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Original article

Free radical production and antioxidant status in brain cortex non-synaptic mitochondria and synaptosomes at alcohol hangover onset

Analía G. Karadayian[a,b], Gabriela Malanga[a,b], Analía Czerniczyniec[a,b], Paulina Lombardi[a,b], Juanita Bustamante[c], Silvia Lores-Arnaiz[a,b,*]

[a] Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Fisicoquímica, Buenos Aires, Argentina
[b] CONICET-Universidad de Buenos Aires, Instituto de Bioquímica y Medicina Molecular (IBIMOL), Buenos Aires, Argentina
[c] Universidad Abierta Interamericana, Centro de Altos Estudios en Ciencias de la Salud, Buenos Aires, Argentina

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ABSTRACT

Alcohol hangover (AH) is the pathophysiological state after a binge-like drinking. We have previously demonstrated that AH induced bioenergetics impairments in a total fresh mitochondrial fraction in brain cortex and cerebellum. The aim of this work was to determine free radical production and antioxidant systems in non-synaptic mitochondria and synaptosomes in control and hangover animals. Superoxide production was not modified in non-synaptic mitochondria while a 17.5% increase was observed in synaptosomes. A similar response was observed for cardiolipin content as no changes were evidenced in non-synaptic mitochondria while a 55% decrease in cardiolipin content was found in synaptosomes. Hydrogen peroxide production was 3-fold increased in non-synaptic mitochondria and 4-fold increased in synaptosomes. In the presence of deprenyl, synaptosomal H_2O_2 production was 67% decreased in the AH condition. Hydrogen peroxide generation was not affected by deprenyl addition in non-synaptic mitochondria from AH mice. MAO activity was 57% increased in non-synaptic mitochondria and 3-fold increased in synaptosomes. Catalase activity was 40% and 50% decreased in non-synaptic mitochondria and synaptosomes, respectively. Superoxide dismutase was 60% decreased in non-synaptic mitochondria and 80% increased in synaptosomal fractions. On the other hand, GSH (glutathione) content was 43% and 17% decreased in synaptosomes and cytosol. GSH-related enzymes were mostly affected in synaptosomes fractions by AH condition. Acetylcholinesterase activity in synaptosomes was 11% increased due to AH. The present work reveals that AH provokes an imbalance in the cellular redox homeostasis mainly affecting mitochondria present in synaptic terminals.

1. Introduction

Acute alcohol consumption, particularly defined as binge drinking pattern, involves huge quantities of alcohol consumed in a short period of time [1,2]. Binge drinking pattern refers to the consumption of more than four and five drinks on one occasion for women and men respectively [3]. Alcohol hangover (AH) is described in humans as a physiological state which involves unpleasant symptoms being the direct consequence of a binge drinking episode [4].

Hangover begins when ethanol (EtOH) is absent in plasma and is characterized by a cluster of psychophysical symptoms which include headaches, nausea, diarrhea, fatigue and tremors [5,6]. We previously demonstrated that AH impaired neuromuscular coordination, locomotion and walking deficiencies during 20 h inducing also anxiety-like behavior, fear-related phenotype and depression signs [7–9].

It is well known that alcohol abuse is associated with medical comorbidities affecting numerous body systems, including the central nervous system [10]. In this sense, we established that AH impaired mitochondrial energetic metabolism which included decrement in oxygen uptake, inhibition in respiratory complexes, changes in mitochondrial membrane permeability, decrease in transmembrane potential, increases in $O_2^{\bullet-}$ and H_2O_2 production and impairment of nitric

Abbreviations: AChE, acetylcholinesterase; AH, alcohol hangover; Ant, antimycin; CAT, catalase; CNS, central nervous system; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; EtOH, ethanol; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, glutathione disulfide; GST, glutathione S-transferase; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; MAO, monoamine oxidase; MitoSOX, red mitochondrial superoxide indicator; NAO, 10 N-nonylacridine orange; NS mit, non-synaptic mitochondria; $O_2^{\bullet-}$, superoxide anion; Prdx, peroxiredoxins; RNS, reactive nitrogen species; ROS, reactive oxygen species; S, synaptosomes; SOD, superoxide dismutase

* Correspondence to: Instituto de Bioquímica y Medicina Molecular, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 2° piso, C1113AAD Buenos Aires, Argentina.
E-mail address: slarnaiz@ffyb.uba.ar (S. Lores-Arnaiz).

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oxide metabolism in brain cortex and cerebellum [11–13]. Some of the detrimental effects of EtOH have been also attributed to the induction of metabolic processes which lead to the generation of excessive levels of reactive oxygen species [14]. In addition, it was widely established that EtOH is capable of inducing reactive oxygen species production and lipid peroxidation in brain in association with the induction of mitochondrial permeability transition, increasing the sensitivity of cells to damage and proapoptotic signals [15,16]. Moreover, years ago, it was evidenced that lipid peroxidation induced by EtOH occurs mainly in synaptosomal fractions [17]. After that, Mayas et al. hypothesized that EtOH, as an amphipathic molecule, produces a physical perturbation of the lipid matrix of neuronal membranes, possibly leading to changes in the activity of membrane-bound proteins [18].

Glutathione (GSH) is a ubiquitous non-protein thiol which plays a cellular protective role under conditions of oxidative stress [19]. Previously, it was demonstrated that chronic EtOH feeding induced the depletion of mitochondrial GSH due to cholesterol loading in mitochondria [20]. Additionally, it was established that chronic EtOH exposure to rats significantly decreased GSH levels and GSH-related enzymes activities in brain homogenates [21].

Mitochondria inside the synaptosomes supply ATP to the cytoplasm and preserve metabolism, plasma membrane excitability, receptors and ion channels functioning and machinery for the exocytosis and reuptake of neurotransmitters [22]. Thus, synaptosomal preparations constitute an appropriate approach to study bioenergetics and mitochondrial function in the synapses in a variety of genetic models of neurodegenerative disorders [23]. Bae et al. proposed that EtOH damage in CNS is provoked due to its site of action within the cell membrane at synapses [24]. In this context, it was proposed that severe EtOH intoxication could produce acute synaptic dysfunction, being acetaldehyde the potential responsible molecule for that effect [10]; however, Boyd et al. demonstrated that the physiological concentrations of acetaldehyde due to EtOH exposure were not the main cause of the increases in neuroactive steroids observed and on the contrary, EtOH resulted to be primarily responsible for those biochemical changes in the rat brain cortex extract [25].

Considering our previous investigations showing that AH produced mitochondrial dysfunction and free radical production in a crude mitochondrial fraction, the aim of this work was to determine free radical production and antioxidant enzymes activities in non-synaptic mitochondria and synaptosomes in AH. This allowed us to deeply study the after-effects of acute EtOH exposure in CNS bioenergetics in two different subcellular fractions.

2. Materials and methods

2.1. Materials

Antimycin, bromophenol blue, $CaCl_2$, catalase, cytochrome c, CNDB, dithiothreitol, DTT, EDTA, EGTA, free fatty acid BSA, Folin reagent, glutamate, glutathione disulfide (GSSG), Hepes, H_2O_2, KH_2PO_4, HRP, KCl, K_2HPO_4, KCN, L-arginine, malate, mannitol, $MgCl_2$, NaH_2PO_4, Na_2HPO_4, NaN_3, NADH, NADPH, oxyhemoglobin, reduced glutathione (GSH), scopoletin, SDS, succinate, sucrose, superoxide dismutase were obtained from Sigma Chemical Co. (Saint Louis, MO, USA). Metanol, perchloric acid, orthophosphoric acid and salts for HPLC mobile phase were from Merck (Darmstadt, Germany). The probe NAO was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and MitoSOX was acquired from Thermo Fisher Scientific Inc. (Massachusetts, USA). Other reagents were of analytical grade.

2.2. Animals

Male Swiss mice (*Mus musculus*) weighing 30 g housed in a soundproof room, with humidity and-controlled temperature (22 ± 2 °C) with a 12:12-h light:dark cycle photoperiod (lights on 0700 h), fed standard rat chow and tap water *ad libitum* were used. 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). Moreover, the present study had the legal ethical accreditation from Ethics Committee for Laboratory Animal Handling of the School of Pharmacy and Biochemistry from Universidad de Buenos Aires where the protocol was performed (Res. 2419). All efforts were made to minimize suffering and reduce the number of animals used.

2.3. Experimental procedures

2.3.1. Alcohol hangover model

Ethanol (EtOH) 15% w/v, was prepared by diluting a 95% stock solution of EtOH with 0.9% saline solution (SS). Animals received an injection (i.p.) of EtOH (3.8 g/kg BW) or saline (8:00 a.m.). Fed standard rat chow and tap water were available and there was no fasting period before or after EtOH injection. The EtOH dose applied in this work was previously used in other studies [26–29]. The choice for i.p. administration was based on our previous research in which we studied alcohol hangover effects in mitochondrial crude fractions isolated from different nervous tissues. According to our previous researches, alcohol hangover onset was considered six hours after ethanol injection when blood alcohol concentration was close to zero [11].

2.4. Isolation of subcellular fractions

Six hours after injection, animals were killed by cervical dislocation in accordance with the directive systems of protection of vertebrate animals for scientific research. Brains were weighed and cerebral cortex was dissected and homogenized in a ratio of 1g cortex/5 ml in a medium consisting of 230 mM mannitol, 70 mM sucrose, 5 mM Hepes (pH 7.4) supplemented with 1 mM EDTA (MSHE). Homogenates were centrifuged at 600*g* for 10 min at 4 °C to discard nuclei and cell debris. A protease inhibitor cocktail (1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.4 mM PMSF and 1 μg/ml aprotinin) was added to the homogenates. The supernatant was decanted and centrifuged again at 8000*g* for 10 min; the resulting pellet was washed and resuspended in the same buffer and the last supernatant obtained was named cytosolic fraction.

Further mitochondrial purification and synaptosomal fraction separation were performed by Ficoll gradient [30] with modifications. The crude mitochondrial fraction was resuspended in MSHE buffer and layered on Ficoll gradients containing steps of 13%, 8% and 3% Ficoll [31]. The gradients were centrifuged at 11,500*g* for 30 min. After centrifugation, the original sample appears separated in two fractions: a pellet in the bottom of the tube corresponds to a fraction of heavy mitochondria, which are mainly non-synaptic and the fraction occurring at 8% contains synaptosomes. All the procedure was carried out at 0–2 °C. Submitochondrial membranes were obtained from mitochondria by twice freezing, thawing and homogenizing by passing the suspension through a 15/10 hypodermic needle [32]. Protein content was assayed by using the Folin phenol reagent and bovine serum albumin as standard [33], and was used to normalize the results obtained for each subcellular fraction.

Mitochondrial yield of subcellular preparations was estimated as previously described by our group by the determination of the activity of monoamine oxidase (MAO) both in total homogenates and in mitochondrial or synaptosomal fractions from control animals, being 30–45 mg mitochondrial protein/g brain tissue, both for non-synaptic mitochondria and synaptosomal fractions. Non-synaptic mitochondria were less than 5% contaminated with synaptosomal components, according to acetylcholinesterase activity determinations [34]. In addition, using a detailed flow cytometry analysis, we recently demonstrated that both fractions contained NAO positive particles indicating

the presence of mitochondria either free or inside the synaptosomes [35].

2.5. Mitochondrial superoxide levels

Non-synaptic mitochondria and synaptosomes superoxide relative production was determined as follows: isolated non-synaptic mitochondria or synaptosomes (25 μg/ml) were incubated at 37 °C for 20 min in MSH buffer (230 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.4) supplemented with 5 mM malate, 5 mM glutamate, 1 mM phosphate and 4 mM $MgCl_2$ in the presence of 2.5 μM MitoSOX Red (λem = 580 nm) a fluorogenic mitochondrial indicator highly selective for superoxide. This probe is readily oxidized by superoxide but not by other ROS- or reactive nitrogen species (RNS)- generating systems. Fresh non-synaptic mitochondria and synaptosomes were prepared for each experiment and samples were protected from light until acquired by the cytometer FACScalibur (Becton-Dickinson) [36]. In addition, 1 μM antimycin, Complex III – ubiquinol cytochrome bc1 oxidoreductase inhibitor, was added as a positive control. MitoSOX fluorescence was analyzed using the median value of the fluorescence events distribution from each treatment. A common marker (M_1) was fixed on control median value representing 50% of the fluorescent events. Histogram differences in MitoSOX fluorescence were quantified in three independent experiments as the number of events which drop under the median value of the distribution corresponding to M_1. Low MitoSOX fluorescence under M_1 would reflect decreased superoxide levels. Quantification of results was shown as bar graph in which data were expressed as the percentage of control MitoSOX relative fluorescence.

2.6. Mitochondrial hydrogen peroxide production

Hydrogen peroxide (H_2O_2) generation was determined in non-synaptic mitochondria and synaptosomes by the scopoletin-HRP method, following the decrease in fluorescence intensity at 365 and 450 nm as λexc – λem respectively, at 37 °C [37]. 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 and 0.3 μM SOD to ensure that all superoxide ($O_2^{\bullet-}$) was converted to H_2O_2; 6 mM malate plus 6 mM glutamate were used as substrates. To determine monoamine oxidase (MAO) contribution to H_2O_2 production, samples from both subcellular fractions were pre-incubated with a MAO inhibitor (10 μM deprenyl) during 2 min. We previously demonstrated that deprenyl was able to inhibit MAO activity [38]. 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.

2.7. Cardiolipin content

The two fresh subcellular fractions (synaptosomes and non-synaptic mitochondria) were stained with the fluorophore 10 N-nonylacridine orange (NAO, λem = 525 nm), in order to evaluate the cardiolipin mitochondrial content [39–41]. It is well known that low NAO fluorescence would be due to oxidation/depletion of cardiolipin [42–44].

Isolated cortical non-synaptic mitochondrial and synaptosomes samples from control and alcohol-treated animals were loaded with 100 nM NAO during 20 min at 37 °C in MSH buffer supplemented with 5 mM malate, 5 mM glutamate and 1 mM phosphate. The procedure was carried out in a dark room. Then, subcellular fractions were acquired by the cytometer as described above. In addition, 1 μM antimycin, an ubiquinol cytochrome c reductase inhibitor, was added as a positive control [45].

NAO fluorescence was analyzed using the median value of the fluorescence events distribution from each treatment. A common marker (M_1) was fixed on control median value representing 50% of the fluorescent events. Histogram differences in NAO fluorescence were quantified in three independent experiments as the number of events which drop under the median value of the distribution corresponding to M1. Quantification of events with low NAO fluorescence reflecting cardiolipin oxidation/depletion is shown as a bar graph of control percentage of NAO fluorescence.

2.8. Enzymes activities

Catalase (CAT) and superoxide dismutase (SOD) activities were determined in subcellular fractions by spectrophotometric measurements. Catalase activity was assayed following absorbance decrease of H_2O_2 at 240 nm, in a medium containing 50 mM phosphate buffer and 10 mM H_20_2 (pH 7.2), and expressed as pmoles enzyme/min mg protein [46]. Superoxide dismutase activity was determined by measuring the inhibition of autocatalytic adrenochrome formation rate in a reaction medium containing 1 mM epinephrine and 50 mM glycine-NaOH (pH 10.2) and expressed as U/mg protein [47]. Monoamine oxidase activity was measured in brain cortex non-synaptic mitochondrial and synaptosomal membranes from control and hangover mice by following spectrophotometrically the oxidation of kynuramine at 30 °C, in a reaction medium containing 50 mM phosphate buffer (pH 7.4). Kinetics were followed at 360 nm (MEC = 4.28 mM^{-1} cm^{-1}) [48].

Acetylcholinesterase activity was performed in synaptosomes according to the modified Ellman spectrophotometric method using acetylthiocholine iodide as the substrate and 5-5′-bis dithionitrobenzoic acid (DTNB) as a colouring reagent [49]. Kinetics were followed at 412 nm during 2 min at 37 °C. Results were expressed as nmol/min mg protein.

2.9. Reduced and oxidized glutathione contents

In order to assess GSH and GSSG contents, non-synaptic mitochondrial and synaptosomal fractions were homogenized in 1 M $HClO_4$ and 2 mM EDTA (1:5) (w/v). The preparations were centrifuged at 10,000*g* and 4 °C for 20 min. The protein free supernatant was filtered with a 0.22 μm nylon membrane and immediately analyzed. The contents of GSH and GSSG were quantified by reverse-phase HPLC analysis (isocratic modality) according to the modified method of Rodriguez-Ariza et al. [50]. The equipment employed was: a Perkin Elmer 250 LC bomb, a fixed phase: Supelcosil LC-18 column (5 μm size particle) 4.6 × 150 mm, a mobile phase: 20 mM NaH_2PO_4, pH 2.7, and an electrochemical detector: ESA Coulochem II with an analytical cell ESA 5011, at an applied potential of +0.45 V and 0.80 V and a 1 ml min^{-1} flow. Quantification of GSH and GSSG was performed through a standard curve with a linear relationship between 0.25 and 10 μg/ml GSH or GSSG. Results were expressed as nmol/mg protein. Also, the ratio GSH/GSSG was calculated considering it as a redox status index.

2.10. GSH-related enzyme systems

Glutathione peroxidase (GPx) activity was measured following NADPH oxidation at 340 nm (MEC = 6.22 mM^{-1} cm^{-1}) in the presence of 0.17 mM reduced glutathione, 0.2 U/ml glutathione reductase and 0.5 mM tert-butyl hydroperoxide and expressed as μmol/min mg protein [51]. Glutathione reductase (GR) activity was assayed by monitoring the oxidation of NADPH at 340 nm in the presence of 1 mM NADPH and 1 mM GSSG and results were expressed as μmol/min mg protein [52]. Glutathione S-transferase (GST) was assayed by the 1-chloro 2-4-dinitro benzene (CNDB) method. To 1 ml reaction mixture containing 0.1 M phosphate buffer (pH 6.5), 0.5 mM EDTA, 1.5 mM CDNB and 1 mM GSH, 30 μg protein (sample) was added and the increase in absorbance at 340 nm was monitored for 5 min (MEC = 9.6 mM^{-1} cm^{-1}). The enzyme activity was expressed as nmol/min mg protein [53].

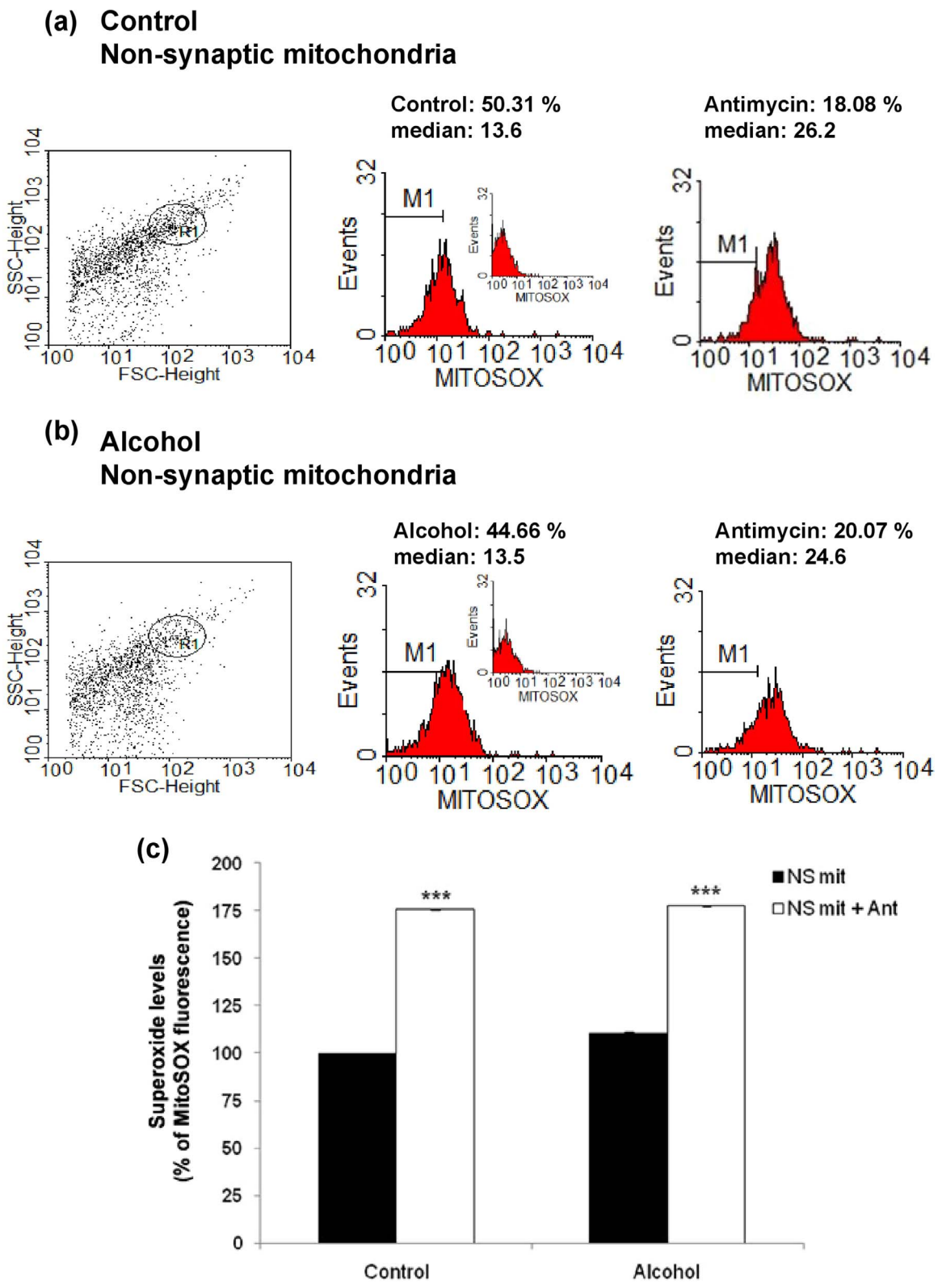

Fig. 1. Mitochondrial superoxide anion levels in non-synaptic mitochondria. Brain cortex non-synaptic mitochondria fractions were loaded with the potentiometric probe MitoSOX and direct measurements of mitochondrial superoxide were obtained by flow cytometry. **(a, b):** Typical dot-blot of FSC-H vs. SSC-H indicating a gated mitochondrial population (R1) and the histograms of gated events versus relative fluorescence intensity (FL-1) corresponding to unloaded or 1 μM antimycin for control (a) and hangover mice (b). Samples without probe used for autofluorescence are shown in the insets. Each histogram represents a typical experiment, which was performed in triplicate. **(c):** Bars graph quantification of the amount of MitoSOX relative fluorescence events. Fluorescence events were quantified as the number of events which drop under a common marker M1 (fixed at the median value of the control histogram) taking control fluorescence events as 100%. ANOVA, Tukey's test ***$p < 0.001$, compared with same group without antimycin. Bars represent the mean ± SEM of 3 different experiments. Antimycin A was used as positive control. NS mit: non-synaptic mitochondria; NS mit + Ant: non-synaptic mitochondria with antimycin.

2.11. Statistical analysis

Prior to each analysis, test variables were checked for normality; all data were evaluated by the Kolmogorov-Smirnov test in order to follow a posterior parametric or nonparametric statistical analysis. As the obtained data followed a parametric distribution, results were presented as mean ± SEM. The analysis of the results was performed using unpaired Student *t*-test or ANOVA and post-hoc Tukey in order to

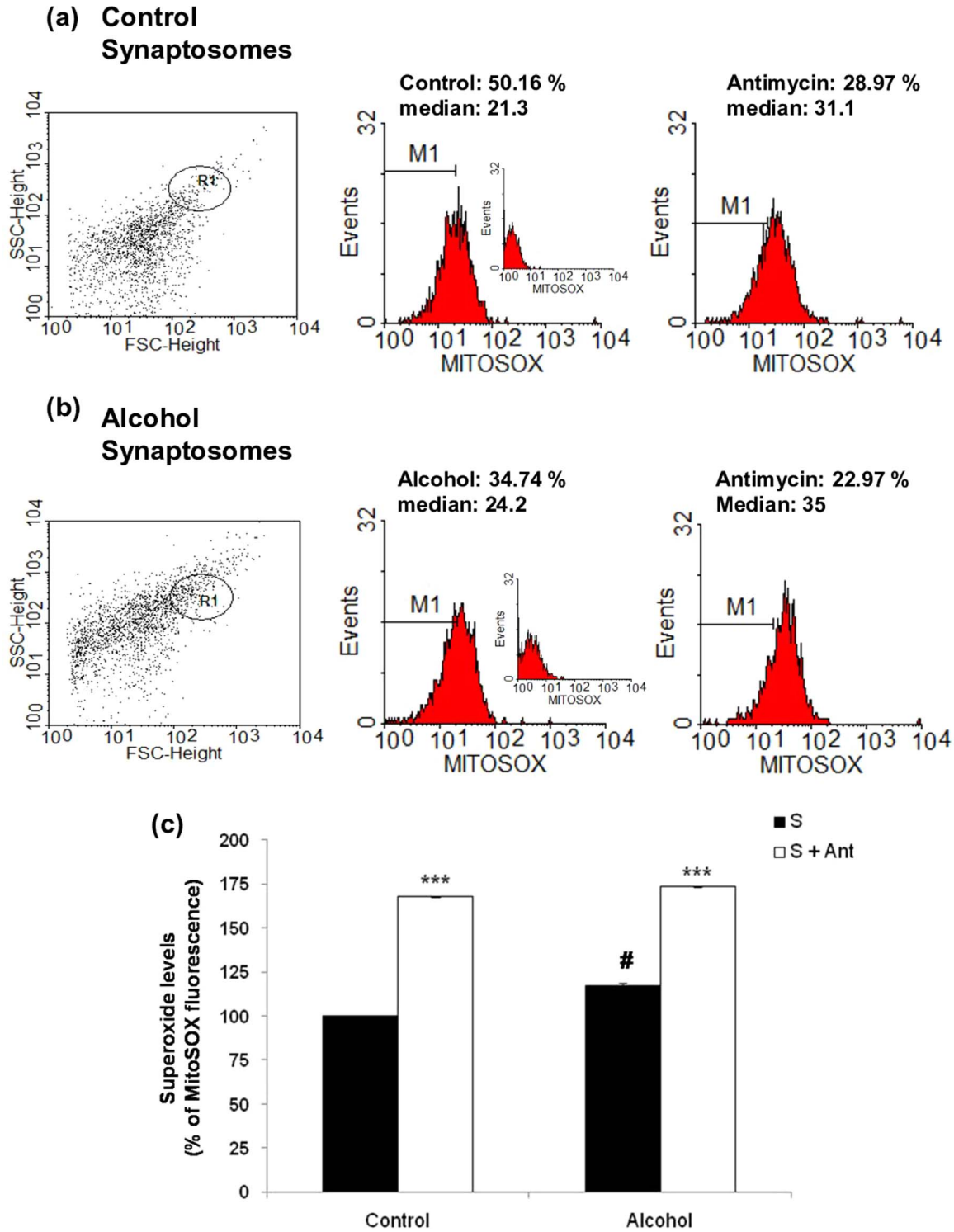

Fig. 2. Mitochondrial superoxide anion levels in synaptosomes. Brain cortex synaptosomes fractions were loaded with the potentiometric probe MitoSOX and direct measurements of mitochondrial superoxide were obtained by flow cytometry. **(a, b):** Typical dot-blot of FSC-H vs. SSC-H indicating a gated mitochondrial population (R1) and the histograms of gated events versus relative fluorescence intensity (FL-1) corresponding to unloaded or 1 μM antimycin for control (a) and hangover mice (b). Samples without probe used for autofluorescence are shown in the insets. Each histogram represents a typical experiment, which was performed in triplicate. **(c):** Bars graph quantification of the amount of MitoSOX relative fluorescence events. Fluorescence events were quantified as the number of events which drop under a common marker M1 (fixed at the median value of the control histogram) taking control fluorescence events as 100%. ANOVA, Tukey's test ***$p < 0.001$, compared with same group without antimycin; #$p < 0.05$, compared control group. Bars represent the mean ± SEM of 3 different experiments. Antimycin A was used as positive control. NS mit: non-synaptic mitochondria; NS mit + Ant: non-synaptic mitochondria with antimycin.

analyze the significance of differences between groups. IBM SPSS Statistics (22.0 version) software was used and a difference was considered to be statistically significant when $p < 0.05$.

3. Results

3.1. Mitochondrial superoxide levels

Dotblot of FSC-H vs. SSC-H indicating the gated mitochondrial population is shown in Figs. 1 and 2 for non-synaptic mitochondria and synaptosomes respectively. Alcohol hangover did not modify MitoSOX fluorescence median in non-synaptic mitochondria compared with control group (Fig. 1a, b). On the other hand, synaptosomes from alcohol-treated mice showed an increase in MitoSOX fluorescence median compared with control group (21.3 vs. 24.2, respectively; Fig. 2a, b).

The quantification of fluorescence events from three independent experiments indicates that although non-significant, non-synaptic mitochondria showed a tendency of 10% to increase superoxide levels (Fig. 1c). In addition, synaptosomes exhibited a significant increase of 18% in superoxide levels ($p < 0.05$; Fig. 2c). As expected, antimycin provoked a significant increase in both subcellular fractions from control and AH conditions ($p < 0.01$; Figs. 1 and 2).

3.2. Mitochondrial hydrogen peroxide production

Malate-glutamate- supported hydrogen peroxide production was measured in brain cortex non-synaptic mitochondria and synaptosomes from the two studied groups in the presence or absence of an inhibitor of MAO (deprenyl). Data are included in Fig. 3. Results show that H_2O_2 production rates in hangover animals were 3-fold increased in non-synaptic mitochondria ($p < 0.01$) and 4-fold increased in synaptosomes ($p < 0.05$) compared to control condition.

Deprenyl did not induce significant differences in H_2O_2 production in control samples from both subcellular fractions. On the other hand, deprenyl induced a 66–67% decrease in H_2O_2 production both in synaptosomes and NS mitochondria from hangover group compared with the same condition without deprenyl ($p < 0.05$). However, while H_2O_2 production in the presence of deprenyl was not different from the control condition for non-synaptic mitochondria, it was still higher than controls in synaptosomal fractions from hangover condition ($p < 0.05$).

3.3. Cardiolipin content

Cardiolipin content was determined by flow cytometry. Results represented by histograms showed that acute ethanol exposure did not modify intact cardiolipin content in non-synaptic mitochondria (Fig. 4a, b). This can also be visualized in the bar graph which represents the quantification of NAO fluorescence events (Fig. 4c). On the other hand, synaptosomes from ethanol-treated mice showed a 55% decrease in NAO fluorescence ($p < 0.01$) which indicated a decrease in cardiolipin content (Fig. 5c). As it was expected, the inhibitor of the ubiquinone-cytochrome c reductase, antimycin, induced a significant decrease in NAO fluorescence in both subcellular fractions from control and hangover animals.

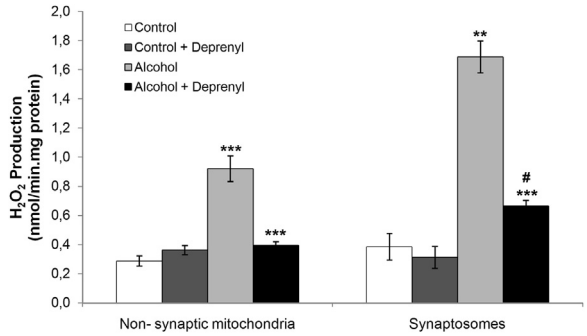

Fig. 3. Mitochondrial hydrogen peroxide production rate in non-synaptic mitochondria and synaptosomes. Mitochondria were supplemented with 6 mM malate plus 6 mM glutamate, as described under Materials and methods. Deprenyl was used as MAO inhibitors. Bars represent the mean ± SEM of four to six individual mitochondria samples, each obtained from a pool of 5 mice brain cortexes. ANOVA, Tukey's test: $^{**}p < 0.01$, $^{***}p < 0.001$, $\#p < 0.05$, compared with control group. Bar colors indicate: white, control; dark gray, control with deprenyl; gray, alcohol; black, alcohol with deprenyl.

3.4. Enzymes activity

Catalase and superoxide dismutase activities were determined in non-synaptic mitochondria, synaptosomes and cytosol from brain cortex by spectrophotometric measurements (Table 1). Superoxide dismutase was 60% decreased in non synaptic mitochondria and 80% and 29% increased in synaptosomes ($p < 0.01$) and cytosolic fractions respectively ($p < 0.05$). Together with this, catalase activity was found 40%, 50% and 60% decreased in non-synaptic mitochondria, synaptosomes and cytosol respectively compared to controls ($p < 0.05$).

On the other hand, monoamine oxidase activity was determined in both subcellular fractions from control and ethanol hangover mice (Table 1). MAO activity was 57% increased in non-synaptic mitochondria ($p < 0.05$) and 3-fold increased in synaptosomes in the hangover condition ($p < 0.001$). In addition, results indicates that acetylcholinesterase (AChE) activity was 11% increased in synaptosomes from ethanol-treated group compared to controls ($p < 0.05$, data no shown).

3.5. Reduced and oxidized glutathione contents

Levels of GSH and GSSG were determined in different subcellular fractions by HPLC. Typical chromatograms for GSH and GSSG are shown in Fig. 6. Quantifications of GSH and GSSG levels and the ratio GSH/GSSG were included in Table 2. Alcohol hangover induced a 43% decrease in GSH levels in the synaptosomes ($p < 0.05$) and a 17% decrease in cytosol ($p < 0.05$). No significant changes were observed in GSH content in non-synaptic mitochondria. On the other hand, GSSG remained unchanged in the three studied subcellular fractions. The ratio GSH/GSSG was calculated in order to evaluate redox status. In this sense, and as a consequence of the decrements observed for GSH levels, synaptosomes and cytosol fractions from AH mice showed decreases of 43% and 18% in the ratio GSH/GSSG respectively ($p < 0.05$) compared with controls.

3.6. GSH-related enzyme systems

Glutathione-related enzymes activities were determined in NS-mitochondria, synaptosomes and cytosol. Results are shown in Table 3. Glutathione peroxidase activity was 40% decreased in non-synaptic mitochondria ($p < 0.01$) and 65% decreased in both synaptosomes and cytosol from AH mice ($p < 0.01$). Glutathione reductase activity was 23% decreased in synaptosomes ($p < 0.05$) while no significant changes were observed in non-synaptic mitochondria and cytosol from AH mice as compared with controls. Glutathione S-transferase activity was 83% increased in synaptosomes ($p < 0.001$); however, the enzyme activity was not different in non-synaptic mitochondria and cytosol from EtOH-treated mice with respect to controls.

4. Discussion

Results from our laboratory demonstrated that alcohol hangover induced a significant mitochondrial dysfunction and oxidative stress evaluated in a crude mitochondrial fraction from mouse brain cortex and cerebellum [11–13]. Considering that some authors have hypothe-

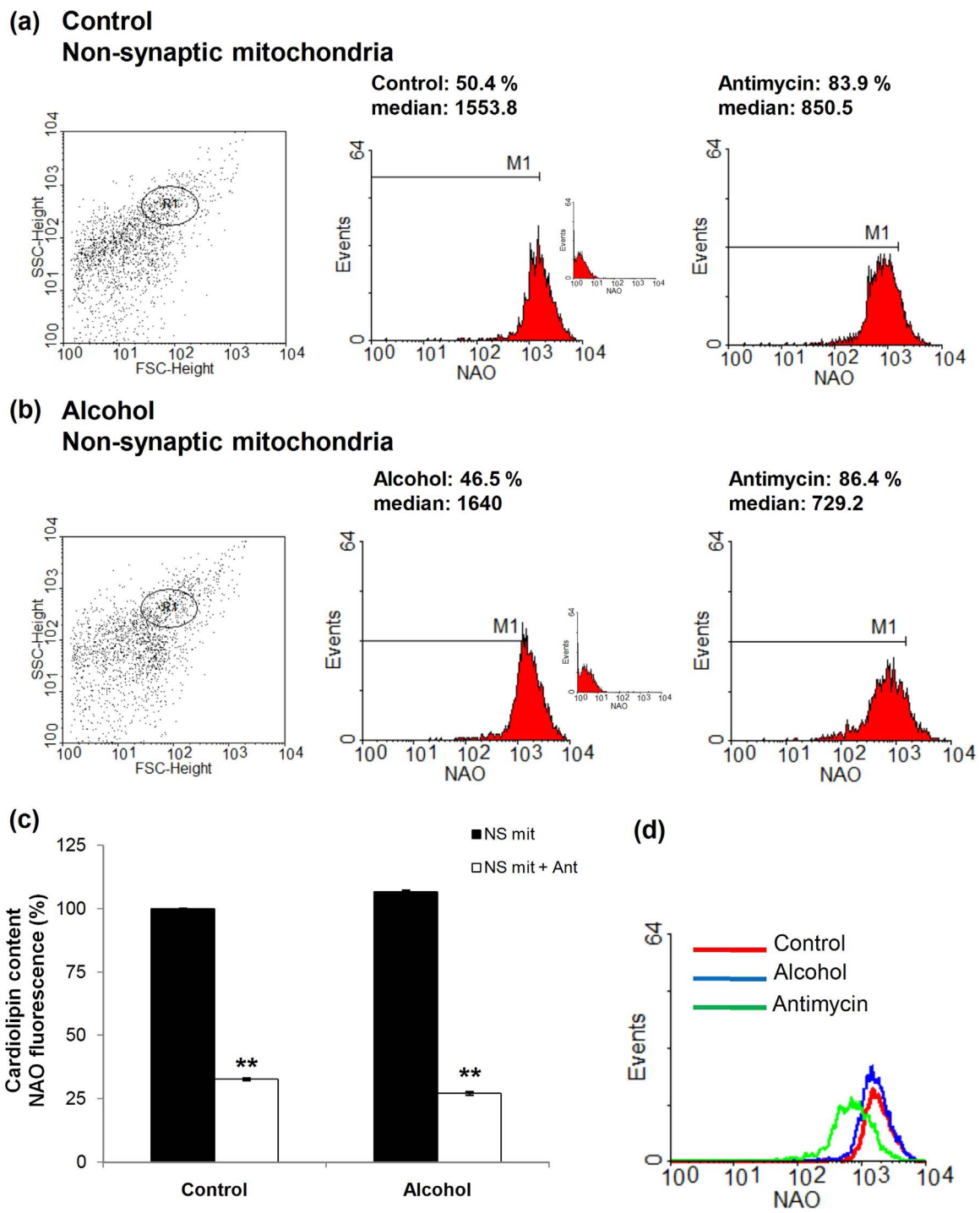

Fig. 4. Cardiolipin content through the changes in NAO fluorescence intensity in non-synaptic mitochondria. (a, b): Typical dot-blot of FSC-H vs. SSC-H indicating a gated mitochondrial population (R1) and the histograms of gated events versus relative fluorescence intensity (FL-1) corresponding to unloaded or 1 μM antimycin for control (a) and hangover mice (b). Samples without probe used for autofluorescence are shown in the insets. Each histogram represents a typical experiment, which was performed in triplicate. **(c):** Bars graph quantification of the amount of NAO relative fluorescence events. Fluorescence events were quantified as the number of events which drop under a common marker M1 (fixed at the median value of the control histogram) taking control fluorescence events as 100%. ANOVA, Tukey's test $^{**}p < 0.01$, compared with same group without antimycin. Bars represent the mean ± SEM of 3 different experiments. Antimycin A was used as positive control of lipid peroxidation. NS mit: non-synaptic mitochondria; NS mit + Ant: non-synaptic mitochondria with antimycin. **(d):** Overlapped histograms showing the effect of the three experimental conditions on NAO relative fluorescence.

sized that the major EtOH effects occurs at synapses [10,17,24], the aim of this work was to study free radicals production and antioxidant enzymes activities in synaptosomes and non-synaptic brain cortex mitochondria at the beginning of alcohol hangover after acute EtOH exposure.

The increased oxidative stress as a consequence of alcohol exposure together with impaired epithelial barrier integrity and depressed immune response have been largely explored [54], however, increment in oxidative stress as part of the after-effect of acute EtOH exposure was not studied in synaptosomes before. The results obtained showed that even though superoxide levels showed a tendency to increase in non-synaptic mitochondria, the effect of alcohol hangover was significantly harmful for mitochondria at nerve terminals as it induced an increment of 18% in superoxide anion levels in synaptosomes. Together with this,

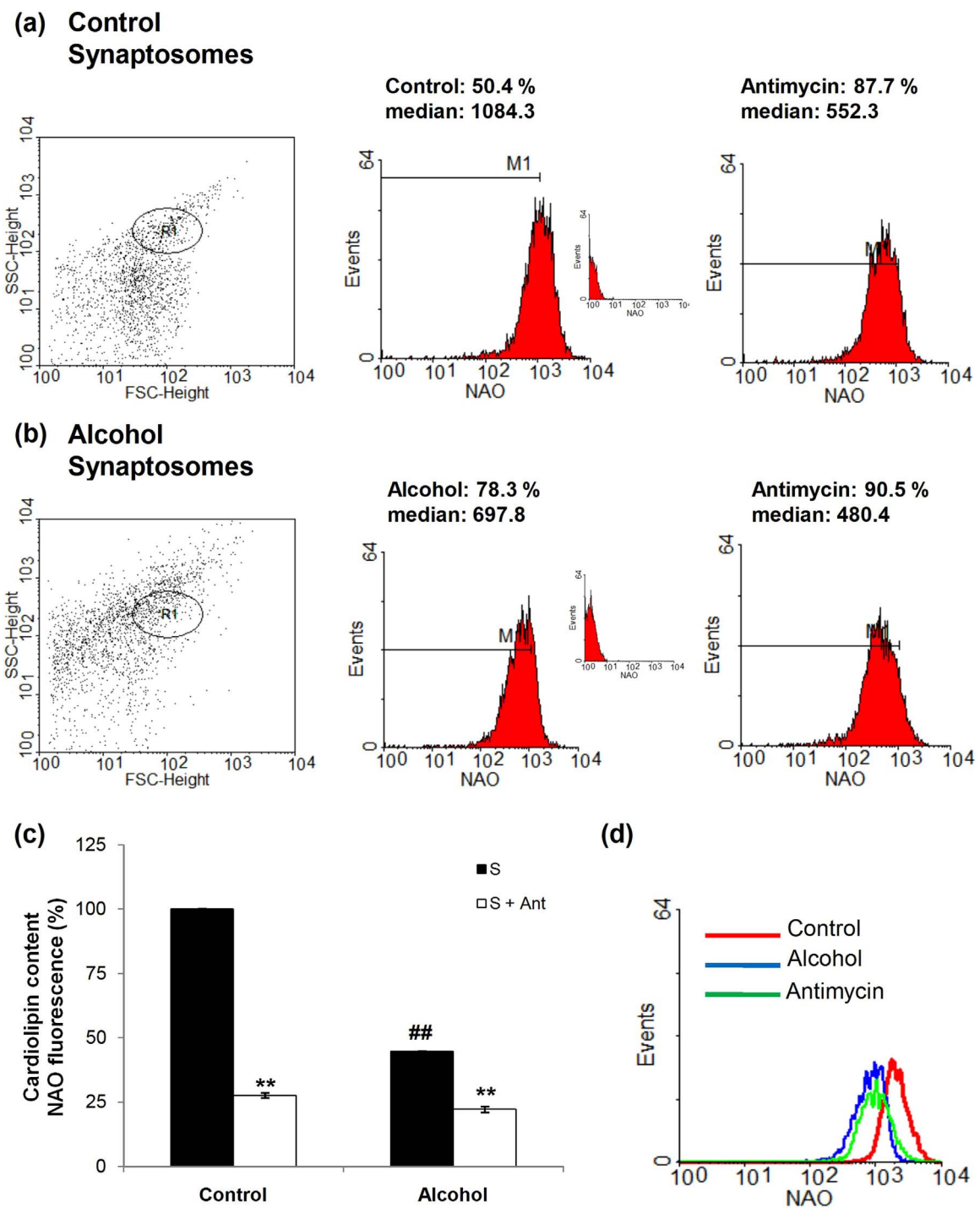

Fig. 5. Cardiolipin content through the changes in NAO fluorescence intensity in synaptosomes. (a, b): Typical dot-blot of FSC-H vs. SSC-H indicating a gated synaptosomes population (R1) and the histograms of gated events versus relative fluorescence intensity (FL-1) corresponding to unloaded or 1 μM antimycin for control (a) and hangover mice (b). Samples without probe used for autofluorescence are shown in the insets. Each histogram represents a typical experiment, which was performed in triplicate. (c): Bars graph quantification of the amount of NAO relative fluorescence events. Fluorescence events were quantified as the number of events which drop under a common marker M1 (fixed at the median value of the control histogram) taking control fluorescence events as 100%. ANOVA, Tukey's test $^{**}p < 0.01$, compared with same group without antimycin; $^{\#\#}p < 0.01$, compared control group. Bars represent the mean ± SEM of 3 different experiments. Antimycin A was used as positive control of lipid peroxidation. S synaptosomes; S + Ant: synaptosomes with antimycin. **(d):** Overlapped histograms showing the effect of the three experimental conditions on NAO relative fluorescence.

hydrogen peroxide production was 3-fold increased in non-synaptic mitochondria and 4-fold increased in synaptosomes at the onset of alcohol hangover.

On the other hand, it is important to recall that the observed EtOH effects on H_2O_2 production in both studied fractions could not be separated from those that can be found in astrocytes since preparations may contain mitochondria from astrocytes as well. Even though astrocytes seem less vulnerable than neurons, studies by Gonthier et al. demonstrated that astrocytes sensitivity to H_2O_2 toxicity is enhanced by EtOH exposure, as shown by a decreased astrocytic viability and DNA damage [55].

Particularly, the use of deprenyl as a MAO inhibitor gave support to the idea that the increment in H_2O_2 production in non-synaptic mitochondria came from MAO activity while in synaptosomes H_2O_2

Table 1
Effect of alcohol hangover on the activity of superoxide dismutase, catalase and monoamine oxidase in brain cortex subcellular fractions.

Enzyme	Subcellular fraction	Control	Alcohol
SOD (U/mg protein)	NS mitochondria	14.40 ± 0.01	6.01 ± 1.03**
	Synaptosomes	19.10 ± 0.04	34.21 ± 0.42**
	Cytosol	44.40 ± 2.12	57.30 ± 2.10*
CAT (pmol/min mg protein)	NS mitochondria	0.14 ± 0.02	0.09 ± 0.01*
	Synaptosomes	0.15 ± 0.05	0.08 ± 0.01*
	Cytosol	0.27 ± 0.01	0.11 ± 0.02*
MAO (nmol/min mg protein)	NS mitochondria	7.02 ± 0.65	21.10 ± 2.80**
	Synaptosomes	7.80 ± 0.37	12.30 ± 0.37**

Results are expressed as mean values ± SEM of 4–6 individual non-synaptic (NS) mitochondria, synaptosomes or cytosol samples, each obtained from a pool of brain cortex from 5 mice. ANOVA, Tukey test.
* $p < 0.05$ compared with control group.
** $p < 0.01$ compared with control group.

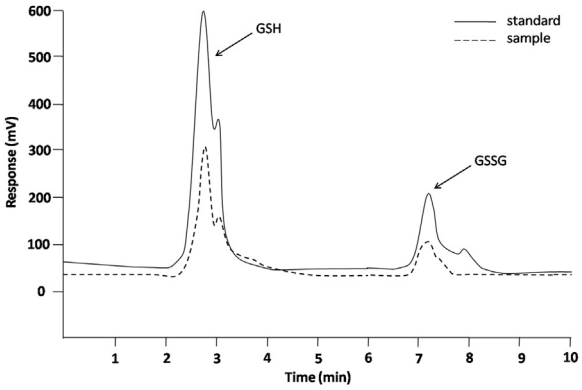

Fig. 6. Typical HPLC chromatogram of GSH and GSSG.The analysis shows GSH and GSSG signals (mV) during time (minutes). The signal peaks for GSH and GSSG standards are shown (solid line). Dotted line corresponds to GSH and GSSG signals for control synaptosomes.

Table 2
Effect of alcohol hangover on GSH, GSSG and GSH/GSSG ratio in brain cortex subcellular fractions.

Enzyme	Subcellular fraction	Control	Alcohol
GSH (nmol/mg protein)	NS mitochondria	8.5 ± 1.8	6.8 ± 1.4
	Synaptosomes	19.1 ± 5.3	10.9 ± 1.7*
	Cytosol	89.1 ± 1.7	74.3 ± 1.2*
GSSG (nmol/mg protein)	NS mitochondria	0.9 ± 0.1	0.7 ± 0.2
	Synaptosomes	1.5 ± 0.5	1.5 ± 0.1
	Cytosol	8.9 ± 0.9	9.1 ± 2.2
GSH/GSSG	NS mitochondria	9.7 ± 0.2	9.5 ± 0.3
	Synaptosomes	12.7 ± 0.7	7.2 ± 0.2*
	Cytosol	10.0 ± 0.8	8.2 ± 0.3*

Results are expressed as mean values ± SEM from triplicates of each of 3 individual non-synaptic (NS) mitochondria, synaptosomes or cytosol samples, each obtained from a pool of brain cortex from 5 mice. ANOVA, Tukey test.
* $p < 0.05$ compared with control group.

production would be a consequence of both MAO and respiratory chain. This hypothesis was also supported by the data obtained for MAO activity which resulted to be increased both in non-synaptic mitochondria and synaptosomes. In this sense, it was previously demonstrated that acute EtOH exposure induced the activation of MAO in rat brain stem and cerebral cortex [56]; however, this is the first evidence of the persistence of MAO activation in the hangover condition when EtOH is absent in plasma.

Cardiolipin content was analyzed in both subcellular fractions in control and hangover animals. Synaptosomes from ethanol-treated animals exhibited a significant reduction in cardiolipin content. This

Table 3
GSH-related enzyme systems in brain cortex subcellular fractions.

Enzyme	Subcellular fraction	Control	Alcohol
GPx (μmol/min mg protein)	NS mitochondria	84 ± 4	49 ± 1**
	Synaptosomes	100 ± 8	35 ± 3**
	Cytosol	222 ± 15	79 ± 6**
GR (μmol/min mg protein)	NS mitochondria	7.5 ± 0.4	7.6 ± 0.7
	Synaptosomes	12.3 ± 0.7	9.7 ± 0.6*
	Cytosol	14.9 ± 1.1	16.6 ± 2.7
GST (nmol/min mg protein)	NS mitochondria	2.9 ± 0.4	3.5 ± 0.3
	Synaptosomes	2.3 ± 0.1	4.2 ± 0.5***
	Cytosol	6.4 ± 1.0	5.7 ± 1.0

Results are expressed as mean values ± SEM from triplicates of each of 3 individual non-synaptic (NS) mitochondria, synaptosomes or cytosol samples, each obtained from a pool of brain cortex from 5 mice. ANOVA, Tukey test.
* $p < 0.05$ compared with control group.
** $p < 0.01$ compared with control group.
*** $p < 0.001$ compared with control group.

result could be understood considering the strong oxidative stress observed in synaptosomes. As it was described above, this subcellular fraction displayed increases in the production of superoxide anion and hydrogen peroxide which in turn induce the oxidation of cardiolipin in the inner mitochondrial membrane. A previous research demonstrated that acute doses of EtOH ingestion enhanced lipid peroxidation in rat brain synaptosomes and microsomes even higher than mitochondria or cytosol [57].

A previous study demonstrated that AChE activation could be correlated with an enhancement of lipid peroxidation in brain cortex and hippocampus [58]. Accordingly, our results showed that AChE activity was 11% increased in synaptosomes from AH condition. This could be detrimental for CNS since the enzyme is responsible for terminating the cholinergic transmission by degradation of acetylcholine into choline and acetate. The increment in AChE activity could lead to a decrease in acetylcholine levels and thus disfavour nervous impulse. Years ago and in contrast to our result, high alcohol concentration exposure was associated with a decline in AChE activity [59]. On the other side, Mathis et al. have discovered that the administration of an acetylcholinesterase inhibitor, immediately enhances sympathetic nervous system activity, restores the neuroendocrine response and improves hemodynamics after acute alcohol intoxication [60].

On the other hand, non-synaptic mitochondria did not show any changes in the mitochondrial phospholipid content. In order to analyze this absence of effect over cardiolipin content in non-synaptic mitochondria, it is necessary to take into account some considerations in relation to MAO and antioxidant enzyme activities, together with glutathione metabolism. In NS-mitochondria, hydrogen peroxide production came mainly from MAO activation; being this enzyme located in the external membrane, it could be hypothesized that hydrogen peroxide could spread outward mitochondria without altering cardiolipin. Nevertheless, hydrogen peroxide diffusion inside mitochondria cannot be discarded and should be analyzed in the context of antioxidant enzymes activity and glutathione balance.

Acute EtOH exposure presents after-effects in synaptosomes and non-synaptic mitochondria which are mainly characterized by oxidative stress due to the enhancement of oxidative species production and decrements in enzymatic and non-enzymatic antioxidants. Some previous studies in rodents implicated oxidative stress as a key mediator of hangover syndrome, and demonstrated that antioxidants could suppress the adverse events caused by alcohol exposure; in fact, the importance of the antioxidant imbalance due to excessive alcohol exposure was exposed by a recent review which showed that natural products could treat hangover and other alcohol use disorders mainly in liver and brain [61].

In non-synaptic mitochondria, catalase and superoxide dismutase

activity were decreased by alcohol hangover showing that hydrogen peroxide detoxification is impaired. On the other hand, glutathione peroxidase activity was 40% decreased while no changes were observed in glutathione reductase and transferase activity in non-synaptic mitochondria. Moreover, no significant alterations were found in GSH and GSSG levels. Thereby, if hydrogen peroxide produced by MAO diffuses to mitochondrial matrix, it could not be efficiently detoxified by glutathione peroxidase. Nevertheless, as no decrements of glutathione content were found, we proposed that the presence of a proper balance GSH/GSSG protects mitochondrial environment preventing cardiolipin oxidation. Indeed, it was suggested that cells with more GSH have a greater redox buffering capacity than cells with low GSH levels [62].

During alcohol hangover, in synaptosomes, catalase and glutathione peroxidase activities were decreased and, in contrast to non-synaptic mitochondria, superoxide dismutase activity was significantly increased. This result together with the data obtained for reactive oxygen species production allow to propose that in synaptosomes, hydrogen peroxide not only comes from monoamine oxidase activity and respiratory chain but its level can also be increased by the driven activity of superoxide dismutase and the diminished detoxification by catalase and glutathione peroxidase. Related to glutathione imbalance, alcohol hangover induced a 43% decrease in GSH content which led to a decrease in the ratio GSH/GSSG in the same proportion. In agreement with our results, Reddy et al. found that oral doses of EtOH decreased GSH concentration in rat brain synaptosomes [57]. The imbalance of ratio GSH/GSSG constitutes a clear sign of an altered redox state in cells [62]. Indeed, the relation GSH/GSSG is the most important and commonly measured couple which allows an estimation of physiological cellular redox state, being this essential to maintain redox status of proteins [63,64]. Besides, it was previously stated that if GSH is depleted to levels below a threshold between 30% and 40%, an increase in H_2O_2 release from mitochondria will occur because GSH is needed by glutathione peroxidase to detoxify H_2O_2 [62]. Additionally, glutathione-related enzymes activities were altered in synaptosomes due to hangover. Particularly, glutathione reductase activity was decreased impeding the regeneration of GSH; even more, glutathione transferase activity was found increased promoting the detoxification of xenobiotics compounds through conjugation with GSH. Particularly, it was well established that the reduction of hydroperoxides by GSH with the concomitant formation of GSSG and its recycling back to GSH by GSH-reductase is an effective means by which the mitochondrial GSH prevents oxidative damage [65].

Cytosolic enzymatic and non-enzymatic antioxidants were determined in order to better understand the redox status in the cytosolic environment and whether or not it was modified by alcohol hangover. For instance, alcohol hangover decreased cytosolic catalase and glutathione peroxidase activity and increased superoxide dismutase activity. Together with this, GSH was 17% diminished generating a decrease in the ratio GSH/GSSG as observed for synaptosomes. No changes were observed in cytosolic glutathione reductase and glutathione transferase activities. Thus, alcohol hangover alters GSH redox status and induced a decrease in antioxidant enzymes activities.

Related to antioxidant enzymes, several studies have demonstrated that EtOH exposure induces decreases in the synthesis of Cu and Zn-SOD, CAT and GPx both in alcoholic humans and animals models of EtOH dependence [66,67]. The reduction in antioxidant enzymes was proposed to be related not only with alterations in the gene expression but also with changes in the protein structure [68].

Other evidences, in the context of antioxidant defenses, indicate that the expression of peroxiredoxins (Prdx), whose activity lies in the reduction and detoxification of H_2O_2, peroxynitrite, and other organic hydroperoxides, was found to be decreased due to alcohol exposure, thus contributing to mitochondrial redox imbalance [69]. Moreover, it was shown that in vitro EtOH exposure of a mouse cell line induces decreases in mRNAs Prdx3 and Prdx5 plus the mitochondrial thiol-disulfide proteins glutaredoxin 2, GR and GPx [70]. In the context of alcohol hangover, it was demonstrated that the oxidation and inactivation of Prx could take place rapidly (as early as 1 h after alcohol exposure) being consistent also with the increased H_2O_2 level detected in E47 HepG2 cells [71].

A differential response of synaptic and NS mitochondria has been previously reported; for instance, we recently have shown that synaptic mitochondria seem to be more susceptible than non-synaptic mitochondria to suffer mitochondrial respiratory dysfunction and alterations in calcium homeostasis due to aging [35].

Considering that the present work shows that synaptosomes suffer more oxidative damage than NS mitochondria after acute EtOH exposure, future experiments will be conducted in order to study mitochondrial bioenergetics in nerve terminals mitochondria. Moreover, additional studies could be necessary to evaluate if the present changes observed both in synaptosomes and non-synaptic mitochondria could be reversed or if a recovery period of time could be determined as we previously established for behavioural alterations in the same animal model [7,8].

The present data support the importance of studying alcohol hangover state since it presents pathological implications in CNS. In fact, in the last few years, it was demonstrated that acute EtOH exposure and its direct consequence, the alcohol hangover, can alter neuronal integrity in vulnerable CNS regions such as the cerebellum, hippocampus and brain cortex by inducing mitochondrial swelling [12,13], membrane potential collapse and decrease of cytochrome c oxidase activity [72]. Moreover, when brain is repeatedly exposed to such chaotic events, neuronal injury can lead to motor, affective and cognitive disturbances as observed in our previous studies of ethanol hangover [7,8] and also during withdrawal from chronic ethanol treatment [73].

5. Conclusions

The present work showed that alcohol hangover induced oxidative stress observed both in mouse brain cortex synaptosomes and non-synaptic mitochondria six hours after of acute ethanol exposure. The data obtained showed that non-synaptic mitochondria exhibited changes in antioxidant enzymes while glutathione balance and its related enzymes were preserved. In contrast, significant increments in oxidative species production and decrements in antioxidant enzymes activities together with an imbalance in glutathione levels and its related enzymes were found for synaptosomes. Thereby synaptosomes resulted to be the most affected subcellular fraction revealing an imbalance in the cellular redox homeostasis mainly affecting mitochondria present in synaptic terminals.

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