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Does chronic alcohol exposure induce neurodegeneration in the central nervous system of rats?

Karla Hernández-Fonseca,[1] Paula Martinell,[1] Cosette Reyes-Guzmán,[1] Milagros Méndez[1]

Original article

ABSTRACT

Background

Chronic alcohol exposure is associated with neurotoxic and neurodegenerative mechanisms that lead to several cognitive and memory dysfunctions. Alcohol-induced damage depends on ethanol consumption patterns. Prolonged alcohol exposure induces damage in distinct brain regions (prefrontal, perirhinal, entorhinal and parahippocampal cortices, thalamus, hypothalamus, hippocampus, and cerebellum) in both alcoholic patients and animal models of alcoholism. However, brain areas of the drug reinforcement and reward circuit have not been investigated.

Objective

To investigate if chronic alcohol exposure induces neurodegenerative damage in the rat brain, particularly in the mesocorticolimbic system and the amygdala.

Method

Male Wistar rats were exposed to ethanol (10% v/v) or water by oral consumption for 30 days. In another set of experiments, animals similarly treated with ethanol were withdrawn from the drug for 24 and 48 hours. At the end of the treatments, the animals were killed, whole blood samples were obtained, and the brains were removed. A fluorescence marker (Fluoro-Jade B) was used to assess neurodegenerative damage in the brain. Blood alcohol concentration was evaluated by spectrophotometry.

Results

We observed a low number of cells positive to Fluoro-Jade B in different brain regions, including the piriform cortex, frontal cortex of association, caudate-putamen, and dorsal thalamus. No differences were found between chronic alcohol- or ethanol-withdrawn groups versus control animals.

Discussion and conclusion

Our results suggest that chronic alcohol exposure does not induce neurodegeneration under the present experimental conditions. Alcohol blood concentrations attained during treatment may not be sufficient to induce cell death.

Key words: Alcohol, ethanol, chronic treatment, cell damage, neurodegeneration.

RESUMEN

Antecedentes

La exposición crónica al alcohol se asocia con procesos neurotóxicos y neurodegenerativos relacionados con disfunciones cognitivas y de memoria. El daño inducido por alcohol depende de los patrones de consumo de etanol. La exposición prolongada al alcohol induce daño en distintas regiones cerebrales (cortezas prefrontal, perirrinal, entorrinal y parahipocampal, tálamo, hipotálamo, hipocampo y cerebelo) en pacientes alcohólicos y modelos animales de alcoholismo. Sin embargo, no se han estudiado las regiones cerebrales asociadas con el circuito de reforzamiento y recompensa de drogas de abuso.

Objetivo

Investigar si la exposición crónica al alcohol induce daño neurodegenerativo en el cerebro de la rata, en particular en el sistema mesocorticolímbico y la amígdala.

Método

Ratas Wistar macho fueron expuestas a etanol (10% v/v) o agua por consumo oral durante 30 días y se les privó de la droga por 0, 24 y 48h. Los animales fueron sacrificados y se les extrajo la sangre troncal y el cerebro. Para evaluar el daño neurodegenerativo, se utilizó el marcador fluorescente Fluoro-Jade B. La concentración de alcohol en sangre se determinó por espectrofotometría.

Resultados

Se observó un escaso número de células positivas a Fluoro-Jade en las cortezas piriforme y frontal de asociación, el caudado-putamen y el tálamo dorsal. No se encontraron diferencias entre el tratamiento crónico o la privación de alcohol *versus* el grupo control.

Discusión y conclusión

La exposición crónica al alcohol no indujo neurodegeneración en las condiciones utilizadas en este estudio. Probablemente, las concentraciones de alcohol en sangre alcanzadas durante el tratamiento no fueron suficientes para inducir muerte celular.

Palabras clave: Alcohol, etanol, tratamiento crónico, daño celular, neurodegeneración.

[1] Department of Neurochemistry, Subdirectorate of Clinical Research, Ramón de la Fuente Muñiz National Institute of Psychiatry.

Correspondence: Dra. Milagros Méndez. Department of Neurochemistry, Subdirectorate for Clinical Research, Ramón de la Fuente Muñiz National Institute of Psychiatry. Calz. México-Xochimilco 101, San Lorenzo Huipulco, Tlalpan, 14370 Mexico City. E-mail: ubach@imp.edu.mx

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BACKGROUND

Use and abuse of alcohol (ethanol) is a health risk factor that can lead to dependence on the drug. According to the World Health Organization,[1] alcohol abuse and dependence is the third highest risk factor for health worldwide. It is estimated that it contributes to 4% of the global risk of death worldwide, only slightly below smoking. Latin American countries (including Mexico) and former Soviet Union countries are those where alcohol consumption presents more than double the global health risk.[2] In Mexico, around 46.3% of the population aged between 12 and 65 ingests alcoholic drinks. This percentage corresponds to an approximate number of 34 million Mexicans. Of this total, around four million present patterns of abuse of, or dependence on, alcohol. And of these, 1.5 million require medical treatment in clinics for detoxification and rehabilitation. In Mexico, three in ten people receive some kind of treatment, which represents a cost to the State of 200 million pesos a year.[3]

The most frequent pattern of alcohol consumption in Mexico is drinking very high quantities in short periods (high consumption per occasion, or binge drinking). This places Mexico among the countries with the highest incidence of conditions and disorders associated with alcohol consumption. It is estimated that alcohol abuse alone represents 9% of the total diseases in the country.[4]

Alcohol consumption represents a risk associated with at least 60 medical disorders or complications, among which are cirrhosis, followed by cardiovascular and renal problems, diabetes, neuropsychiatric disorders, neoplasms of the mouth, esophagus, and liver, and homicides or accidental deaths.[2] Variations in the proportion of deaths attributable to alcohol consumption are due to the absolute quantity of alcohol ingested, as well as to consumption patterns.

Given that alcohol consumption has increased considerably in Mexico in recent years, it is important to understand the effects and mechanisms of action that this substance has on the body, particularly the brain.

Alcohol addiction is a chronic disorder that is characterized by the compulsive seeking out and use of the substance, loss of control in consumption, and the emergence of a negative emotional state when access to alcohol is impeded (abstinence syndrome). Abuse of, and dependence on, alcohol is a syndrome that can be described as a three-phase cyclical process: intoxication (use of elevated doses of the drug in short periods or *binge drinking*), deprivation (negative emotional state), and anticipation (compulsion for the drug, which again leads to intoxication). The three stages interact, becoming even more intense and finally leading to the pathological state known as addiction.[5]

In the brains of mammals, there is a neuronal system that regulates the effects of natural stimulants which allow the survival of the species (food, water, and sex). This system, known as the reinforcement and reward circuit, is also affected by another type of non-natural stimulant, such as drugs of abuse.[6-8] This neuronal circuit comprises the mesocorticolimbic dopaminergic system (ventral tegmental area [VTA], nucleus *accumbens* [NAcc] and prefrontal cortex [PFC]), and the central nucleus of the amygdala.[6,9] The ethanol increases the frequency of these dopaminergic neurons being fired in the VTA, which results in a powerful release of dopamine (DA) in the NAcc.[10] This event is fundamental in the reinforcing effects of alcohol and other drugs of abuse. As well as DA, ethanol affects other systems of neurotransmitters and neuromodulators in the brain, including gamma-aminobutyric acid (GABA), glutamate, serotonin, and opioid peptides.[6,9]

Furthermore, alcohol affects various functions in the body including temperature regulation, motor coordination and behavior, circadian rhythms, and sleep patterns. Behaviorally, it brings biphasic effects which have been observed in both animals and humans. Low doses of ethanol induce locomotive stimulation in animals, which equates to a psychomotor and euphoric activation in humans. High doses of ethanol reduce locomotive activity and induce sedation.[11]

Excessive use of alcohol can cause structural and functional damage to the brain, which has been demonstrated in alcoholic individuals through various techniques, such as nuclear magnetic resonance (NMR), and positron emission tomography (PET). In these studies, a reduction has been observed in the weight and volume of the brains of alcoholic patients, as well as an increase in the size of the ventricles and a reduction in the thickness of the cerebral cortex.[12,13] Furthermore, it has been observed that the cerebral regions that suffer the most damage in these patients are the cerebral cortex (prefrontal, perirhinal, entorhinal, and parahippocampal), the thalamus, the hypothalamus, and hippocampus, and the cerebellum. The cortical region which seems to be invariably affected by alcohol is the frontal cortex. The damage to these structures is associated with a wide range and variety of cognitive and memory dysfunction.

Alterations in neurological functions commonly observed in alcoholic patients comprise deficits in abstract problem-solving, verbal and visuo-spatial learning, memory, and motor skills.[14,15] However, there are alcoholic patients with serious liver alterations as well as the aforementioned deficits. These individuals show a more deteriorated state of health, as they present Wernike-Korsakoff syndrome.[16-18] This neurological illness is associated with a deficiency of vitamin B1 (thiamine). The acute phase of this syndrome is Wernicke's encephalopathy, which is characterized by mental confusion, incapacity in spatial organization, ataxia, and loss of short-term memory. The symptoms of this phase reduce or disappear with abstinence and the administration of high doses of thiamine. Around 25% of people who have Wernicke's encephalopathy develop serious memory alterations, which characterizes Korsakoff's syndrome or psychosis.[18]

The severity of the damage caused by alcohol consumption depends on various factors, among which are genetic predisposition, gender, starting age for consumption, and diet. However, the factors with the greatest influence are alcohol consumption patterns (frequency and quantity of consumption) and the time between these behaviors.[17,19,20]

In rodents, blood alcohol levels reached and variations in terms of time are affected by a large number of factors, such as the breed of the animal, age, gender, prandial state, dosage, duration of exposure, and method of administration of alcohol.[21] Some authors have suggested that neurological alterations induced by alcohol could be due to the loss of neurons, which would cause neuronal death.[18,22]

Some studies have reported that the *in vivo* administration of ethanol to rodents produces damage depending on the protocol used. However, not all studies have shown neuronal damage due to alcohol.[23,24] For example, chronic treatment of alcohol with a liquid diet at 9% for four months induces a reaction in the number of hippocampal cells in the CA1 region.[25] Ethanol consumption at 22% for 12 months induces loss of cholinergic neurons in various regions of the anterior basal brain, among which the medial septum and Broca's area are the most affected.[26] Other studies have shown that the greater the quantity of alcohol ingested and the longer the exposure time to the substance, the greater the severity and extension of damage to the brain.[7,12,18,23,27,28] These works reveal that the cerebral regions present a vulnerability that is differential to the neurodegenerative effects of ethanol, because of which there are areas which are immediately affected by moderate doses of alcohol, while others do not show damage despite being continually exposed to high quantities of the substance. In rodents, the regions most vulnerable to alcohol-induced damage are the cerebral cortex (frontal, piriform, perirhinal, and entorhinal regions), regions CA1 and CA3 of the hippocampus, the olfactory bulb, and the cerebellum.[18,23,27,29-32] In spite of the neurotoxic effect of alcohol having been described on different regions of the brain, it is not known whether the areas which form part of the reinforcement and reward circuit of ethanol are vulnerable to the neurotoxic effects of the drug. Because of this, the aim of the present study was to investigate the neurodegenerative effects of chronic exposure to alcohol in different regions of the rat brain, emphasizing the DAergic mesocorticolimbic system and the amygdala.

METHOD

Treatment of the animals

Adult male Wistar rats (180-200g) maintained in a 12-hour light-darkness cycle (7:00-19:00hrs) were chronically treated with a 10% (v/v) ethanol solution for 30 days. The animals had free access to food and either to water (control group) or to the ethanol solution as the only source of liquid. Their weight and the amount of food and liquid they consumed were measured every day throughout the treatment. In a parallel series of animals, the ethanol solution was replaced with water for 24 and 48 hours after the chronic treatment. This was done with the aim of studying a group of rats deprived of the drug. At the end of the treatment, the rats (8 per group) were killed by decapitation; the brains were removed and core blood was collected. The brains were immediately frozen and maintained at -94°F for later analysis. The blood was processed to obtain the plasma, which was kept refrigerated. The experiments were carried out according to the regulations of the Ethics Committee of the Ramón de la Fuente Muñiz National Institute of Psychiatry and the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996).

Processing of tissue

The coronal section (20µm) of the animals' brains was obtained in a cryostat (Leika CM3050), according to the Paxinos and Watson atlas[23] (coordinates from -6.80 through 5.70 mm, in terms of bregma). The sections were stored at -94ºF until the following histological analyses were made: cresyl violet, Fluoro-Jade B and 4', 6-Diamidino-2-Phenylindole Dilactate (DAPI). Tissue integrity was analyzed through tinting with cresyl violet. In order to study the process of neurodegeneration, Fluoro-Jade B tint was used. The cellular nuclei were identified with DAPI tinting.

Histological analysis

Cresyl violet

The sections of frozen tissue were progressively brought to temperature, hydrated, and fixed in paraformaldehyde at 4%. The sections were later cleaned with a phosphate-buffered saline solution (PBS), dyed with cresyl violet (Sigma-Aldrich),[23] cleaned, and dehydrated in a series of alcohol solutions (60, 80, 95, and 100%). Finally, the sections were cleaned, introduced to xylol, and mounted with Permount (Fisher Scientific).

Fluoro-Jade B and DAPI

Cells in the process of neurodegeneration were studied following the Chemicon protocol,[34] with some modifications. The sections of tissue were dehydrated at 122ºF (30 min) and were passed sequentially through alcohol at 80% and 70%. They were later cleaned and covered with potassium permanganate at 0.06% for 10 min, and gently, continuously shaken. After this procedure, the sections were cleaned again and incubated in darkness with a Fluoro-Jade B solution at 0.0004% and DAPI at 0.0001% (in 0.1% acetic acid)[35] for 20 min. Finally, the sections were cleaned, dried at 122ºF and passed through xylol to later be mounted with Per-

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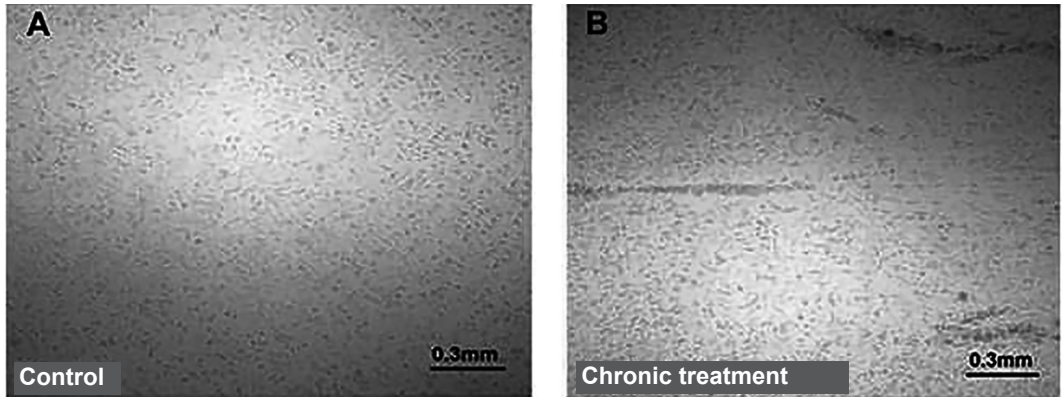

Figure 1. The effect of chronic exposure to alcohol on cellular morphology in the rat brain. Animals were kept in *ad libitum* (A) conditions or treated chronically with ethanol at 10% (v/v) (B) for 30 days. Coronal sections were obtained of 20 µm thickness and tinted with cresyl violet. A) frontal cortex of a control brain; B) mediodorsal thalamus of a brain with chronic ethanol treatment. Images were captured at 100x increase by an inverted microscope.

mount (Sigma-Aldrich). The sections were observed under an epiflourescent microscope (Olympus BX51) at a wavelength excitation of 450-490nm (Fluoro-Jade B) or 358nm (DAPI).

Determination of blood alcohol

The plasma was obtained through centrifusion of the core blood sample in the presence of ethylenediaminetetraaceitic acid (EDTA) (6 mM final) (Sigma-Aldrich) for 30 min at 3000 rpm a 39.2ºF. The supernatant was deproteinized with trichloroacetic acid (TCA) 0.38N, according to the indications for the enzymatic determination of ethanol in biological fluids.[36]

To determine the blood alcohol levels, a spectrophotometric method of enzymatic activity of the alcohol dehydrogenase (ADH) (Sigma-Aldrich) was used, according to that described by Poklis and Mackell,[36] with some modifications. This method quantifies the production of nicotinamide adenine dinucelotide reduced (NADH), whose absorbency is detected at a wavelength of 340nm. The reaction was carried out in the presence of a buffered solution of glycine-hydrazine 0.5 M, pH 8.8, NAD+ (1.8 µM) and ADH (1500 U/ml) for 30 min. The change in absorbency was quantified in a spectrophotometer (Thermo Spectronic, BioMate3). The ethanol levels in the sample were calculated through extrapolating the optical densities into a standard curve of known concentrations of ethanol.

Quantification of cells in degeneration

A total of 10-15 fields of 28 to 35 brain sections were analyzed. Cells positive to Fluoro-Jade B were identified in distinct cerebral regions. In the same way, the total number of nuclei tinted with DAPI was counted, which corresponds to the total number of cells/field/section/brain. The number of cells positive to Fluoro-Jade B is expressed as the percentage of the total of nuclei quantified by DAPI. Four animals per group were assessed in this study.

Statistical analysis

The blood alcohol data was analyzed by means of an ANOVA of two routes, considering the treatment and the deprivation time as sources of variation. The differences between groups were determined with the *post hoc* test by Tukey-HSD.

Table 1. Number of cells positive to Fluoro-Jade B in different cerebral regions of rat brains chronically exposed to alcohol for four weeks.

Control				Ethanol			
Rat	Number of cells positive to Fluoro-Jade B	Cells positive to Fluoro-Jade B (%)	Region with cells positive to Fluoro-Jade B	Rat	Number of cells positive to Fluoro-Jade B	Cells positive to Fluoro-Jade B (%)	Region with cells positive to Fluoro-Jade B
1	1	0.013	Granular insular cortex (left hemisphere)	1	1	0.030	Piriform cortex (right hemisphere)
2	–	–	–	2	2	0.019	Dorsal caudate putamen (CPu) (right hemisphere)
3	–	–	–	3	1	0.18	Paraventricular nucleus of thalamus
4	–	–	–	4	–	–	–

Table 2. Number of cells positive to Fluoro-Jade B in different cerebral regions of rat brains chronically exposed to alcohol for four weeks and deprived of the drug for 24 hours.

Control				Ethanol + Deprivatio			
Rat	Number of cells positive to Fluoro-Jade B	Cells positive to Fluoro-Jade B (%)	Region with cells positive to Fluoro-Jade B	Rat	Number of cells positive to Fluoro-Jade B	Cells positive to Fluoro-Jade B (%)	Region with cells positive to Fluoro-Jade B
1	–	–	–	1	–	–	–
2	–	–	–	2	0.003	0.004	Dorsal CPu (left hemisphere)
3	0.007	0.01	Mediodorsal thalamic nucleus (left hemisphere)	3	0.005	0.005	Hippocampus area CA1 (right hemisphere)
4	–	–	–	4	–	–	–

RESULTS

Chronic exposure to ethanol does not alter neuronal morphology

The histological analysis of the sections tinted with cresyl violet showed that chronic treatment with alcohol does not alter cellular morphology and that neuronal integrity is preserved (figure 1).

Chronic exposure to ethanol does not induce neurodegeneration in the rat brain

The quantification of the number of cells positive to Fluoro-Jade B in the sections coming from control rats and rats treated with ethanol for 30 days shows a mild effect of alcohol in some regions of the rat brain (table 1). This table specifies the percentage of cells positive to Fluoro-Jade B, as well as the cerebral regions where they were identified. In the control animals, we only observed one cell positive to Fluoro-Jade B in the insular granular cortex of one of the brains. In the animals treated with alcohol, we observed cells positive to Fluoro-Jade B in the piriform cortex, the caudate-putamen, and the paraventricular nucleus of the thalamus in three of the brains. These results indicate that in the experimental conditions used, and given the small number of cells positive to Fluoro-Jade B detected in the present study, alcohol does not induce neurodegeneration in any region of the rat brain.

Deprivation of ethanol in rats chronically treated with alcohol does not induce neurodegeneration in the rat brain

The quantification of the number of cells positive to Fluoro-Jade B in the sections coming from control rats and rats treated with the drug (24 and 48 hrs) after chronic treatment with alcohol for 30 days shows a mild neurodegenerative effect in some regions of the rat brain (tables 2 and 3). These tables specify the percentage of cells positive to Fluoro-Jade B, as well as the cerebral regions where they were identified.

In the control animals from the 24-hour deprivation experiment, we observed a cell positive to Fluoro-Jade B in the mediodorsal thalamic nucleus of one of the brains. Animals treated with alcohol and deprived of the drug for 24 hours had cells marked with Fluoro-Jade B in two of the brains. The regions where these cells were localized were the caudate-putamen and the CA1 region of the hippocampus (table 2). Furthermore, we observed that two of the brains of the control animals from the 48-hour deprivation experiments had cells positive to Fluoro-Jade B in the primary somatosensorial cortex and the dorsal raphe nucleus. In one of the brains of the animals treated with alcohol and deprived of the drug for 48 hours, we observed a cell marked with Fluoro-Jade B in the frontal association cortex (table 3). This set of data indicates that deprivation of ethanol in animals previously exposed to alcohol for 30 days does not induce neu-

Table 3. Number of cells positive to Fluoro-Jade B in different cerebral regions of rat brains chronically exposed to alcohol for four weeks and deprived of the drug for 48 hours

Control				Ethanol + Deprivation			
Rat	Number of cells positive to Fluoro-Jade B	Cells positive to Fluoro-Jade B (%)	Region with cells positive to Fluoro-Jade B	Rat	Number of cells positive to Fluoro-Jade B	Cells positive to Fluoro-Jade B (%)	Region with cells positive to Fluoro-Jade B
1	0.002	0.002	Primary somatosensory cortex (right hemisphere)	1	0.017	0.020	Frontal association cortex (left hemisphere)
2	–	–	–	2	–	–	–
3	0.390	0.430	Rostral raphe nucleus (right hemisphere)	3	–	–	–
4	–	–	–	4	–	–	–

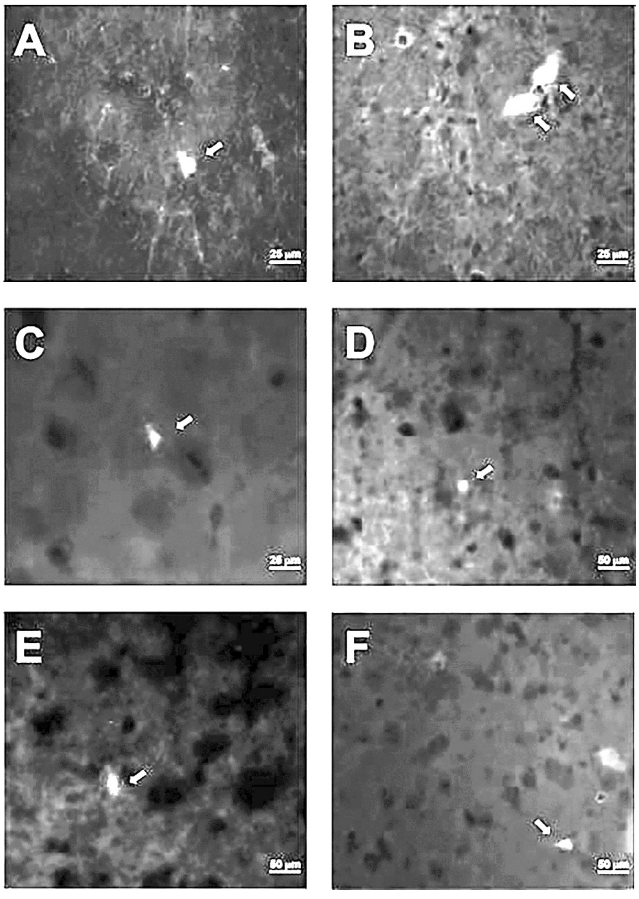

Figure 2. Effect of chronic exposure to alcohol and deprivation of the drug on neurodegeneration in the rat brain. Animals were kept in *ad libitum* (A, C, and E) conditions or treated chronically with ethanol at 10% (v/v) (B, D, and F) for 30 days. Images were also shown of sections from animals with chronic treatment and deprived of the drug for 24 (C, D) and 48 (E, F) hours. After the treatment the brains were removed, coronal sections of 20μm thickness were obtained and dyed with Fluoro-Jade B. Cells positive to Fluoro-Jade B (indicated with arrows) were found in the insular granular cortex (A), the dorsal caudate-putamen (B and D), the mediodorsal thalamic nucleus (C), the somatosensorial primary cortex (E), and the frontal association cortex (F). The images were captured at an increase of 400x.

rodegeneration. Figure 2 shows images of sections with cells positive to Fluoro-Jade B from animals chronically treated with ethanol and deprived of the drug for 24 and 48 hours.

Blood alcohol levels in rats chronically exposed to ethanol and deprived of the drug for 24 and 48 hours

The blood alcohol levels in animals chronically treated with ethanol for 30 days and deprived of the drug for different lengths of time were significantly higher than those of the control animals (figure 3). The increase in blood alcohol levels was similar in animals treated with ethanol and those deprived of the drug for 24 hours. The average values reached were 4.2 and 4.4 mg/dl, respectively. In contrast, the alcohol levels detected in animals deprived of ethanol for 48 hours (average value of 2.67 mg/dl) were lower than those treated with the drug for 30 days and deprived of it for 24 hours (figure 3). The blood alcohol concentration values in the control animals are at the limit of the trial's sensitivity.

DISCUSSION AND CONCLUSION

Alcohol is a substance which has a wide range of effects on various tissues. The damage to health caused by the use and abuse of this drug is very varied. Among the organs and systems most affected are the liver, kidneys, gastrointestinal system, and Nervous System.[4,18,20,37]

The damage caused by alcohol depends on various factors, such as the quantity ingested, the length of time of intoxication, the number and duration of periods of abstinence, as well as the ingestion of other psychoactive substances. Other factors also have an important effect, such as sensitivity and vulnerability to the drug, diet, gender, and age.[7,18,31] The extent to which an individual's health is affected by the drug directly depends on the aforementioned factors, the way in which the magnitude and irreversibility of the same are subject to the state of the individuals, and their history with the drug.

Alterations produced by ethanol in the CNS can lead to a reduction or loss of cognitive or motor functions, which threatens the life of the organism. Some studies in humans and animal models have shown that short exposures to high doses of ethanol can cause moderate lesions in specific regions of the brain, though the disappearance of the damage through a short period of deprivation (3-7 days) has also been reported.[27,38-40] Conversely, chronic exposure to intoxicating doses of ethanol affects the majority of cerebral structures. In spite of there being a recovery from damage and of cerebral functions if the drug's use is suspended, the recovery is not total.[12,18,41]

The results obtained in the present work indicate that the number of cells positive to Fluoro-Jade B is similar between treatments. The absence of a significant number of fluorescent cells in the brains of animals treated with ethanol in terms of controls could be due to the treatment protocol with ethanol used not producing damage to the CNS. Another explanation of the absence of damage is that the cells had died before the moment the tissue samples were obtained, given that Fluoro-Jade B only marks neurons that are in the process of degeneration.

The absence of cells positive to Fluoro-Jade B in the brains of animals chronically treated with ethanol for 30 days could be due to the blood alcohol levels produced by the doses of ethanol consumed not reaching concentrations capable of inducing damage.[23,30,42] It is also possible that the blood alcohol levels were not elevated for long enough to produce neuronal degeneration.

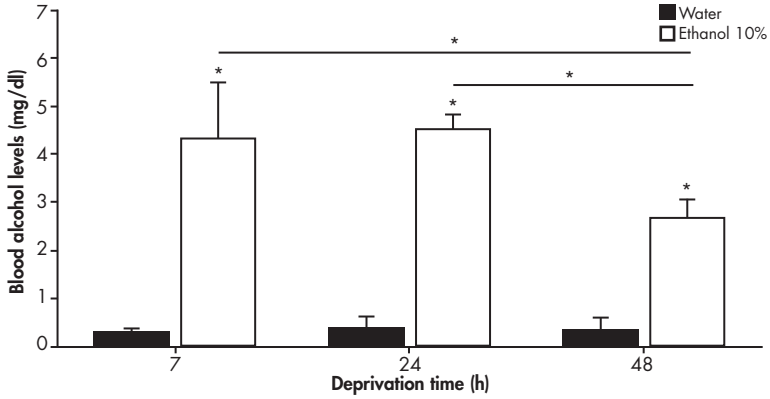

Figure 3. Blood alcohol levels in rats chronically exposed to ethanol for 30 days and deprived of the drug for 24 and 48 hours. Blood alcohol levels were determined by a spectrophotometric method of ADH in the animals' plasma Values are expressed as the average ± EEM of 6-8 animals; *p<0.001 *versus* water in the same time. Significant differences were also obtained in the blood alcohol levels between the chronic animals and those which were chronic with deprivation for 24 hours *versus* chronic animals with deprivation for 48 hours (*p<0.001).

In the model of chronic treatment developed in this study, the animals had the 10% v/v ethanol solution as their only liquid option. In previous laboratory studies, we observed that during the first week of treatment, the rats consumed low quantities of alcohol, which may be due to the animals' natural aversion to the substance. However, from the second week, the ingestion of the drug increased progressively, such that on the final day of the treatment, alcohol consumption reached values of 68 ± 7.8 ml of ethanol (equivalent to 5.4g of alcohol), which suggests the development of a tolerance.[43] In spite of the alcohol consumption for this study lasting for 30 days, the blood alcohol levels at the end did not reach elevated levels (4.4 mg/dl on average). After 24 hours of abstinence from ethanol, the blood alcohol concentrations remained at similar levels in the chronic animals, while they diminished significantly after 48 hours of abstinence. It is possible that the blood alcohol levels detected in the three groups of animals for this treatment had not been sufficiently elevated to be considered intoxicating at the end of the experiment.[44] Given that in this study, the animals were progressively increasing their exposure to alcohol, it is possible that the blood alcohol concentrations had reached intoxication in the early and middle stages of the treatment. As such, it is imperative to carry out a time course with the aim of determining the blood alcohol concentration throughout the treatment.

On the other hand, it is possible that metabolic adaptations were produced in the animals during the chronic treatment and the abstinence. In this sense, some studies have shown that chronic exposure to ethanol (treatment over 24 days) promote the induction of cytochrome P450 2E1 (CYP2E1), which causes an increase in the rate of metabolism of alcohol.[45-49] Furthermore, it has been observed that isoform 3 of the ADH contributes to accelerating the metabolism of ethanol when blood alcohol levels are raised.[50] The fact that similar blood alcohol levels were maintained in our study 24 hours after deprivation of the drug would not seem to be congruous with an increase in the metabolic rate of ethanol caused by the over-activity of CYP2E1. However, it has been observed that the acceleration of metabolism due to CYP2E1 is only relevant to high concentrations of blood alcohol (90-250 mg/dl),[51,52] (Alderman et al., 1987). Blood alcohol levels recorded at the end of the chronic treatment in our study were found to be far below the range at which CYP2E1 significantly accelerates the metabolism of alcohol. This data suggests that during our chronic treatment, there was a metabolic adaptation which would progressively lead to the reduction of the blood alcohol level. In this way, the blood ethanol levels at the end of the chronic treatment had reached concentrations at which CYP2E1 was not a significant contributing factor to the metabolism of alcohol. As mentioned above, it is necessary to perform a time course of the blood alcohol level throughout the treatment with the aim of monitoring the changes produced. It is also important to study the participation of other enzymes involved in the metabolism of alcohol (*i.e.*, ADH and catalase).

The absence of a significant number of cells positive to Fluoro-Jade B could be due to the start of the neurodegeneration observed with that marker preceding the time at which the histological analysis was made. The damage caused by alcohol can trigger apoptotic or necrotic death mechanisms.[23,53] The time in which these death mechanisms are completed depends on the cell type, the inducing agent, and the analysis technique. Various *in vivo* studies have reported that the apoptotic process can last for an hour in nervous retinal cells exposed to staurosporine.[54,55] Studies *in vitro* have also shown that the apoptotic process induced by the tumoral necrosis factor (TNFα) in fibroblastic cells of mice lasts for 3 to

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6 hours,[56] while the damage caused by potassium chromate in human larynx cells happens over a period of 24 hours.[57] On the other hand, necrotic death has a variable range in the duration of the process, and it is noted that it can happen in shorter times than apoptosis.[58] Until this time, there are no existing studies which have assessed the duration of the processes of apoptotic or necrotic death induced by ethanol. It is important to stress that the process of degeneration is activated at different times depending on the susceptibility of cells. As such, it is necessary to carry out a time course of the neurodegenerative effects of alcohol, which would give more precise information of the time when death occurs.

The set of data obtained in this work indicates that chronic exposure to alcohol for 30 days in Wistar rats does not produce significant neurodegeneration detectable with the Fluoro-Jade B marker at the end of treatment. Furthermore, the deprivation of this drug after chronic treatment also does not generate a process of degeneration that can be detected 24 or 48 hours after the last ingestion of alcohol. These results suggest the need to carry out other studies which promptly identify the changes produced throughout chronic exposure to alcohol, in particular on the concentration of alcohol in blood and the proteins associated with neuronal damage. We also consider it important to carry out experiments with other histological techniques *i.e.*, propidium iodide, TUNEL, etc.), which complement the information obtained in this study. Finally, we consider it relevant to research the participation of distinct enzymes involved in the metabolism of alcohol.

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Conflict of interest

The authors do not declare any conflict of interest.

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