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Alterations of motor performance and brain cortex mitochondrial function during ethanol hangover

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ABSTRACT

Ethanol has been known to affect various behavioral parameters in experimental animals, even several hours after ethanol (EtOH) is absent from blood circulation, in the period known as hangover. The aim of this study was to assess the effects of acute ethanol hangover on motor performance in association with the brain cortex energetic metabolism. Evaluation of motor performance and brain cortex mitochondrial function during alcohol hangover was performed in mice 6 hours after a high ethanol dose (hangover onset). Animals were injected i.p. either with saline (control group) or with ethanol (3.8 g/kg BW) (hangover group). Ethanol hangover group showed a bad motor performance compared with control animals ($p < .05$). Oxygen uptake in brain cortex mitochondria from hangover animals showed a 34% decrease in the respiratory control rate as compared with the control group. Mitochondrial complex activities were decreased being the complex I–III the less affected by the hangover condition; complex II–III was markedly decreased by ethanol hangover showing 50% less activity than controls. Complex IV was 42% decreased as compared with control animals. Hydrogen peroxide production was 51% increased in brain cortex mitochondria from the hangover group, as compared with the control animals. Quantification of the mitochondrial transmembrane potential indicated that ethanol injected animals presented 17% less ability to maintain the polarized condition as compared with controls. These results indicate that a clear decrease in proton motive force occurs in brain cortex mitochondria during hangover conditions. We can conclude that a decreased motor performance observed in the hangover group of animals could be associated with brain cortex mitochondrial dysfunction and the resulting impairment of its energetic metabolism.

Introduction

It is well known that chronic alcohol consumption has a variety of serious consequences on health. The different effects are widespread, altering numerous physiological, endocrine and behavioral functions. However, less is known about a state that exists after acute alcohol consumption: the ethanol hangover. In fact, often the terms ethanol hangover and ethanol withdrawal are confused and used without distinction (Swift & Davidson, 1998). In humans, hangover begins when ethanol (EtOH) is absent in plasma and is defined as the sum of unpleasant physical and psychological symptoms like headaches, nausea, diarrhea, fatigue, tremor combined with decreased occupational, cognitive, or visual-spatial skill performances (Kim, Yoon, Lee, Choi, & Go, 2003; Wiese, Shlipak, & Browner, 2000). In the case of experimental animals, EtOH induces voluntary consumption if it is first administrated by intubations or injections (Sinclair, Walker, & Jordan, 1973); hypo-activity (Doremus-Fitzwater & Spear, 2007) or anxiety-like behavior (Zhang, Morse, Koob, & Schulteis, 2007) are observed during withdrawal. Indeed, other works confirm different kind of alterations in various behavioral parameters, body temperature, wheel running activity and pain perception during the hangover period (Brasser & Spear 2002; Sinclair & Gustafsson 1987; Varlinskaya & Spear 2004). Although, in humans, alcohol hangover is the most frequently reported alcohol-related consequence, it received remarkably little attention from the scientific community even when it is related with serious implications for activities such as job performance and driving (Frone, 2006; Verster & Penning, 2010).

Ethanol produces a wide variety of behavioral and physiological effects in the body, but exactly how it acts to produce these effects is still poorly understood. Although ethanol was long believed to act nonspecifically through the disordering of lipids in cell membranes, proteins are at the core of most current theories of its mechanisms of action. Ethanol affects various biochemical processes such as neurotransmitter release, enzyme function and ion channel kinetics.

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With respect to behavioral effects, probably the most relevant of the neuronal actions of ethanol on ion channels are inhibition of *N*-methyl-D-aspartate (NMDA)–type glutamate receptors and its enhancement of γ-aminobutyric acid type A (GABAA) and glycine receptor function (Harris, Trudell, & Mihic, 2008). In addition, it has been observed that EtOH induces the production of oxygen reactive species (ROS) (Comporti et al., 2010) which are known to be harmful for the cell homeostasis.

Several animal models of hangover have been developed in order to provide insights into the physiological and behavioral changes that occur in the period immediately after intoxication (Gauvin, Cheng, & Holloway, 1993; Gauvin et al., 1997). Acetaldehyde is known to be a primary metabolite of ethanol and is the main cause of hangover (Penning, van Nuland, Fliervoet, Olivier, & Verster, 2010). Lieber reported acetaldehyde to be more toxic to the body than ethanol itself (Lieber & De Carli, 1973). It has been reported that acetaldehyde causes steatohepatitis, hepatic cirrhosis and downregulation of ALD2 (aldehyde dehydrogenases) expression via mitochondrial dysfunction (Helander & Tottmar, 1988). Decreased locomotor activity during acute ethanol hangover has also been reported in adult animals tested on the elevated plus maze (Lal, Prather, & Rezazadeh, 1991). At the moment there is no clear knowledge about how hangover affects the energetic metabolism in brain tissue, and how alcohol or its metabolites could be associated with cellular dysfunction. Thus, studies of the physiopathological effects of alcohol and the adaptive responses in brain will lead to a better comprehension of the complex molecular mechanisms induced in brain cells soon after ethanol disappear from blood. The aim of this work was to assess the effects of acute ethanol hangover on motor performance in mice and its relation with the brain energetic metabolism.

Materials and methods

Materials

Ethylenediaminetetraacetic acid, oxyhemoglobin, mannitol, Hepes, malate, succinate, glutamate, catalase, superoxide dismutase, and Folin reagent were obtained from Sigma Chemical Co. (Saint Louis, MO, USA). The potentiometric probe, DiOC6, was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Animals

A total of 41 male Swiss mice (*Mus musculus*) weighing 30–40 g housed in a soundproof room, with humidity and-controlled temperature (22 ± 2 °C) with a 12:12-hour light: dark cycle photoperiod (lights on 0700 hours), fed standard rat chow and tap water 'ad libitum' were used. The animals were purchased from the Animal House of the School of Pharmacy and Biochemistry from the University of Buenos Aires. Animal handling and treatment, as well as all experimental procedures were reviewed in accordance with the guidelines of the National Institute of Health (USA), and with the 6344/96 regulation of the Argentinean National Drug Food and Medical Technology Administration (ANMAT). The animals were grouped as follows: 9 mice for the blood alcohol determinations, 20 mice for the behavioral test and 12 mice for the biochemical assays. All efforts were made to minimize suffering and reduce the number of animals used.

Hangover model and experimental procedures

Solutions preparation, injections and blood ethanol concentration

Ethanol (EtOH), 15% w/v, was prepared by diluting a 95% stock solution of ETOH with 0.9% saline solution (SS). Male mice ($n = 9$) received an i.p. injection of ethanol (EtOH) at a dose of 3.8 g/kg BW, (Fee et al., 2004, Brasser & Spear, 2002, Gilliam & Kotch, 1990; Mollenauer, Bryson, Speck, & Chamberlin, 1992). Three from each group were decapitated 60, 180 and 360 min after ethanol injection. Blood was collected from the trunk and plasma ethanol level was measured by gaseous chromatography (Livy, Parnell, & West, 2003; Swift, 2003) in order to determine the animals' response to ethanol together with the onset of alcohol hangover (plasma ethanol concentration = 0). Experiments were carried out during the morning (9:00 h).

Evaluation of the motor performance during ethanol hangover

Animals were divided in two groups (control and ethanol treated) receiving tap water during 7 days and in the morning of the eighth day, animals from control group ($n = 10$) were injected i.p. with saline solution (SS) and ethanol treated animals ($n = 10$) injected i.p with EtOH (3.8 g/kg BW). Both groups were evaluated for motor performance six hours after the injection (acute ethanol or saline), in the early afternoon (15:00) with a modified tightrope test (Boveris & Navarro, 2008; Pallarés, Scacchi Bernasconi, Feleder, & Cutrera, 2007). Briefly, the procedure consisted in placing the animal on the middle of a 60 cm long horizontal rope suspended 30 cm above the floor and time was recorded until the animal either reached the end of the rope or fell down. A score was assigned accordingly: animals reaching the end of the rope in ≤6 s were given 1 point and an additional point was given for every additional 6 s needed to complete the test. Animals that stayed on the rope for 60 s without reaching the end were given 11 points. Mice falling down before 60 s were given 1 point in addition to the 11 points for every 6 s falling earlier than 60 s. Therefore, animals that scored the lowest performed the best. The test evaluates the motor performance of the animal as a mean of its' intrinsic neuromuscular coordination (Navarro, Sánchez del Pino, Gómez, Peralte, & Boveris, 2002).

Isolation of mouse brain cortex mitochondria

A different group of animals ($n = 12$) were killed by cervical dislocation in accordance with the directive systems of protection of vertebrate animals for scientific research. Control ($n = 6$) and ethanol hangover ($n = 6$) animals were used in three different experiments. Brain cortex was rapidly removed and minced on ice, resuspended in MSH buffer (210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.4) supplemented with 1 mM EDTA, and homogenized with a glass Dounce homogenizer and Teflon pestle. Homogenates were centrifuged at 600× *g* for 10 min at 4 °C. The supernatant was decanted and centrifuged again at 8000× *g* for 10 min; the new mitochondrial pellet was washed several times in MSH without EDTA, in order to avoid the calcium chelation by this compound. Mitochondria were stored on ice prior to the experiments. Protein was determined by the Lowry assay (Lowry, Rosenbrough, Farr, & Randall, 1951). The isolated mitochondrial fraction corresponds to synaptic and non synaptic cortex mitochondria mainly from neurons and glyal cells.

Mitochondrial respiratory function

Oxygen consumption by isolated brain cortex mitochondria was measured with a high-resolution respirometry (Oroboros Oxygraph, Paar KG, Graz, Austria). Mitochondrial protein (0.5–1 mg/ml) was placed in a reaction medium consisting of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris-HCl, 5 mM malate plus glutamate, 5 mM PO_4H_2K, 4 mM $MgCl_2$ (pH 7.4), and 0.2% bovine serum albumin, at 30 ° C. State 3 was estimated by the addition of 1 mM

ADP and the respiratory control ratio was calculated from the ratio of the state 3/state 4 respiratory rates with and without ADP, respectively (Chance & Williams, 1956; Estabrook, 1967). The mitochondrial fraction obtained from brain cortex tissue showed a respiratory control ratio (RCR) between 4.0 and 6.0 ($n = 5$) determined with malate plus glutamate as substrates, and was not affected by the concentration of the potentiometric probes used.

Mitochondrial hydrogen peroxide production

H_2O_2 generation was determined in intact brain cortex mitochondria by the scopoletin-HRP method, following the decrease in fluorescence intensity at 365–450 nm (λ exc–λ em) at 37 °C (Boveris, 1984). The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris-HCl (pH 7.4), 0.8 μM HRP, 1 μM scopoletin, 0.3 μM SOD to ensure that all superoxide ($O_2^{\bullet}$) was converted to H_2O_2; 6 mM succinate plus glutamate were used as substrates. Calibration was made using H_2O_2 (0.05–0.35 μM) as standard to express the fluorescence changes as nmol H_2O_2/min mg protein. Hydrogen peroxide production was highly sensitive to catalase addition (3.500 U/ml).

Mitochondrial transmembrane potential

Mitochondrial transmembrane potential was determined as follows: isolated mitochondria (25 μg/ml) were incubated at 37 °C for 20 min in MSH buffer supplemented with 5 mM malate, 5 mM glutamate, 1 mM phosphate and 4 mM $MgCl_2$ in the presence of 30 nM DIOC6, a potentiometric probe that can be used for direct measurement of transmembrane potential in cells and isolated mitochondria from different sources. The fluorescence changes were determined by cytometric measurement. Fresh mitochondria were prepared for each experiment and samples were protected from light until acquired by the cytometer. Auto-fluorescence of the mitochondrial preparation was measured as a probe loading control, and 0.5 μM of the depolarizing agent FCCP as a positive control. A common marker, indicating the relative fluorescence intensity of the mitochondrial population was used to quantify the resulting changes in membrane potential (Bustamante et al., 2011). The RCR was not affected by the concentration of the potentiometric probes used.

Evaluation of mitochondrial respiratory complexes I–III, II–III and IV

NADH-cytochrome *c* reductase activity (complex I–III) was measured in brain submitochondrial membranes by following spectrophotometrically the reduction of cytochrome *c* at 550 nm ($\varepsilon = 19.6\ mM^{-1}\ cm^{-1}$) in a reaction medium containing 100 mM phosphate buffer (pH 7.4), 0.2 mM NADH, 0.1 mM cytochrome *c* and 0.5 mM KCN at 30 °C. Enzyme activity was expressed in nmoles cytochrome *c* reduced per minute per mg of protein. Succinate cytochrome *c* reductase activity (complex II + III) was similarly determined and expressed, except that NADH was substituted by 20 mM succinate. Cytochrome oxidase activity (complex IV) was assayed spectrophotometrically at 550 nm by following the rate of oxidation of 50 μM ferrocytochrome c (Yonetani, 1967). The activity was expressed as nanomoles cytochrome *c* oxidized per minute per mg of protein.

Nitric oxide production associated to the mitochondria

Nitric oxide production was measured in SMP (submitochondrial particles) (0.2–0.5 mg/ml) by using a double-beam dual-wavelength spectrophotometer, following the oxidation of oxyhemoglobin (HbO_2) (25 μM in heme) to methemoglobin at 577–591 nm ($\Delta\varepsilon_{577-591} = 11.2\ mM^{-1}\ cm^{-1}$), sensitive to L-NNA inhibition (Boveris et al., 2002; Boveris, Valdez, Zaobornyj, & Bustamante, 2006; Bustamante, Czerniczyniec, Cymeryng, & Lores-Arnaiz, 2008) at 37 °C. In order to avoid the presence of $O_2^{\bullet-}$ and H_2O_2, Cu–Zn SOD and catalase at 4 μM, 0.1 μM, were used respectively (Bustamante et al., 2002; Bustamante, Bersier, Romero, Badin & Boveris, 2000). The results were expressed as nmol of NO per minute per milligram protein. NO production associated to the mitochondria, is also strongly dependent on the availability of arginine; this compound is present in brain mitochondria in non-limiting concentrations (Lores Arnaiz et al., 2004).

Statistical analysis

Values in Tables and figures are mean values ± SEM. Three independent experiments were performed for each experimental condition. Results were compared using an unpaired Student *t*-test.

Results

Blood alcohol levels and hangover onset

In order to determine the onset of alcohol hangover, blood alcohol concentration (BAC) was measured 60, 180 and 360 min after the acute injection of ethanol. The results showed that the levels of blood ethanol tend to fall between 60 and 180 min as expected, but both levels are not significantly different (289 ± 41 mg/dl vs. 246 ± 15 mg/dl). However a marked decay was observed after 360 min post- injection being 13.67 ± 1.81 mg/dl) ($p < .001$) as described in Fig. 1. Interesting to note, is that the observed blood alcohol level obtained during the hangover condition, in this study, was similar to the ethanol level obtained by other laboratories (Livy et al., 2003). This result indicates that at this point, the alcoholemia was 95% decreased from its starting value. Thus, the criteria used in this work was that the *alcohol hangover onset* six hours after (360 min) the acute injection of ethanol. So, all behavioral and biochemical assays were carried out 6 h after ethanol treatment.

Evaluation of the motor performance during the alcohol hangover

As described previously a lower score implies a better motor performance. Mice from the ethanol group obtained a higher score

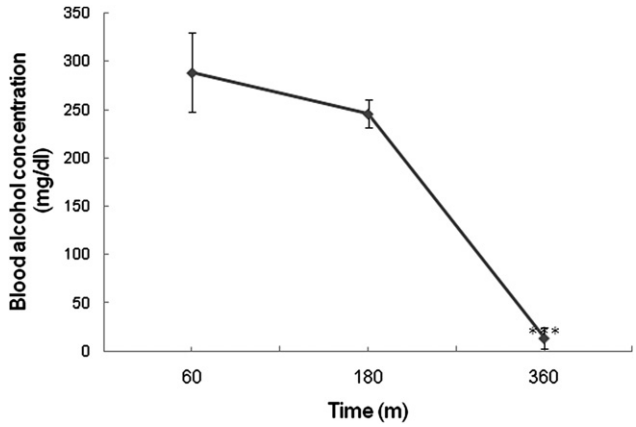

Fig. 1. Blood alcohol concentration during and after alcoholemia. Blood alcohol concentration in Swiss male mice was evaluated 60, 180 and 360 minutes after acute ethanol injection ($n = 9$). Values are expressed as mean ± SEM ***$p < .001$ T independent sample test.

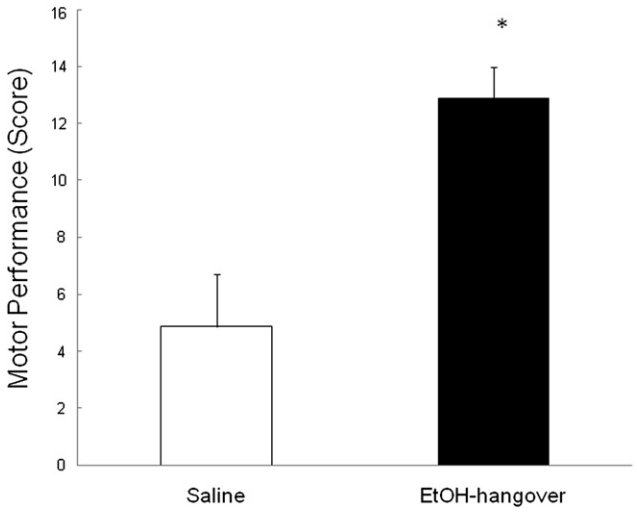

Fig. 2. Male tightrope performance during ethanol hangover (Tightrope test). Lower score represents a better performance during the test. White bars represent score for control animals, filled bars correspond to ethanol treated animals. Values are expressed as mean ± SEM (**$p < .01$ T independent sample test).

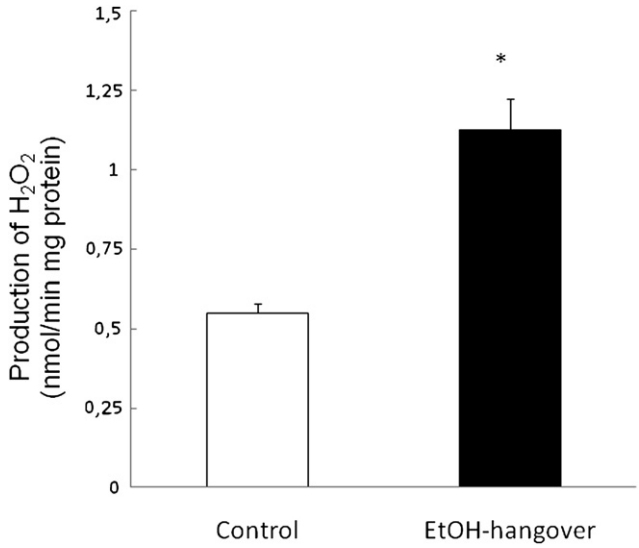

Fig. 3. Mitochondrial hydrogen peroxide production during ethanol hangover. Brain cortex mitochondria were evaluated for the hydrogen peroxide production by the scopoletin-HRP method. White bars represent control animals and filled bars correspond to ethanol hangover animals. Values are expressed as the mean ± SEM *$p < .001$.

than control saline injected animals showing a significant difference ($p < .01$) as described in Fig. 2; the results followed a parametric statistical analysis (Kolmogorov–Smirnov test).

Mitochondrial respiratory function

The results of the respiratory function showed that animals exposed to hangover condition presented an impaired mitochondrial oxygen uptake. The respiratory rate in the resting state (state 4) in the mitochondria from hangover animals did not show a significant difference as compared with the control animals. However, the oxygen uptake in the active metabolic mitochondrial condition (state 3) was 47% decreased in the hangover animals as compared with control animals. As a result, the RCR of brain cortex mitochondria from the animals in hangover condition showed a 34% decrease as compared with the control group, as described in Table 1, when malate-glutamate were used as substrates.

Hydrogen peroxide production

Brain cortex mitochondria from hangover animals showed a hydrogen peroxide production rate of 1.12 nmol/min mg protein as compared with 0.5 nmol/min mg protein produced by control animals, when succinate and glutamate were used as substrate, indicating a 51% increase in hydrogen peroxide production by brain cortex mitochondria from the hangover group as compared with the untreated animals (Fig. 3).

Mitochondrial transmembrane potential

In order to investigate in detail the transmembrane potential changes that occur in brain cortex mitochondria during the ethanol hangover, we evaluated fluorescence after loading mitochondria with $DIOC_6$ a fluorescent staining potentiometric probe. A clear decrease in mitochondrial fluorescence was observed in the histograms from ethanol treated animals as compared with saline injected animals, for a chosen- mitochondrial population in a typical flow cytometric assay (Fig. 4A). Comparative fluorescence histograms are shown in Fig. 4B. Quantification of the mitochondrial fluorescence changes indicates that ethanol injected animals presented 17% less fluorescence intensity as compared with mitochondria from control animals. As expected, a 51% decrease in mitochondrial fluorescence was observed after treatment with the depolarizing agent FCCP. These results indicate that a clear decrease in $\Delta\rho$ (proton motive force) occurs in brain cortex mitochondria at 6 hours after ethanol injection.

Table 1
Respiratory rates of isolated of brain cortex mitochondria during hangover conditions.

Oxygen uptake (ng-atom O/min mg protein)			
Condition	State 4	State 3	RC
Control	7.7 ± 0.5	32 ± 3	4.1 ± 0.2
EtOH-hangover	8.0 ± 1.0	17 ± 2[a]	2.7 ± 1.2[a]

[a] $p < .05$, significantly different from control.

Mitochondrial electron transport chain components

It has been suggested that different types of cell stressors could impair the electron transport flow through inhibition of some of the respiratory complexes. In this study the activity of the complex I–III, II–III and IV presented in Table 2 indicate that ethanol hangover induces a dysfunction in the electron transport chain. The three complex activities were decreased being the complex I–III the less affected by the hangover condition; complex I–III was 17% decreased during ethanol hangover, the activities of complex II–III and Complex IV were markedly decreased during the hangover condition showing 50% and 42% less activity respectively, than the control animals.

Nitric oxide production associated to the mitochondrial fraction

The NO production by NOS attached to the mitochondria in the hangover animals showed an important decrease as compared with controls, being 0.28 ± 0.03 and 0.62 ± 0.08 nmol/min mg proteins respectively. These results showed a 55% decrease in NO production by the mitochondria from animals during hangover conditions as compared with the control animals.

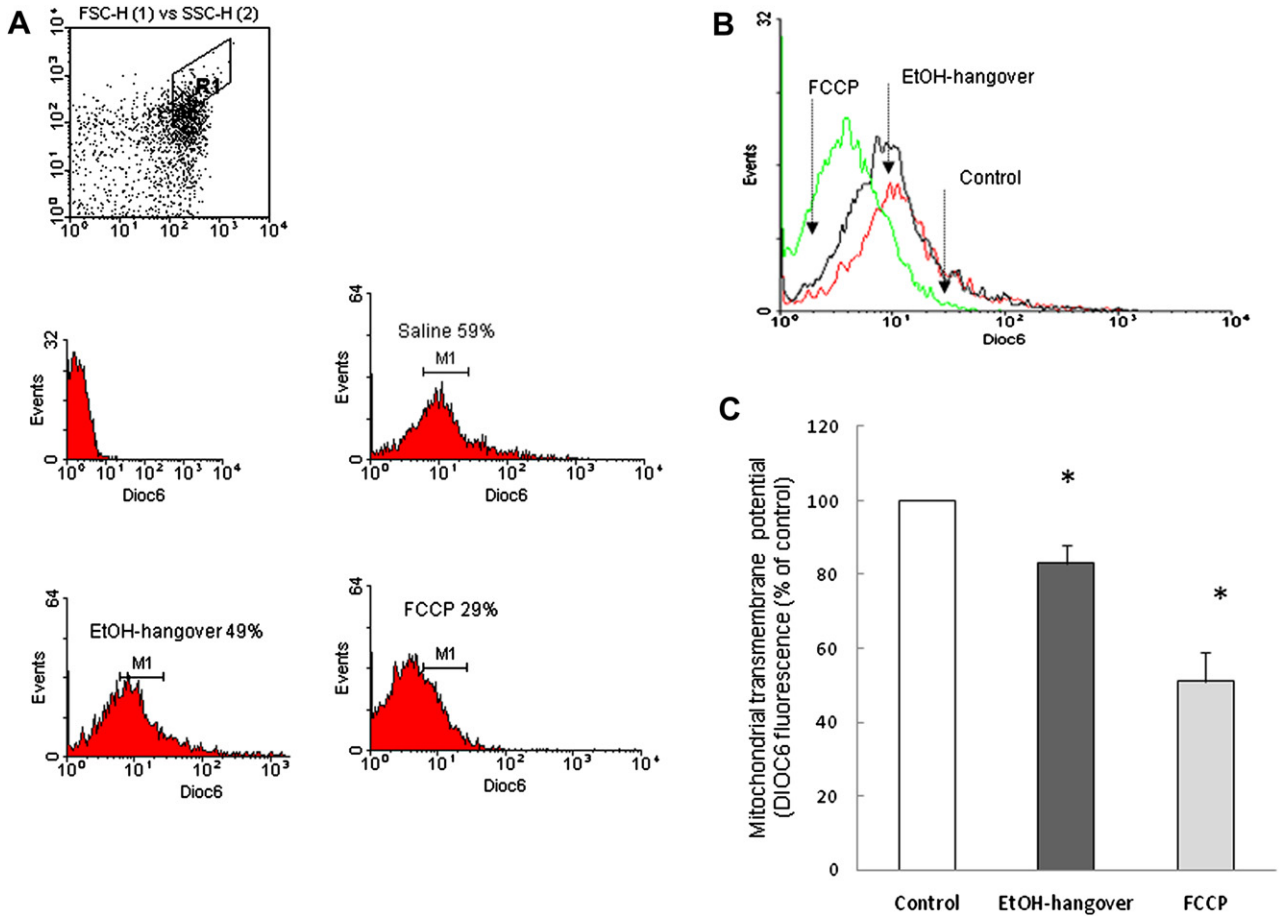

Fig. 4. Mitochondrial transmembrane potential during ethanol hangover Mitochondrial polarization was evaluated by changes in fluorescence of specific mitochondrial population after loading with 30 nM DiOC6. A) A typical experiment is shown indicating DIOC6 r.f.i. in control, ethanol hangover condition and after FCCP treatment. B) Comparative histograms of the three mitochondrial conditions. C) Bar graph quantification of the transmembrane potential. Values were expressed as the mean ± SEM $^{*}p < .001$.

Discussion

Ethanol hangover symptoms have been attributed to several causes. In fact, the removal of alcohol after an acute ethanol exposure, or the physiological effects of alcohol metabolite, mainly acetaldehyde, may be the responsible of the undesirable hangover effects (Swift & Davidson, 1998).

It has been observed that ethanol can either increase or decrease the excitability of different neurons populations and either reduce or potentiate fast synaptic transmission. For example, it potentiates responses mediated by $GABA_A$ receptors in most brain areas but it reduces them in the ventral tegmental area (Blomeley, Cains, Smith, & Braci, 2011; Weiner & Valenzuela, 2006). Furthermore, ethanol has been associated with an uncontrolled production of ROS that could be associated with the induction of the permeability transition, increasing the sensitivity of cells to other proapoptotic or damage signals (Hoek, Cahill, & Pastorino, 2002). In addition, mitochondrial permeability transition in brain tissue has been frequently observed during other damaging signals such as calcium overload (Bustamante & Lores Arnaiz, 2010), and ethanol withdrawal (Jung et al., 2008).

The aim of this work was to study the effects of the hangover condition after an acute ethanol experimental exposure (i.p. injection) on the mice motor performance and the metabolic mitochondrial function in brain cortex. In this model the *alcohol hangover onset* starts six hours after the injection of ethanol, when alcoholemia decreased 95%. Our results showed impairment in the neuromuscular coordination as well as a clear brain cortex mitochondrial dysfunction six hours after a unique ethanol injection. Our study showed that motor performance was markedly impaired by ethanol hangover. Animals from the ethanol group showed a higher score than controls indicating a bad performance that could be the result of disturbed motor control. This fact is in agreement with Philibin, Cameron, Metten and Crabbe, 2008, who described a motor incoordination as a new phenotype of alcohol withdrawal in mice. In addition the work of McQuarrie and Fingl, 1958, describing the central nervous system hyperexcitability induced by a single dose of ethanol, supports the idea of this work, stating that during ethanol hangover an altered motor performance could be due to the central nervous system excitation. This impaired motor performance was associated with a clear mitochondrial dysfunction observed in brain cortex. This mitochondrial dysfunction was characterized by an altered electron transfer, impairment of the components of the electron transport chain, decreased NO

Table 2
Activity of respiratory complexes I–III, II–III and IV from isolated cortex mitochondria from hangover animals.

	Complex I–III	Complex II–III (nmol cyt c/min mg protein)	Complex IV
Control	168 ± 8	46 ± 4	67 ± 3
EtOH-hangover	140 ± 11[a]	23 ± 3[a]	39 ± 8[a]

[a] $p < .05$, significantly different from control.

production, mitochondrial membrane depolarization and marked generation of reactive oxygen derived substances such as hydrogen peroxide. This indicates that ethanol hangover could act as a provoking stressor inducing oxidative signaling pathways. Indeed, the effect of hangover, on the respiratory control rate (RCR) in brain cortex mitochondria, indicates that mitochondrial respiration is an important target of this adverse condition and that brain cortex is a vulnerable brain region. The NO decrease was associated with an impaired mitochondrial metabolism as observed in other models of mitochondrial dysfunction (Lores Arnaiz et al., 2005). Moreover, the observed proton motive force ($\Delta\rho$) decrease in the hangover condition indicate a loss of energy to synthesize ATP. Furthermore, the increased hydrogen peroxide production could clearly perturb the redox balance and the cellular energy supply. Together, these results could indicate that brain cortex mitochondria from hangover animals could be more prone to suffer alterations such as that observed in $\Delta\Psi_m$ and reactive oxygen species, as a consequence of the opening of the permeability transition pore (MPT) with dramatic consequences for neural cell surviving (Bustamante & Lores Arnaiz, 2010). Recent reports showed that unmanaged neuronal sudden withdrawal of the excessive consumption of alcohol adversely alters neuronal integrity in vulnerable brain regions such as cerebellum, hippocampus and cortex (Jung & Metzger, 2010).

Thus we can conclude that the alterations in motor performance observed during the hangover conditions could be also associated with the observed mitochondrial dysfunction inducing a typical oxidative stress associated with an impairment of mitochondrial oxidative phosphorylation and decrease of proton motive force, in the whole brain cortex of these animals. The direct consequence of this mitochondrial metabolic impairment is the failure of the brain cortex energetic metabolism compromising the neural cellular homeostasis even when ethanol is already absent from the plasma. Further experiments will be necessary in order to determine the molecular mechanism of the hangover condition effects and to understand the association of motor impairment and brain energetic metabolism.

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