as well the association group reduced the grooming time [Ethanol: $F_{(1, 42)} = 5.374$, $P < 0.05$; Methylmercury: $F_{(1, 42)} = 18.35$, $P < 0.001$; Interaction: $F_{(1, 42)} = 10.55$, $P < 0.01$; Fig. 4a], which is related to anhedonia-like behavior. In addition, all tested groups increased equally the immobility time on FST [Ethanol: $F_{(1, 33)} = 8.854$, $P < 0.01$; Methylmercury: $F_{(1, 33)} = 12.03$, $P < 0.01$; Fig. 4b], which is related to depressive-like behavior.

In cognitive function, baseline parameters of step-down latency were similar between the analyzed groups (baseline 1, Fig. 5a). One and half hour after electrical stimulus (baseline 2), the step-down latency was reduced in the ethanol *per se* and ethanol plus MeHg-treated groups, but not in the MeHg *per se* individuals [Ethanol: $F_{(1, 43)} = 16.47$, $P < 0.001$; Fig. 5b]. These results suggest that MeHg plus ethanol, as well as ethanol *per se* display short-term memory deficits in the step-down inhibitory avoidance test.

3.3. Low doses of MeHg causes interference on the peripherical antioxidant system without promoting oxidative stress, but not when associated to ethanol binge drinking administration

Regarding the antioxidant parameters (Fig. 6, panel a), intoxication with low doses of MeHg increased CAT activity and sulfhydryl production compared to control and basal groups, although it has been observed that CAT activity was higher than for the EtOH-treated group (Fig. 6, panel a). On the other hand, treatment with MeHg plus ethanol reduced SOD activity and did not alter thiol production compared to MeHg or EtOH-treated groups, while CAT activity of the group of MeHg plus ethanol was decreased compared to the MeHg-treated group, reaching similar values as the EtOH-treated group (Fig. 6, panel a).

For oxidizing parameters, the control group that performed behavioral tests showed increased NOx and MDA levels compared to the basal group (Fig. 6, panels b and c). However, MeHg exposure inhibited NOx production induced by behavioral stress, obtaining lower NOx levels compared to the basal group (Fig. 6, panel b). Regarding MDA, low doses of MeHg only displayed lipid peroxidation when associated to ethanol paradigm, which appears to be resulted from pro-oxidant action of ethanol (Fig. 6, panel c).

4. Discussion

In the current study, we investigated whether a low dose of MeHg, alone or in association with EtOH binge drinking, would produce behavioral alterations and peripheral oxidative stress in female rats.

To demonstrate that CELDS of MeHg was sufficient to concentrate in the body tissues, we measured total mercury dosage in hair and CNS regions of the animals. Our data show that mercury elicited tropism by cortical structures and the low affinity for central structures (*i.e.*, hippocampus). According to other studies, MeHg in the CNS has more affinity for the gray portion than the white, and displays tropism by certain neuronal groups such as cerebellum, spinal cord and midbrain (Ramírez, 2008; Kong et al., 2013). Surprisingly, ethanol intoxication reduced mercury content in the samples. Hair is a biomarker widely used to identify mercury intoxication in humans and was extrapolated for the rodent hair analyses, which showed a correlation with mercury stored in the brain (Yasutake et al., 2004; Yasutake et al., 2003). Ethanol interference in mercury body accumulation occurred in CNS tissues (prefrontal and motor cortex), as well as in hair.

A few studies have investigated the association between MeHg and ethanol. Firstly, Tamashiro et al. (1986) found that ethanol administration increased MeHg and total mercury concentration in brain, liver and kidneys. However, Tamashiro et al. (1986) study used high doses (5 mg/kg/day for 10 days), which are one hundred and twenty-five times higher than those used here. Conversely, Maia et al. (2009) showed that co-intoxication with ethanol plus a single dose of MeHg resulted in a decrease of total mercury in Wistar rat hair in a model of pregnancy exposure. The detoxification of MeHg is traditionally linked to the S-conjugation of this heavy metal with gluthathione to form MeHg-SG, which may be excreted using multidrug resistance-associated

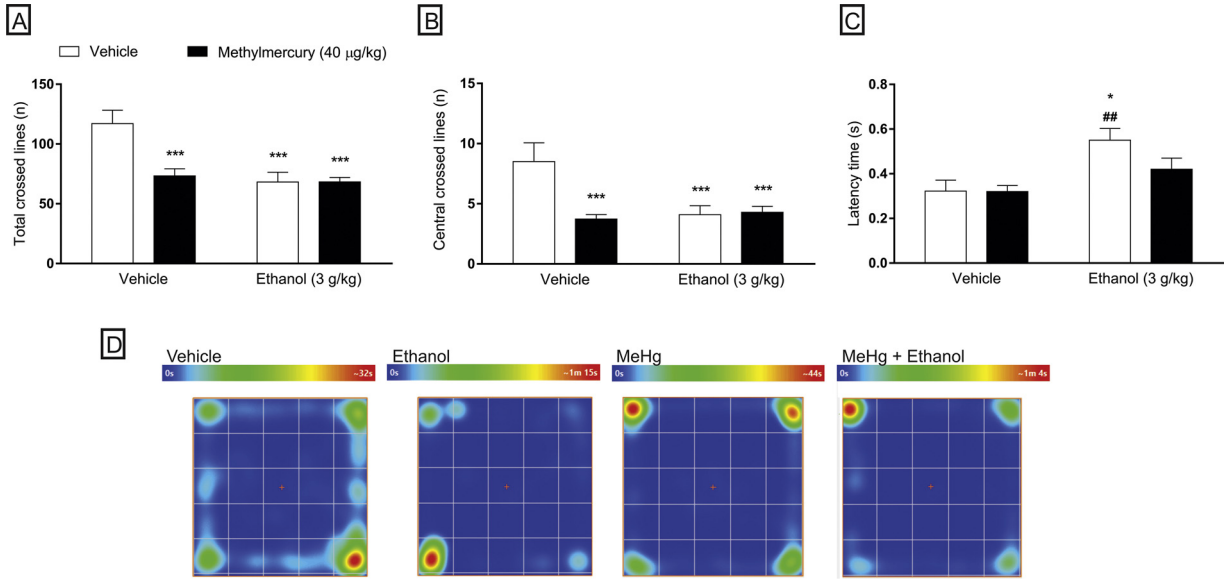

Fig. 2. Effect of chronic exposure to low doses of methylmercury (40 μg/kg/day for 5 weeks) and/or ethanol binge drinking (3 g/day at 3 days per week for 5 weeks) on the behavior of rats in open field test. Control group received distilled water (1 mL/kg/day). Panel A represents total locomotion; panel B represents central area locomotion; panel C represents the latency to start the movement; panel D represents the illustrative figures of groups locomotion. Results expressed as mean ± S.E.M. (n = 10–13). *p < 0.05*versus* control group; ***p < 0.001*versus* control group; ##p < 0.01*versus* methylmercury group. Two-way ANOVA followed by Tukey's *post hoc* test.

protein transporters. However, the content of thiol groups was similar for MeHg and/or ethanol-exposed animals, pointing to different mechanisms to explain the prevention of MeHg accumulation in ethanol-treated animals. One hypothesis includes enzymes responsible for the endogenous synthesis of hydrogen sulfide (H_2S). H_2S is a more reactive nucleophile than glutathione. Recent studies showed that ethanol induces the expression of cystathionine-γ-lyase (CSE, one of the major enzymes in H_2S synthesis), as well as cytathionine-β-synthase (CBS). In addition, the presence of these enzymes are able to significantly reduce MeHg cytotoxicity through the formation of $(MeHg)_2S$, an inactive metabolite of MeHg (Medeiros et al., 2013; Yoshida et al., 2011; Hwang, 2012), which stabilizes the MeHg, preventing that the metal does not bind to the thiol groups of protein in body (Hwang, 2012). Of interest, both CSE and CSB are expressed differently across brain regions, which may explain the different levels of Hg deposit on the analyzed areas, and consequently behavioral alterations found (Yoshida et al., 2011; Awata et al., 1995).

Previous results showed that both toxicants might cause behavioral disturbances. Zemolin et al. (2012) found reduced locomotor activity in an OF paradigm caused by CELD of MeHg for 21 days. Similarly, ethanol causes motor damage, including reduced locomotor activity (Teixeira et al., 2014). Such data reveal that both toxicants impair motor function in a *per se* manner. In addition to spontaneous locomotor activity, in the present study, ethanol exposure increased the latency to start movement, a parameter that has been related to cortical damage (*e.g.* motor cortex) promoted by ethanol consumption (Fontes-Júnior et al., 2016). In this sense, we investigated whether the exposure to CELD of MeHg plus ethanol in a binge drinking protocol could intensify ambulation impairment in the OF assay. Our results demonstrated that the association of the two toxicants might not result in a negative synergistic effect related to spontaneous locomotor activity, although the concomitant administration of MeHg plus ethanol resulted in motor impairment, as well as CELD of MeHg and ethanol *per se*.

In addition, anxiety has been reported as a consequence of mercury and ethanol exposure (Bourdineaud et al., 2011; Zhao et al., 2013). Thus, we explored the effects of the CELD of MeHg, as well as the concomitant administration with ethanol. It is well documented that anxiety consists of a multifactorial disorder, in which the environment, physiological state, CNS diseases, increased vulnerability, aging, cognitive decline, and drug misuse can produce the behavior process. In addition, studies highlight the role of several CNS structures, such as the prefrontal cortex, amygdala, norepinephrine and serotoninergic pathways, and the HPA-axis in anxiogenic conditions (Belzung et al., 2014; Etkin, 2012; Gomoll & Kumar, 2015). Our results revealed that CELD of MeHg seems to have more affinity for the prefrontal cortex than the motor cortex or hippocampus. Such increased mercury concentration in the prefrontal was related to anxiogenic-like behavior accessed on OF and EPM tests. It is noteworthy that MeHg plus ethanol intake presented anxiogenic-like effects, at the same magnitude as CELD of MeHg exposure, even with a significantly reduced concentration of mercury in the prefrontal tissue.

Other ethological measures were analyzed in the EPM test (*i.e.*, the risk assessment). Such parameters have been linked to anxiety disorders and were reduced in MeHg treated groups, *per se* or associated with ethanol intake. Maia et al. (2010a,b) have shown that risk assessment is one of the behavioral parameters that was reduced in offspring submitted to heavy chronic ethanol consumption and/or heavy acute MeHg intoxication, with the greatest values provoked in MeHg individuals.

Beyond anxiogenic behavior, depressive status was evaluated through the splash test and forced swimming test models (Porsolt et al., 1977; Willner, 2005). Our data revealed a negative consequence on depressive criteria, such as increased immobility time and decreased grooming time in all groups analyzed. In the CELD of MeHg exposure, our results reflect that chronic low doses of MeHg subject individuals to a depressive profile. Clinical work has reported depressive syndromes in pediatric individuals after mercury exposure (Maghazaji, 1974). Studies have found an increase in immobility time in a model of gestational intoxication with chronic low (0.5 mg/kg/day) and acute high (8 mg/kg/day) doses of MeHg (Onishchenko et al., 2007; Maia et al., 2009). Our data reveal that even at lower doses of MeHg than Onishchenko et al. study, CELD of MeHg protocol may induce depressive-like behavior in adolescent female rats. In addition, hedonic like-behavior has been related to ethanol moderate binge drinking in rats, in which dopamine, serotonin and norepinephrine neurotransmitter turnover was reduced, contributing to negative reinforcement (Smith et al., 2008).

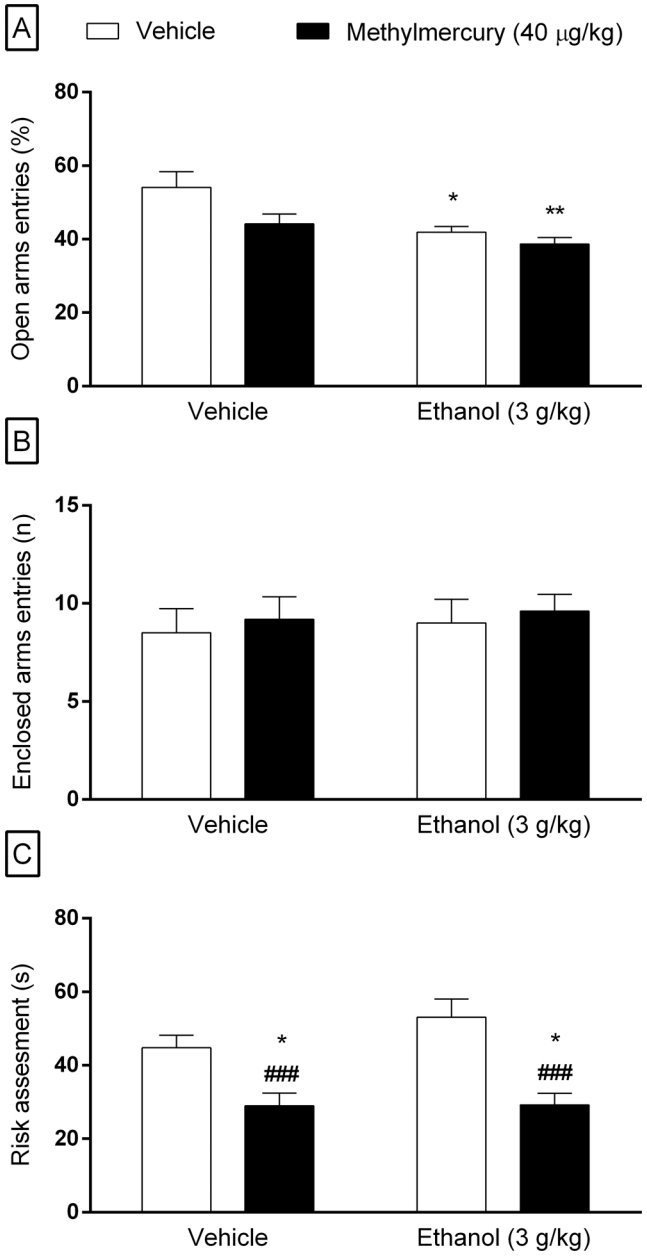

Fig. 3. Effect of chronic exposure to low doses of methylmercury (40 μg/kg/day for 5 weeks) and/or ethanol binge drinking (3 g/day at 3 days per week for 5 weeks) on the behavior of rats in elevated plus maze test. Control group received distilled water (1 mL/kg/day). Panel A represents % of open arms entries; panel B represents number of enclosed arms entries; panel C represents the risk assessment behavior in seconds. Results expressed as mean ± S.E.M. (n = 10–13). *p < 0.05*versus* control group;**p < 0.01*versus* control group; ###p < 0.001*versus* ethanol group. Two-way ANOVA followed by Tukey's *post hoc* test.

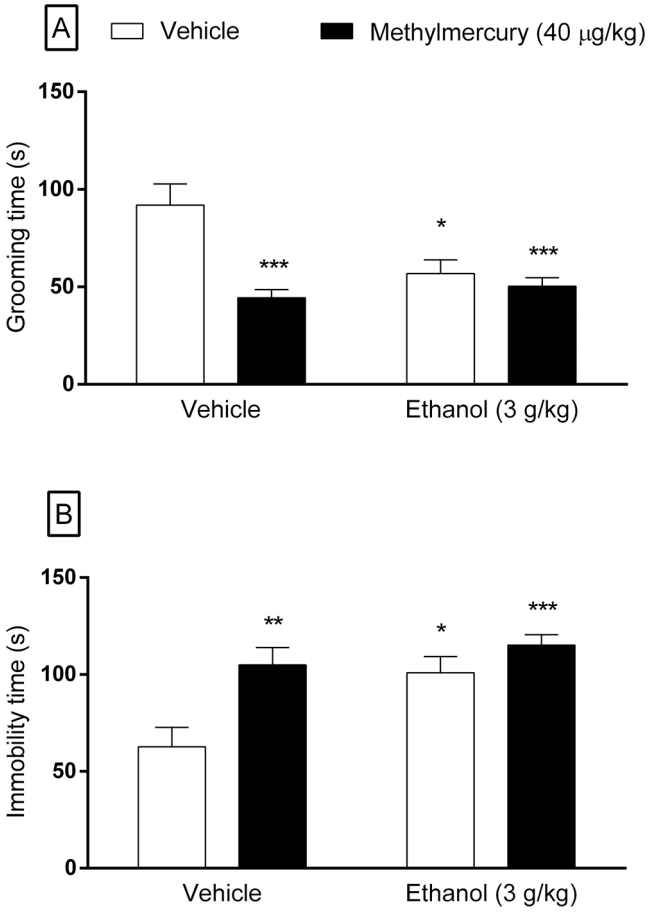

Fig. 4. Effect of chronic exposure to low doses of methylmercury (40 μg/kg/day for 5 weeks) and/or ethanol binge drinking (3 g/day at 3 days per week for 5 weeks) on the behavior of rats in (A) splash test and (B) forced swimming test. Control group received distilled water (1 mL/kg/day). Results expressed as mean ± S.E.M. (n = 10–13). *p < 0.05, **p < 0.01, ***p < 0.001*versus* control group. Two-way ANOVA followed by Tukey's *post hoc* test.

Besides, several studies have postulated a relationship between ethanol binge drinking and depressive symptoms in humans (Choi and Dinitto, 2011; Paljärvi et al., 2009). Our results demonstrate this negative behavior in a binge drinking model in adolescent rats. Several research studies have reported that adolescent ethanol exposure shows an increase in dopamine release in the nucleus accumbens (Badanich et al., 2007; Pascual et al., 2009). However, during an abstinence period there is a reduction of dopamine levels, which results in severe craving, as well as negative behavior symptoms in alcoholics (Heinz et al., 2005; Rossetti et al., 1992). In addition, although both ethanol and MeHg have been pointed out as responsible for depressive disorders, the administration of MeHg plus ethanol elicits depressive behavior in the same manner as the *per se* groups.

In the cognitive assay, low-doses of MeHg was not able to disrupt cognition damage. However, when ethanol binge drinking paradigm was employed, individuals displayed poor performance in cognitive tasks, *e.g.* short-term memory impairment. In fact, an ethanol binge drinking protocol for 4 days (5 g/kg/day) resulted in learning and memory difficulties (Cippitelli et al., 2010; Obernier et al., 2002). The paradigm of Obernier et al. disrupted the cortico-limbic pathway through a neurodegeneration process in the olfactory bulb, piriform cortex, perirhinal cortex, entorhinal cortex and dentate gyrus of the hippocampus, which suggests that even a short-term ethanol binge exposure can lead to long-term dysfunction in cognition (Obernier et al., 2002). However, CELD of MeHg during adolescence was not able to disrupt memory functions. Conversely, studies have revealed that acute low doses of MeHg at an early age (7th postnatal day) promoted memory and learning impairment related to hippocampus damage (Falluel-Morel et al.,2007; Sokolowski et al.,2013). In the design of Tian et al. (2016), 0.4 mg/kg/day from the 5th postnatal day until the 33rd postnatal day (28 days of exposure) elicited spatial learning and memory disruption as a consequence of reduced production of dentate gyrus neurons. We believe that in the former studies, the low doses of MeHg were administered at an early age, before the adolescence period, which could intensify the vulnerability to MeHg poisoning. In addition, in the latter, Tian and coworkers used a MeHg dose 100-fold higher than that was employed in our study, which may enhance MeHg

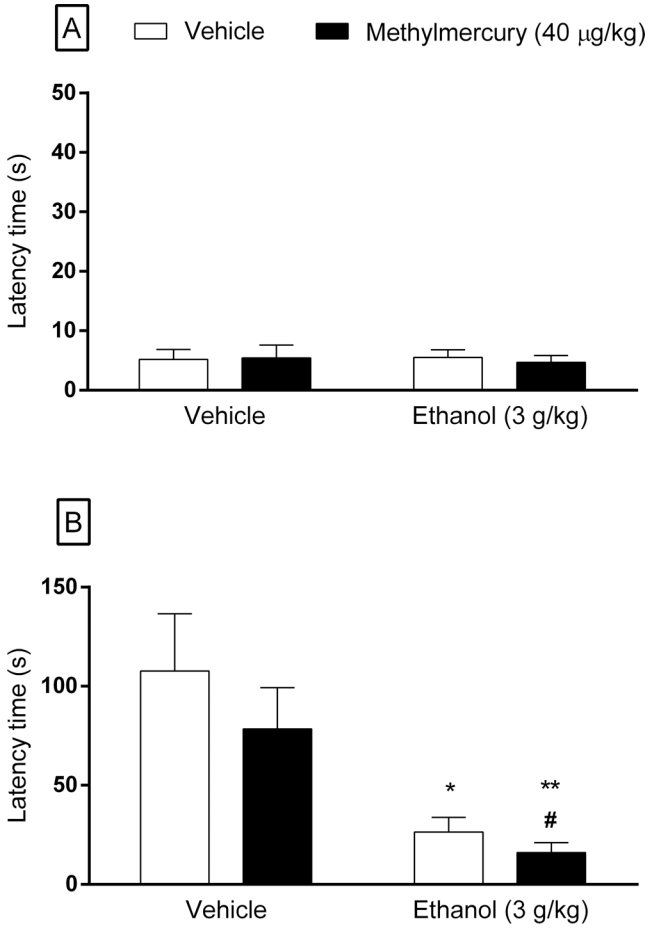

Fig. 5. Effect of chronic exposure to low doses of methylmercury (40 μg/kg/day for 5 weeks) and/or ethanol binge drinking (3 g/day at 3 days per week for 5 weeks) on the behavior of rats in inhibitory avoidance test. Panel A represents the training phase; panel B represents the short-term memory evaluation. Control group received distilled water (1 mL/kg/day). Results expressed as mean ± S.E.M. (n = 10–13). *p < 0.05 *versus* control group; **p < 0.01*versus* control group; #p < 0.05*versus*methylmercury group. Two-way ANOVA followed by Tukey's *post hoc* test.

cognitive dysfunction. In fact, our CELD of MeHg design during adolescence was not sufficient to increase the deposit mercury in the hippocampus, which justifies the absence of cognitive deficits. In this context, we attribute to ethanol the effects of cognitive impairment observed in the ethanol plus MeHg exposure animals.

In this sense, we propose that concomitant intoxication with ethanol reduces the necessity of mercury tissue levels to display psychiatric disorders profile or to provoke cognitive impairment, which would be not seen in the absence of ethanol exposure.

Regarding the oxidative parameters caused by toxicants, no change in antioxidant parameters was present in a behavioral stress model (control group *versus* basal group). Ethanol exposure rats have an increase in catalase activity over the control and basal groups; however, this enzymatic activity was much lower than the MeHg group. The increase in the CAT parameter induced by ethanol may be explained by an increased formation of H2O2 and $O2\cdot -$, and such reactive oxygen species (ROS) are detoxified by antioxidant enzymes (*i.e.*, CAT and SOD). Nevertheless, beyond the CAT enzyme playing a key role in the degradation of H2O2, this enzyme also oxidizes other compounds including ethanol-using H2O2. Therefore, CAT is responsible for around 2% of ethanol metabolism (Cederbaum, 2012), which is important for production of acetaldehyde in the CNS (Correa et al., 2012; Zimatkin et al., 2006). Therefore, ethanol intake may divert CAT activity for ethanol metabolism, thus generating an excess of H2O2 in the organism. Such an excess of H2O2 can facilitate the production of hydroxyl radicals (OH·), the most powerful oxidant molecule, thereafter leading to oxidation and damage of biomolecules (Halliwell, 2006). In addition, thiol content was also increased in the ethanol-intake group. Among thiol forms, GSH is a nonprotein thiol that is involved in the antioxidant defense against the toxic effects of ROS from xenobiotic metabolism, as well as acting as a cofactor for antioxidants enzymes, such as glutathione peroxidase and glutathione-S-transferase (Meister, 1982). Therefore, GSH can be necessary for metabolism of ethanol, to reduce both free radicals and acetaldehyde generated during alcohol metabolism (Wu and Cederbaum, 2009). We postulate that the increased thiol groups probably were not sufficient to balance free radicals or acetaldehyde injury, thus promoting cell damage. In fact, the increase of CAT activity in the ethanol-intake individuals (per se or combined with MeHg) resulted in MDA level elevation, suggesting that lipid peroxidation increased. Our results therefore highlight ethanol toxicity, which during its metabolism leads to marked oxidative stress due to powerful oxidant molecules in the organism. In this context, Tajuddin et al. (2014) described ethanol metabolism (cytochrome P450, catalase, peroxidases) as one of the main ROS sources. In addition, these authors showed that repetitive ethanol binge drinking leads to astroglial swelling-related activation/expression of phospholipase A2 (cPLA2 and sPLA2), which evokes excessive arachidonic acid mobilization, loss of endogenous docosahexaenoic acid (DHA) turnover due to decreased iPLA2, and greater increases in neuron-damaging ROS levels, concomitant with PAR-mediated glial activation.

Regarding exposure to Hg, CAT activity was upregulated peripherally over all other experimental groups, including ethanol and MeHg plus ethanol groups. In agreement with our results, Zemolin et al. (2012) reported that MeHg intoxication also led to antioxidant enzyme activity increases (*i.e.*, CAT and SOD) in the cerebellum, but only CAT

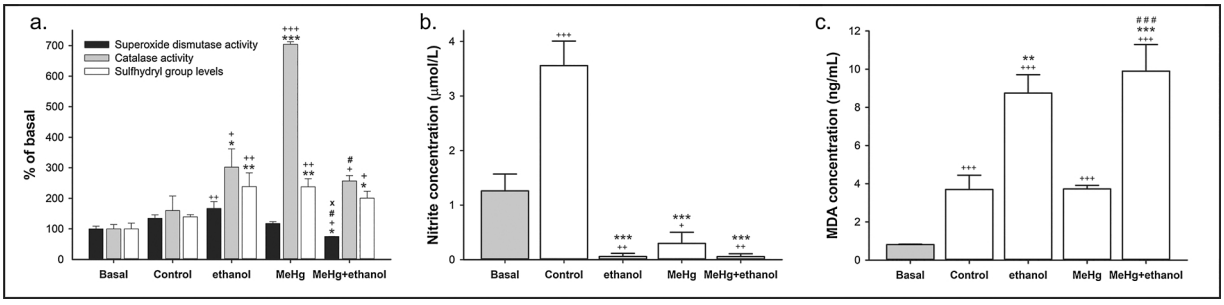

Fig. 6. Effect of chronic exposure to low doses of methylmercury (40 μg/kg/day for 5 weeks) and/or ethanol binge drinking (3 g/kg/day at 3 days per week for 5 weeks) on (a.) antioxidant parameters; (b.) nitrite concentration; (c.) lipid peroxidation (malondialdehyde (MDA) concentration). Control group received distilled water (1 mL/kg/day). Results expressed as mean ± S.E.M. (n = 4–5). +p < 0.05 *versus* Basal group; ++p < 0.01 *versus* Basal group; +++p < 0.001 *versus* Basal group; *p < 0.05 *versus* Control group; **p < 0.01 *versus* Control group; *** p < 0.001 *versus* Control group; #p < 0.05 *versus* MeHg group; ###p < 0.001 *versus* MeHg group; x p < 0.05 *versus* Ethanol group. Two-way ANOVA followed by Tukey's *post hoc* test.

in the cerebral cortex in a mouse model. It seems that such over expression of antioxidant enzymes is a body defense mechanism against the toxicant through the activation of the NF-E2-related factor 2- antioxidant responsive element (Nrf2-ARE) pathway and consequent overexpression of CAT, contributing to antioxidant defense (Zemolin et al., 2012). Accordingly, the Nrf2 signaling pathway is a main protective mechanism against inorganic Hg or MeHg toxicity in the body (Ni et al., 2010, 2011; Deng et al., 2014). Under excessive ROS conditions, for example generated by this metal, the Nrf2 protein dissociates from Kelch-like ECH-associated protein 1 (Keap1) and translocate into the nucleus, in which dimerizes with musculoaponeurotic fibrosarcoma (maf) or other bZIP proteins, forming heterodimer Nrf2-MAF, that binds to AREs. Thus, AREs that are promoters of target genes, initiate the transcription of a number of phase II detoxification enzymes, such as NAD(P)H: quinine reductases (NQO1), c-glutamyl cysteine synthetase (c-GCS), glutathione S-transferase (GSH) and heme-oxygenase (HO-1) and CAT in several tissues (Ishii et al., 2002). In this regard, studies have shown that Hg-exposure displays dose-dependent upregulation of Nrf2 expression of mRNA and protein levels, as well as of HO-1 and c-GCS antioxidant enzymes in liver and cerebral cortices, reducing GPx-1 mRNA in cortex (Liu et al., 2017; Deng et al., 2014). This upregulation of Nrf2 expression might be caused to Hg2 + -induced oxidative modifications due the Hg2 + binds covalently to Cys151 of Keap1 protein thiol (indirect mechanism), or by a direct disruption of the thiol groups by Hg2 + (Hwang, 2012; Liu et al., 2017). Previous *in vitro* study has also reported that MeHg induces acute oxidative stress and Nrf2 nuclear translocation in primary microglial cells (Ni et al., 2010), however an Nrf2 overexpression attenuates MeHg-induced cytotoxicity in SH-SY5Y neuroblastoma cells (Toyama et al., 2007). Therefore, our data that showed an excessive increase in CAT activity and baseline levels of MDA in animals exposed to MeHg may be explained by an upregulation of the Nrf2 signaling pathway and other intracellular factors that also contribute in reducing MeHg toxicity, such as heat shock factor protein 1 (Hsf1) and generation of *in vivo* metabolites such as hydrogen sulfide (H2S) (Yoshida et al., 2011; Hwang, 2012). Thus, these protective factors may lead to the reduction of oxidative stress and inflammation, as well as increased generation of detoxifying molecules that contribute in reducing Hg toxicity in the CNS and in other tissues, as reported by Zhao et al. (2015).

In our data, animals exposed to MeHg presented sulfhydryl groups similar to ethanol and MeHg plus ethanol groups. Thus, we hypothesize that hazardous effects provoked by elevated mercury concentration in the body is minimized by production of H2S and high levels of CAT activity in these animals (Farina et al., 2011), which may also explain the reduced NOx and MDA levels in Hg-exposed subjects similar to the control group. Moreover, biological substances that possess dissociative thiol groups, *e.g.*, glutathione (GSH), a low molecular weight thiol compound that is abundant in the body, plays a pivotal role in the reduction of MeHg toxicity (Hwang, 2012).

Overall, we suggested that the ethanol leads a decrease in mercury levels from hair and CNS tissues by despite the probable induction of CBS and CSE by ethanol and consequent increase of the formation of $(MeHg)_2S$. In addition, considering that ethanol *per se* intoxication has been extensively recognized by several researchers, including our group, as a neurotoxicant, that may be more hazardous in the adolescence period (for review see Pascual et al., 2017; Teixeira et al., 2014). Highlighted, due to ethanol intake, increased MDA levels were also observed in the association-treated groups in a non-synergistic manner. In fact, solely MeHg plus ethanol-intoxicated animals had reduced SOD activity compared to controls, which could increase the vulnerability to oxidative damage. SOD reduction signals an accumulation of superoxide anion (O2.-), which in excess can cause peroxidation of membranes (Mondola et al., 2016). Besides, we suggest that PLA-2 activation induced by ethanol increased MDA content in the association group, but without a significant difference from the EtOH treated group. Thus, we propose that, despite the reduced content of MeHg in brain tissues, the harmful effects of treatment with ethanol led to a reduction in CAT activity due to its metabolic process. These reactions promote accumulation of H2O2, $O2^-$ and other reactive species, which also lead to a reduction in SOD activity and increase MDA levels peripherally, possibly reflecting on the CNS, leading psychiatric disorders profile or cognitive impairment, as shown in our data. Thus, these findings indicate that the ethanol played a crucial role on the peripheral oxidative status observed in our co-intoxication paradigm.

Toxicological effects of MeHg and ethanol have been studied, however, the risk related to female gender are scarce. Of interest, a double risk is present, one for the women itself that are exposure to these compounds, and other to the offspring when the woman is pregnant or at the future could be pregnant. Behavioral tests showed a difference gender and age-dependent in rats exposed to MeHg during the developmental period, especially in motor activity, when females young adults (2–3 months age) exposed to MeHg reduced vertical exploration (Cauli et al., 2013).

Additionally, Wallin-Miller et al. (2017) explored gender differences on the risk-taking behavior related to a low dose of ethanol exposure paradigm. Such interesting study examined ethanol intake *versus* sex, as well as the involvement of the hormonal milieu on risk decision making. The authors concluded that ethanol elicits impulsivity behavior in females, but not males. However, females seem to be less vulnerable to risk taking behavior than their counterparts. Overall, gonadal hormones really play a pivotal role on risk behavior in both gender, contributing to ethanol positive effects, however, it seems that females and males are equally affected by steroids effects related to behavior variability (Becker et al., 2016). Actually, in view of this point, the neuroprotective effectiveness of the female gonadal hormone requires specific conditions, *e.g.* brain area or pathological status (Jung and Metzger, 2016).

Sex differences related to oxidative parameters are also detected. In an ethanol-withdrawal induced oxidative stress model, the estradiol was suggested as a neuroprotector factor which contributes to oxidative damage and behavioral alteration resistance on cerebellum-dependent assays, but not on hippocampus in female rats. Such findings suggest that there is an innate sex difference that is singular to the different brain areas, which predispose female to the protective effects of the gonadal female hormone (Jung and Metzger, 2016). In line with this, we might affirm that oxidative stress, as well as other mechanisms brain area specifics may modulate the behavioural impairment and tissue gender-specific damage, as such observed in drug addiction studies (Eubig et al., 2013; Jung and Metzger, 2016; Wallin-Miller et al. 2017).

5. Conclusions

In conclusion, these results show for the first time that CELD of MeHg during the adolescence period provokes motor dullness and psychiatric-like disorders behavior in rat models. In addition, low-doses of MeHg displayed tropism for hair and brain cortices areas, in comparison to other regions, however presented low reflexes on peripheral oxidative biochemical. The co-intoxication paradigm (CELD of MeHg plus ethanol binge drinking) exhibited interaction in the toxicokinetic of the metal, reducing the contents in hair and brain samples. Finally, ethanol intake reduces the CNS mercury levels required to elicit psychiatric and cognitive disorders, as well as elicited peripheral oxidative damage on MeHg-intoxicated individuals. The present work raises the question: Low levels of MeHg during adolescence is safe? Clinical studies have concerned about slight and silent behavioral alterations among the riverside communities exposed to this metal. The clinical studies have concerned about slight and silent behavioral alterations among populations environmentally exposed to MeHg though high fish consumption (*e.g.* riverside communities in Amazon and other island inhabitants)? The style of life (*i.e.*, consume of alcohol) has been considered when analyzing such population?

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Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.etap.2018.04.021.

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