Postnatal Effects of the Ethanol on the Encephalon Mass and Neocortex Architecture in Wistar Rat Offspring Submitted to Acute Prenatal Injection [E_{12}] and *in vitro* Ethanol Effects on Wistar Rat and Human Erythrocytes

ABSTRACT

Aims: To verify the ethanol effects of the acute injections in pregnant Wistar rats (E_{12}) on the offspring in P_8. Additionally, measures of the brain and corporal masses and the stability of erythrocytes were performed in rats and humans.
Study design: Histological analysis and a spectrophotometer were used to count neural cells and osmotic fragility in erythrocytes.
Place and Duration of Study: The immunohistochemistry study was partially performed at the Federal University of Uberlândia, and at the University of Toyama-Japan, the experiments on the brain, body masses, and stability of erythrocyte membranes in humans were performed from 2012 to 2014.
Methodology: Eighteen pregnant female rats (180-230g) were housed in cages at 22 ± 0.4°C on a 12 h light/dark cycle with free access to food and water. On the 11th day of pregnancy (E_{12}), 12 rats received three intraperitoneal (i.p.) injections of a 20% ethanol solution (3 g of ethanol/kg of body weight) at 8 hours intervals.
Results: The number of neural cells was lower in rats treated with ethanol than to controls to each lobe and total count in all lobes (P = 0.001). Significant alterations of neocortical tissue in ethanol-treated were observed, as the decrease of brain mass (P = 0.05) in linear regression. The stability of erythrocyte membranes was verified with D_{50} equal to 0.46 $g \cdot dL^{-1}$ (± 0.05).
Conclusion: According to this work, the ethanol injection in the rats showed marked brain tissue destruction relative to the control, and ethanol's effects on erythrocytes indicated that membrane destruction could be one of the causes of brain cell disruption in neural migration.

Keywords: Neocortex; drug effects; ethanol; erythrocytes.

1. INTRODUCTION

The chronic effects of ethanol on the development of the brain has been associated, *inter alia*, to damages in the following: cortical migration and generation of neurons [1-3], heterotopy and ectopia [4], neuronal depopulation and neuroapoptosis [5,6], disruption in callosal projection neurons [7], glial alterations [8,9], disruption on the second messengers and protein phosphorylation [10], alterations of the genetic expression and growth factors [11-14], and neurotoxicity of homocysteine [15], as well as alterations in the*substantia nigra* [16], the neural crest [17], the hypothalamic-pituitary-adrenal axis [18], the cholinergic

development [19,20] along with decreases in Purkinje cell density [21] and hippocampus neurons [22,23].

Different regions of the encephalon are not uniformly vulnerable to ethanol and other drug effects during development [17,24], mainly because of the absence or inefficiency of a protective fetal blood-brain barrier [25,26].

Furthermore, the deleterious effects of ethanol on the organism in general and specifically on the development of the encephalon are scarcely understood [27,28] and the *modus operandi* behind the ethanol effects on biological structures is uncertain, putatively because ethanol effects are ubiquitous on cell constituents as well as receptors, growth cones, nucleic acids, and membranes, which could affect migration routes, synaptogenesis, and the structure of tissues and organs as cited above.

Most studies about the effects of ethanol on the neural system are focused on chronic effects, and just a few study acute effects from a single day of exposure to this drug on prenatal development [29]. The acute assays can be made on a specific day in the gestation while prioritizing crucial periods such as gastrulation, migration of cells, for instance, neural and glial migration, or synaptogenesis, hypothetically destroying the basis of the cortical structure and consequently decreasing the cerebral mass [30].

In this way, effects on the birth of neurons in the neocortex that occur from gestational day 11 to 13 in rats [31,32] could be studied, because that is a crucial time for the construction of neocortex layers and corresponds to first trimester-equivalent gestational days in humans [33,34] for the cerebral mass. Thus, the first purpose of this work was to acutely inject ethanol (single day) in the for neocortex when the neuronal migration is starting (neuron birthday) and to measure the evolution of the brain mass in some days after birth, using rats as a model.

On the other hand, ethanol is a known chaotropic that acts by denaturing proteins [35,36] and modifying the membrane bilayer [28]. It has been verified that chronic ethanol exposure in rats changes membrane fluidity [37] and increases the osmotic fragility of erythrocytes [38], producing hemolysis [39,40].

In vitro effects on erythrocytes indicate that ethanol destabilizes the membranes [28,41,42] generates pores [43], and modifies erythrocyte shapes [42]. Nevertheless, membrane stability is amply dependent on the environment that cells live in [44] and the entropy of the aqueous solution-lipid system [45].

Indeed, before the ethanol acts on internal cellular structures, it must pass through extracellular environment and penetrate the cells by going through the plasma membrane and disrupting it [41,42]. Thus, it is reasonable to think, hypothetically, that the membrane rupture should generate apoptosis and migration errors in the cells because the membrane stability is essential to maintaining cell integrity.

Water and saline solutions containing ethanol were tested separately to verify the direction and under what physiological conditions the effect of these solutions on plasma membranes would occur under the ethanol concentration injected into the rats in this experiment. To reach these objectives, the main model used was the erythrocytes because of their particular constitution, i.e. a cytoplasm without nucleus and organelles surrounded by a plasma membrane, such that the plasma membrane is most of the membrane in this structure. Rat and human erythrocytes were used.

In summary, the effects of the ethanol on the neocortex histology and the brain mass were verified. Then, the acute injection of ethanol (single day) on the offspring´s neurons (E_{12}) in Wistar rats was studied, and effects on the offspring were observed on the eighth postnatal day (P_8). Additionally, measures of the brain and corporal masses were made after birth until the 50th postnatal day to verify the effect of ethanol on brain mass. Then, *in vitro* tests were performed to verify the stability of erythrocyte membranes under aqueous and saline ethanol solutions. Finally, the results and other data from the literature were used to understand the general effect of ethanol on brain tissues.

2. MATERIAL AND METHODS

2.1. Immunohistochemistry and encephalon/body masses

Female Wistar rats (180–230 g) were housed from 3:00 p.m. to 6:00 a.m. the next day with male rats. The presence of a vaginal plug and sperm in the vagina confirmed successful mating and indicated the first day of gestation (E_0). Eighteen pregnant female rats were housed in cages at 22 ± 0.4°C on a 12 hours light/dark cycle with free access to food and water. On the 11th day of pregnancy (E_{12}), 12 rats received three intraperitoneal (i.p.) injections of a 20% ethanol solution (3 g of ethanol/kg of body weight), at 8 hours intervals. Ethanol was administered intraperitoneally because this route ensured that all rats received an equal volume and amount of drug, and there was almost 100% absorption. In addition, at E_{12}, six control pregnant rats received saline injections of 0.9% at the same intervals as the ethanol-treated rats. Proliferating cells were labelled as described elsewhere [32]. All rats received a single i.p. injection of BrdU (5 mg/mL in 0.9% NaCl, containing 70 mM NaOH) at a dose of 60 mg/kg 2 h after the last injection of ethanol or saline solution.

On the eighth day of postnatal life (P_8), 18 litters were chosen randomly and anesthetized with sodium pentobarbital (50 mg·kg^{-1}, i.p.) and perfused with saline followed by 70% ethanol. The brains were removed and processed as described elsewhere (Miller 1993). Briefly, the brains were embedded in paraffin, sectioned sagittally (7 µm thick sections) and mounted on gelatine-coated slides. The sections were deparaffinized, hydrated in a graded ethanol series, and treated with 1 molar (M) NaOH followed by 1 M sodium borate buffer (20 min each), before washing with phosphate buffer. After blocking non-specific sites with serum, the sections were incubated for 2 h with a monoclonal anti-BrdU antibody (diluted 1:500; Sigma, St. Louis, MO, USA), followed by incubation for 1 h with a goat anti-rat secondary antibody (diluted 1:200; Vector, Burlingame, CA, USA), and then treated with an avidin-biotin complex (Vector) and incubated with 3,3'-diaminobenzidine (Sigma). Sections were cover slipped with Entellan. Some sections were not incubated with monoclonal antibodies to verify the possible background. Finally, the slides were contrasted with safranin.

The presence of anomalous neuron clusters in the neocortex areas was indicative of heterotopia, and the presence of unusual neurons in the neocortex layers was indicative of ectopia.

The lobes were identified via anatomical region and type of neurons for each layer, according to Caviness Jr [46]. The number of cells immunohistochemically labelled in cortices was obtained from a trinocular microscope, Olympus BX40-F4, coupled to an Oly-200 (Olympus American) camera and IBM computer via an Olympus U-SPT coupling accessory. The images were processed with the HL image ++97 software. Six areas of 15,000 µm^2 from each lobe for each encephalon were captured and analyzed.

The same proceedures used in the immunohistochemistry assays were done with eight female rats prior to mating, ethanol and BrdU injections. The encephalon and body masses of offspring were obtained on postnatal days 1, 8, 17, 26, 33, 45, and 50 after the perfusion (as the perfusion, described before). Between four and six weight measures were obtained from each litter, depending on the number of individuals born to the nest. An effort was made to cut the head between the atlas vertebrae and the occipital using a fine scissor, and the encephalon was removed carefully. All materials were weighed on an analytical balance (Mettler Toledo mod. AG245).

2.2 Osmotic fragility

Blood samples (3 mL) were collected, with a syringe containing heparin, from the antecubital vein of male human volunteers (14) after overnight (8–12 h) fasting and from 2 adult Wistar rats (198 and 230g) via the left ventricle.

The stability of erythrocytes was tested in solutions with different compositions: 1) ethanol from 0.1 to 20 $g \cdot dL^{-1}$ in 0.9% NaCl, 2) NaCl from 0.1 to 0.9 $g \cdot dL^{-1}$ and 3) ethanol from 0 to 19.8 $g \cdot dL^{-1}$ in deionized water for humans, and 4) ethanol from 3.9 to 74.1 $g \cdot L^{-1}$ in 0.9% NaCl. The general conditions of the assays were adapted from the literature [47]. To each unit of a duplicate set of Eppendorf flasks, 1 mL of the testing solution and 10 µL of blood were added. After homogenization and incubation at 37°C, the flasks were centrifuged for 10 minutes at 2000 rpm, and the supernatant fractions were analyzed by visible spectrophotometry at 540 nm with a Micronal B442 spectrophotometer.

Aliquots of 10 µL of blood were added to concentrations of 1.56, 3.9, 7.8, 11.7 and 5.6 $mg \cdot dL^{-1}$ ethanol (equivalent to 2, 5, 10, 15 and 20% of ethanol) in solutions prepared in 0.9% NaCl. After homogenization and incubation at 37°C for 10 minutes, the mixtures were added to histological slides and labelled with May-Grünwald-Giemsa stain and Sudan black. The slides were analyzed in a trinocular microscope, Olympus BX40-F4, coupled to an Oly-200 (Olympus American) camera and IBM computer via an Olympus U-SPT coupling accessory. The images were processed with the HL image ++97 software.

2.3 Statistics

For the cell count, the normality test of the data was performed first. Data submitted and approved by Kolmogorov-Smirnov/Liliefor and Shapiro-Wilk W tests were considered normal. After acceptance, normality was applied to a T-test to compare means between the control and treated groups for each lobe and afterwards for both complete groups. Differences were considered significant at $P = .001$.

Data from the encephalon and body weight were submitted to curve fitting to verify the type of regression that had a better fit for the data. Logarithmic and linear regressions were used in the regressions. Logarithmic regression was used to compare the brain and body weights for both treated and control rats, and linear regressions were used to compare the control and treated brain and body weights. The comparison between the relationship between brain and body weight for both the treated and control groups was performed using linear regression. In all cases, the ANOVA was calculated with $P = .05$.

For both experiments' cell counts and encephalon-body weights, the statistical analyses and graphical plots were performed via the StatPlus program (AnalystSoft Inc. v.7.3.3).

Data obtained for ethanol/saline concentrations and only saline concentration were adjusted to a sigmoidal curve according to the Boltzmann equation, and the D_{50} was obtained by 50% erythrocyte lysis.

3. RESULTS AND DISCUSSION

3.1 Immunohistochemistry of acute ethanol effects on the neocortex

In quantitative terms, the number of cells was lower in rats treated with ethanol than in controls for each lobe and the total count in all lobes (frontal, parietal, temporal, and occipital) considering absolute numeric data and after statistical analysis of means comparison with a t-test to *P = .001* (Fig 1).

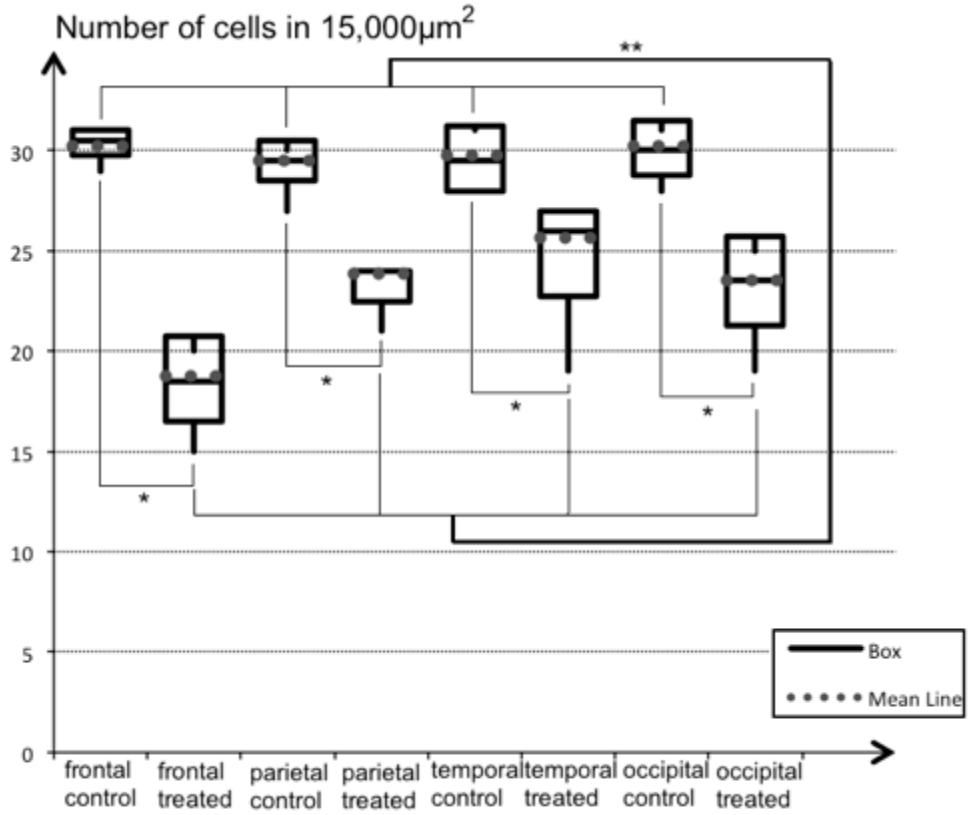

Fig 1. Box graphic of the number of cells in the neocortex (area: 15,000 µm2). The asterisk (*) indicates a significant difference between control and treated groups for each lobe, and two asterisks (**) indicate a significant difference between all controls and treatments by comparing means (T-test) to P = 0.001.

In qualitative terms, significant alterations of neocortical tissue in ethanol-treated rats relative to control ones were observed. The characteristic pyramidal neurons were used as a marker

to define brain layers in Wistar rats (Fig 2A). The BrdU stained nuclei; however, many times, the content of the nucleus may leak during the proceedings, and the cytoplasm shape is visualized in black or shades of grey. On the other hand, the safranin stains permit a pale visualization of the cytoplasm.

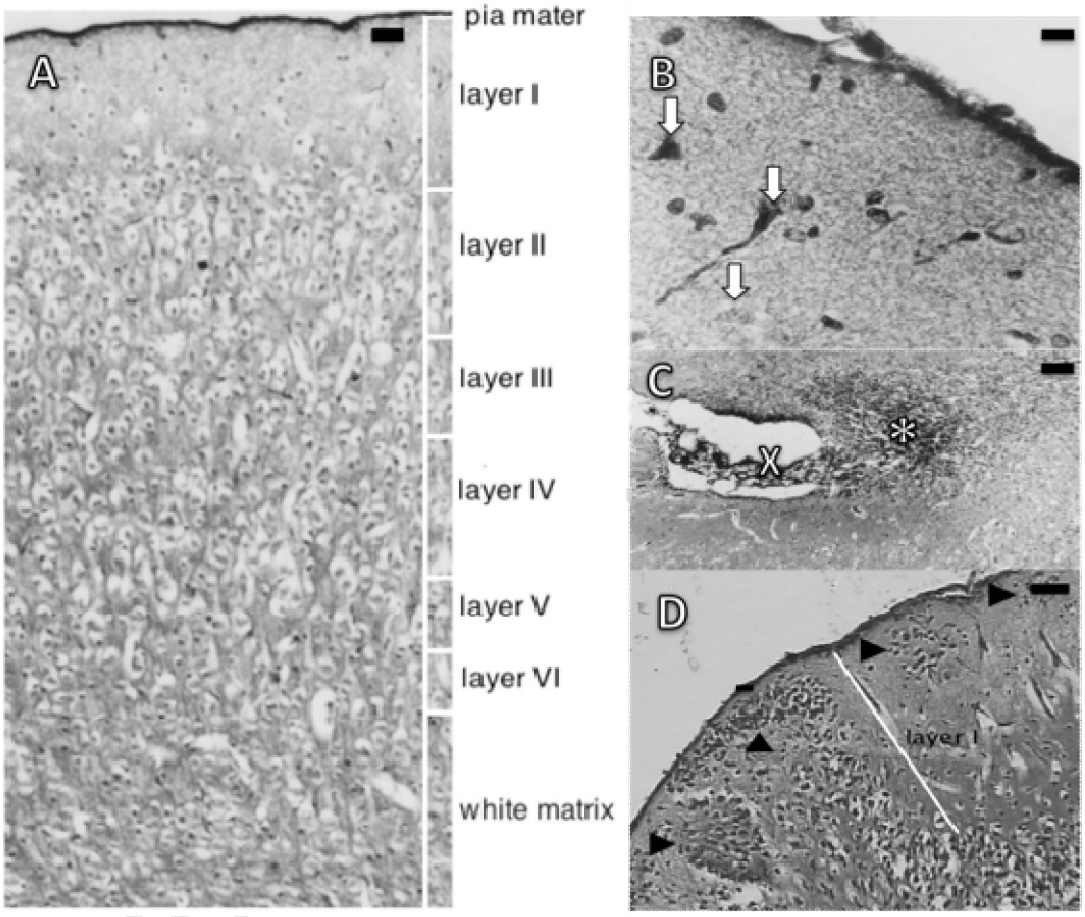

Fig 2. Photomicrographs of the neocortex (prefrontal lobe) of Wistar rats with cells labelled with BrDU. A) photomicrography of the histology of layers in the parietal lobe of a control (Bar = 200 μm); B) photomicrography of layers I and II of a frontal lobe of a treated animal indicating (white arrows) the unusual large cells (ectopia) with pyramidal shape in layer I (Bar = 20 μm); C) photomicrograph of the tangential route from the ventricular zone to the olfactory bulb generating a heterotopic group of cells (*) under fissure between the frontal lobe and olfactory bulb (X) (Bar = 240 μm); D) photomicrograph of superficial layers of the temporal lobe of a treated animal indicating (arrowheads) anomalous group of cells (heterotopy) in layer I close to the pia mater (Bar = 200 μm).

The main alterations observed in ethanol-treated animals were ectopic neurons with pyramidal shapes found in layer I (molecular layer) (Fig. 2B) and heterotopic groups into the molecular (Figs. 2C, 2D, and 3A) and external granular (layer II) layers (Fig. 3C). Cell depopulation in deep layers, manly V and VI (pyramidal internal and fusiform layers, respectively) was observed qualitatively (Fig. 3D) and quantitatively (Fig. 1). Apoptotic cells characterized by pyknotic nuclei were observed in heterotopic clusters (Fig. 3A) and a putative radial pathway to the pia mater was found by the observation of a line of nuclei (Fig. 3B).

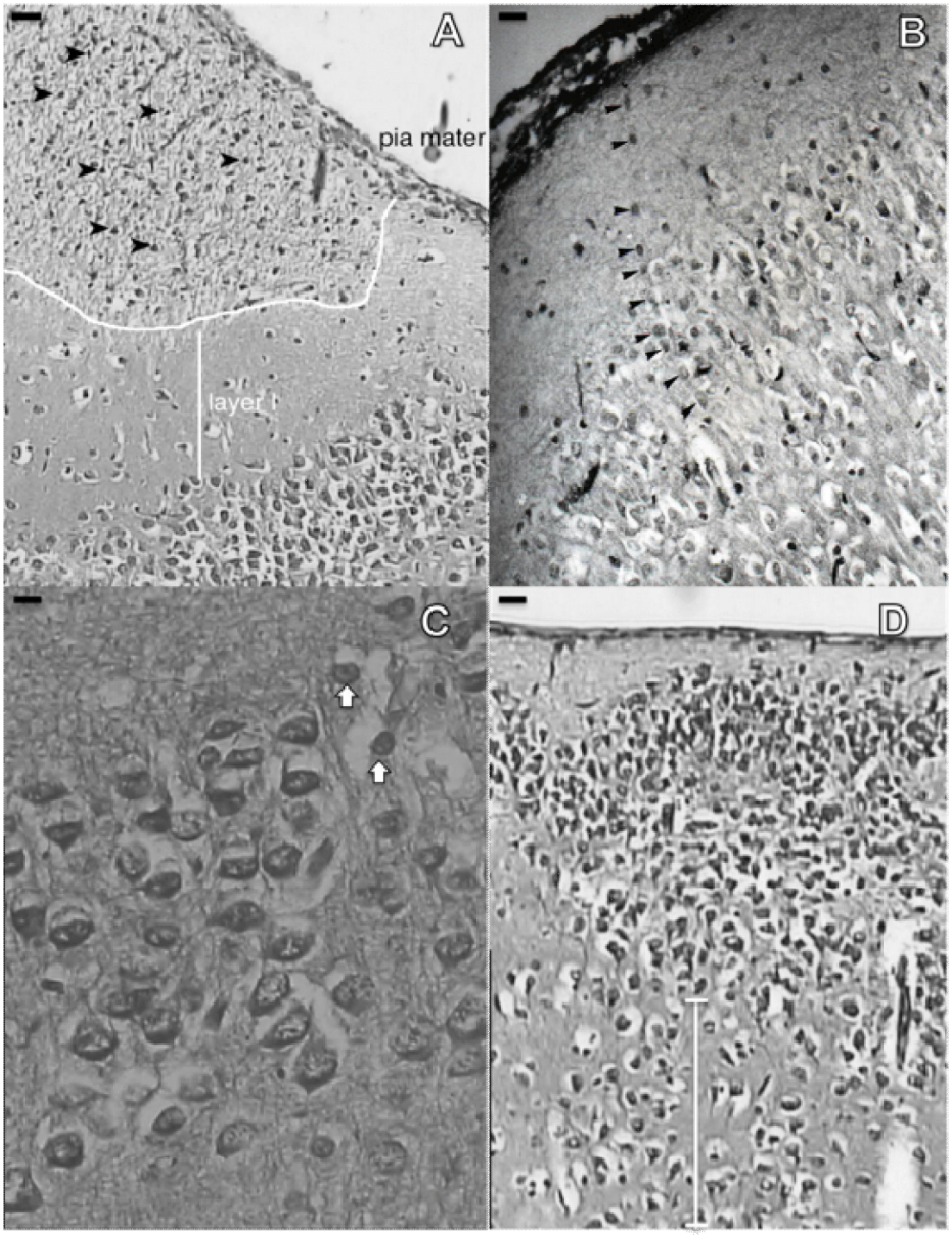

Fig 3. Photomicrographs of the neocortex of treated Wistar rats with cells labelled with BrDU. A) photomicrography of the histology of superficial layers in the parietal lobe with a large heterotopic group of cells presenting pyknotic nuclei (arrowheads) (Bar = 90 µm); B) photomicrography of layers of a frontal indicating (arrowheads) a

group of cells in line seeming to follow a pathway to the pia mater (Bar = 70 µm); C) photomicrograph of superficial layers I, II and part of III of the parietal lobes indicating an anomalous group of cells (heterotopy) in layer II; the normal nuclei expected to this layer are shown by head arrows (Bar = 25 µm); 4) photomicrography of layers of an occipital lobe showing (white line) a few populations of cells in deep layers (V and VI) relative to other more superficial ones (Bar = 50 µm).

3.2 Acute ethanol effects on encephalon mass

The relation of the encephalon weight to body weight demonstrated a statistical difference according to the ANOVA from the 1st day forward until the 50th post-natal day to $P = .05$ (Fig. 4). The statistical logarithmic regression was calculated to compare the brain and body weight for both treated ($R^2 = 0.95504$) and control ($R^2 = 0.9407$) rats. The linear regressions were calculated to compare control and treated brain weights ($R^2 = 0.7166$) and body weights ($R^2 = 0.8212$), as well as the relation between brain and body weights for both treated and control groups ($R^2 = 0.0052$), at $P = 0.05$ (Fig. 4).

The ANOVA shows the acceptance of H_0 for the comparisons between the brain and body weight for both treated and control groups as well as the control and treated brain and body weights; however, for the comparison between the relation between brain and body weight for both treated and control groups, H_0 was rejected at $P = .05$.

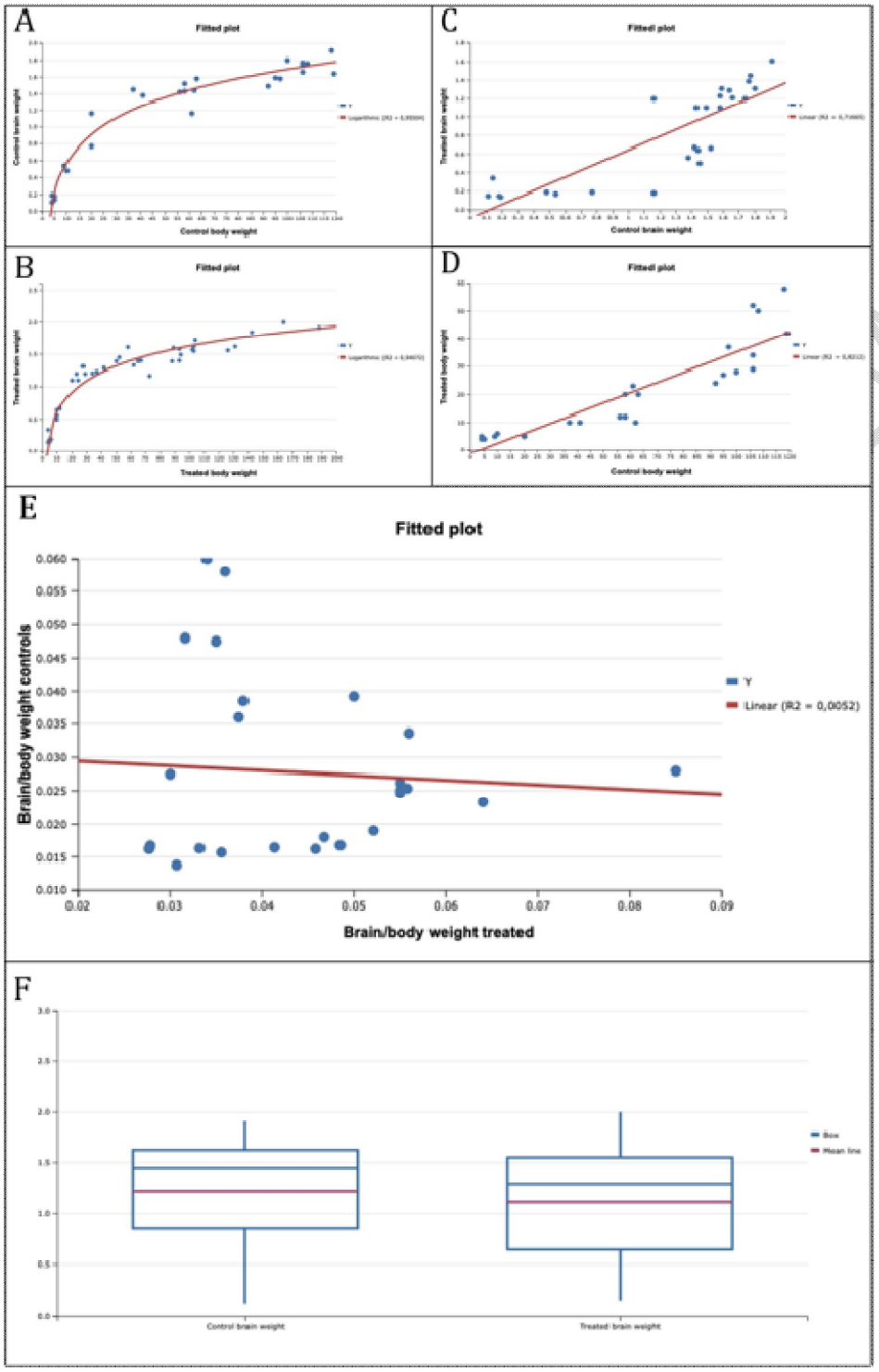

Fig 4. Graphs showing the acute ethanol effects on encephalon mass. A) showing the logarithmic regression to compare the brain and body weight for both treated (R^2 = 0.95504) and B) control (R^2 = 0.9407) rats; C) showing the linear regressions to compare control and treated brains weight (R^2 = 0.7166) and D) body weights (R^2 =

0.8212); E) also the comparison between the relation between brain and body weights for both treated and control groups was performed using linear regression (R^2 = 0.0052) in all cases, to P=0.05; F) the box graph indicating the brain mass difference between the control and treated rats.

3.3 Ethanol effects on osmotic fragility (membrane stability) of human erythrocytes

The stability of erythrocyte membranes was verified under different concentrations of NaCl from 0.1 to 0.9 g·dL-1 (Fig. 5A) with D50 equal to 0.46 g·dL-1 (± 0.05). It was performed for at least two reasons: 1) to verify the device accuracy and 2) to represent an osmotic parameter to study the ethanol osmotic effects on the membranes of erythrocytes. Crescent ethanol concentrations from 0 to 19.8 g·dL-1 were used to verify the membrane stability in erythrocytes (Fig. 5B), demonstrating membrane rupture for all analyzed concentrations.

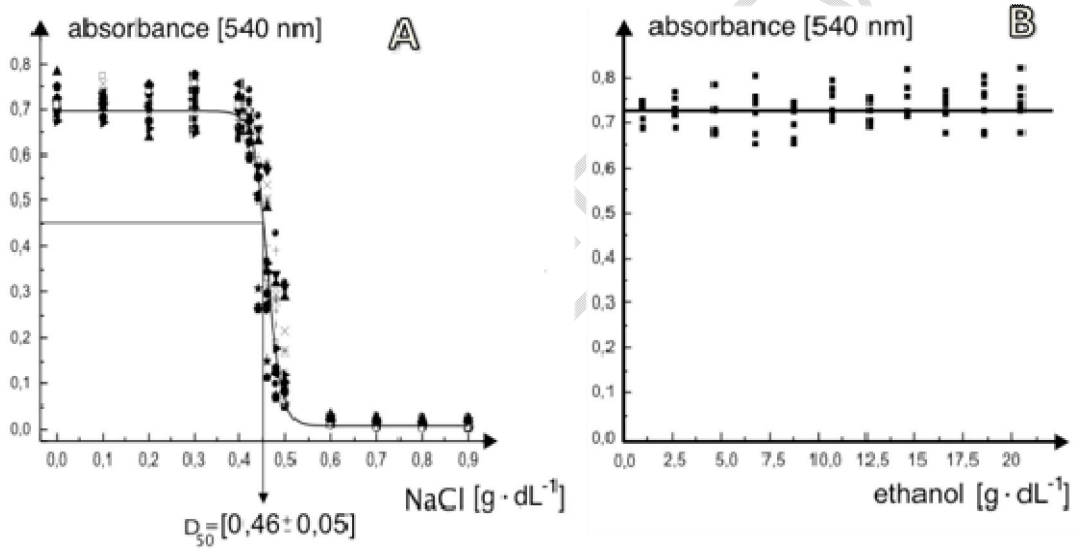

Fig 5. Data from the crescent concentration of NaCl and ethanol on human erythrocyte membranes. A) Data from the crescent concentration of NaCl on human erythrocyte membranes; observe that the membrane stability of erythrocytes starts at D50 (0.46 ± 0.05 g·dL-1); B) data from the crescent concentration of ethanol on erythrocyte membrane stability; observe that for these concentrations membrane stability for erythrocytes is not present.

The membrane stability of Wistar rats and human erythrocytes was tested under the effect of association among crescent ethanol concentrations (0.1 to 20.0 g·dL^{-1} in humans and 3.9 to 74.1 g·dL-1 in Wistar rats) diluted in a constant concentration of NaCl (0.9%) with a D50 equal to 11.05 ± 0.25 g·dL^{-1} for humans (Fig 6A) and a D50 equal to 11.31 ± 0.04 g·dL^{-1} for Wistar rats (not shown graphically). According to these data, the 0.9% NaCl in solution with ethanol stabilizes erythrocyte membranes until 11.05 g·dL^{-1} for humans and 11.31 g·dL^{-1} for

Wistar rats. The average concentration of the ethanol in the blood of Wistar rats for the histological study was approximately 24.4 $g \cdot dL^{-1}$; therefore, the destruction of erythrocytes by ethanol in rats is considered the rats' average mass of 205 mg and the blood volume of approximately 13 mL [48].

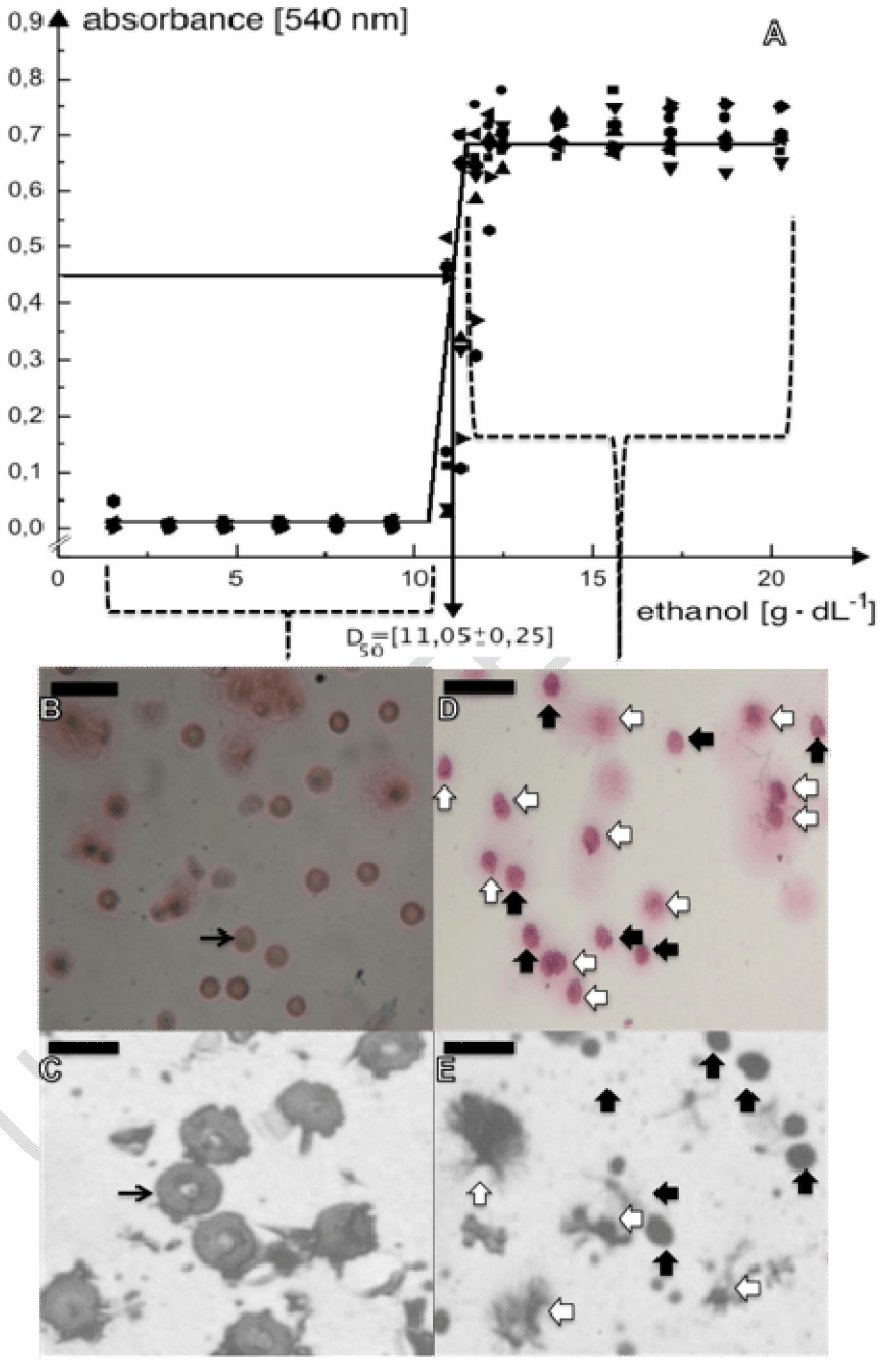

Fig 6. Ethanol effects on membrane stability of human erythrocytes. A) Plotted data of the crescent ethanol concentration effect on the erythrocyte's membrane keeping a

constant concentration of the NaCl (0.9%) generating a sigmoidal curve adjusted to the Boltzmann equation. The pre-transition region must contain most of the erythrocytes intact and the post-transition region must contain most of the destroyed erythrocytes. The calculated D50 (a point that represents 50% of erythrocytes intact) was 11.05 ± 0.25 g·dL^{-1}. B) Photomicrography of erythrocytes labelled by May-Grünwald-Giemsa obtained from samples that generate pre-transition curve (2% or 1.56·g·dL^{-1}) indicating the intact or normal shape of erythrocytes (the thin black arrow indicates an example of intact erythrocyte) (Bar = 35 µm). C) Photomicrography of erythrocytes labelled by Sudan black obtained from samples that generate pre-transition curve (2% or 1.56 g·dL^{-1}) indicating the intact or normal shape of erythrocytes in detail (the thin black arrow indicates an example of intact erythrocyte) (Bar = 10 µm). D) Photomicrography of erythrocytes labelled by May-Grünwald-Giemsa obtained from samples that generate post-transition curve (20% or 15.6 g·dL^{-1}) indicating small (black arrows) and destroyed (white arrows) erythrocytes (Bar = 38 µm). E) Photomicrography of in detail erythrocytes labelled by Sudan black obtained from samples that generate post-transition curve (20% or 15.6 g·dL^{-1}) indicating small (black arrows) and destroyed (white arrows) erythrocytes (Bar = 10 µm).

To verify the shape of the erythrocytes, histological assays were performed using May-Grünwald-Giemsa (Figs. 6B, 6D) and Sudan black (Figs. 6C, 6E) stains. Most erythrocytes shown in Fig. 6B and with more detail in Fig. 6C at a concentration of 2% ethanol (1.56 g·dL^{-1}) presented intact membranes. Meanwhile, at a concentration higher than 11.05 g·dL-1, specifically 20% ethanol (15.6 g·dL^{-1}) in Fig. 6D and with more detail in Fig. 6E, the erythrocyte membranes ruptured and/or the erythrocytes presented small sizes in comparison with the erythrocytes in Figs. 6B and 6C.

4. DISCUSSION

4.1 Immunohistochemistry of acute ethanol effects on the neocortex and loss of the cerebral mass

In the developing neocortex, post-mitotic neurons migrate from ventricular pseudostratified epithelium from days E_{11} to E_{13} [32] to generate, in order, the deepest to superficial layers in the adult neocortex [31,32,47]. This migration phenomenon obeys spatial and temporal patterns dependent on the formation and differentiation of the radial glia stimulated via chemical signals [49,50] for which molecular mechanisms are scarcely known [51-53].

The horizontal and vertical layers of organization in the adult neocortex are essential to its normal function and histological/molecular alterations during the development can affect learning, attention, behavior, motor abilities [11,54] and memory [55,56] in animals and humans.

Chronic ethanol effects are associated with the desynchronization of cortical development and consequently the establishment of neuronal circuits, generating a neocortex reorganization characterized by heterotopic groups of neurons [7] as well as destabilization of the matrix, molecules, and other components in glia [53] and neurons [58,59], including processes such as gene expression [11,60] and apoptosis [53,61-63].

General and more complete studies about the acute effects of ethanol on the brain in development are necessary, considering molecular and genic aspects; indeed, most of the few acute studies are focused on histological aspects [64-67].

The acute studies indicated similar effects in the neocortex of Wistar rats in relation to those verified in chronic ones, mainly in the presence of heterotopic groups of cells and

depopulation in deep layers [66] and apoptosis in primate brains [67]. In rats, however, episodic prenatal exposure to ethanol in macaques has recently been shown to affect neurogenesis [68].

The literature refers mainly to chronic ethanol treatment problems, with some exceptions, as in the studies of the lobes for acute effects of ethanol specifically in P_8 with pregnant rats exposed in E_{12} [65-67].

These causes of brain disorders could be caused by several factors, such as disorganization of the cytoskeleton [49,69], problems in the expression of molecular clues (GGF, RF60, laminin, proteoglycans), or even some metabolic alterations, generating heterotopia, which is one of the most important derangements of migration in the neural system [70-74].

The presence of evident heterotopic groups forming cell mass of a nodular type, determined by chronic exposure to ethanol [72], was also verified in this work as the deviation of the migratory route of neurons, i.e. putatively because the cells lost the ability to locate in the correct space-time place, perhaps they did not obtain the necessary connections to avoid apoptosis.

The neuroblast, when it leaves the germinal lamina to start its migration, has already specified its type, location, and shape [50] and ethanol could disrupt these organizations. Indeed, in this work, the presence of ectopic neurons was observed in all rats acutely treated with/exposed to ethanol.

When neurons lose chemical contact with glial fibers, they continue migrating following the radial pathway until the *pia mater*, where they undergo apoptosis [74,75]. This certainly justifies the neuronal depopulation mainly in the deep layers observed in animals treated with ethanol (Fig. 4). In this sense, putatively, the ethanol inhibits the chemical relationship between glia and neuroblasts necessary for correct neuron localization in the correct cortex layer.

As ethanol is chaotropic, it can affect the chemical environment of the cell matrix or the receptors and disrupt cellular components, in addition to promoting changes in some or several of the basic activities of cells, such as gene expression [60,76]

Molecular, cellular, and histological changes in the neural system in initial development may affect general functions such as learning, inhibition, attention, regular behavior and motor skills [54,77,78] in the adult brain, and ethanol promotes these kinds of disturbances both in chronic and acute exposure, as shown in this work in the last case.

Accordingly, histophysiological modifications from ethanol administration may be responsible for problems such as memory loss reported in adults who had fetal alcohol syndrome [29,56,79]; however, this kind of analysis was not performed here.

Indeed, our data, i.e. the effects of the acute exposure to ethanol, are in agreement with reports of chronic effects studies [4,7], including migration problems in the cortex, with the layers having their natural order reversed, and neuronal depopulation in the deep cortical layer, inter alia.

Hypothetically, the deep neuronal depopulation can be justified by the fact that the cells that form these layers are the first to initiate the migration [48,50,57,74] and, therefore, were subjected to a higher concentration of ethanol on the twelfth day of intrauterine life when the neurons started their migrations.

Another explanation is linked to horizontal or Cajal-Retzius cells that are responsible for the way the direction and stimulation of neuronal migration guide the astrocyte fibers, until their anchorage in the *pia mater* [50]. Therefore, the disorganization of the structure of the cytoskeleton of those cells by ethanol's chaotropic effects [53] could alter, at least secondarily, the time of fiber formation or fiber competence and, consequently, compromise neuron migration.

In summary, most works in the literature report the chronic effects of ethanol, with few studies about the acute effects on specific days of embryonic or fetal development [27]. However, this work shows the severity of acute ethanol intoxication at the beginning of neuronal migration in rats, around the twelfth day of rat intrauterine life.

It seems that the earlier the biological structure of the tissue is affected, the larger the consequences are [29], since the complex structures derived from the movement of cells during the development and differentiation into many cell subtypes depend directly on the initial basic structure of the tissue or organ, mainly the matrix integrity.
As the chronic studies about the ethanol effects are performed in rats [9,48,75,80], and the consequences are projected for humans, the same might be done here, i.e. the acute effects of the ethanol on the cortex are valid for humans, since the toxic concentrations of ethanol in rats and humans are equivalent, and the neuronal densities existing between different regions of the same brain and between brains of different species are also equal for all mammals.

4.2 Brain mass alterations

Neural apoptosis is a normal process for cerebral depopulation after birth in animals [51,81]. The measures of the brain mass diminish across time; however, the corporal mass increases simultaneously [82]. Thus, the relative measure of brain mass/body mass was performed to verify the ethanol effects on the brain mass, because when the numbers of neurons decrease, the brain mass must also decrease. However, the brain mass is not only dependent on neurons, the glia can divide and substitute for the dead neurons [83].
The chronic studies about ethanol effects, including in the FAS, indicate a decrease in cerebral mass [30] relative to controls; however, this was not verified here except for the relation between brain and body mass for both control and treated groups with linear regression, with R^2 indicating no correlation and ANOVA indicating the H_0 rejection. Some correlation was expected for this last case and a deeper study in trying to fit the curve for other parameter such as logarithmic, exponential, square, or cubic regressions also indicates a null correlation. The expected regression for other parameters indicated good precision in the data obtained, i.e., the body and brain growth for both the control and treated groups. Thus, some alterations seem to occur with a decrease in the brain mass of the treated according, to the box graph (Fig. 4), following the information from the FAS, but weakly.

4.3 Disruption of the erythrocyte membrane by ethanol

The literature cites the disruption of the cytoskeleton as well as DNA and protein disarrangement to explain the ethanol injuries in the brain [36]; however, logically, the ethanol first acts on the environment, or cell matrix and the membranes.
In this work, the study of erythrocyte fragility was done to verify ethanol's effects on the membranes. The concentration used for brain histology was 20% (24.4 gdL^{-1}), which was in zone of the ethanol destruction of erythrocytes. Accordingly, it is plausible to think that membrane destruction of the neurons and glia could generate the histological problems in the migrated and dead cells. A study using neurons and glia under the effect of ethanol in comparison with histological data could be performed to clarify this hypothesis.
Other important information from the experimentation is that NaCl can act as a protector of erythrocyte membranes. It behaves in a proportion to the concentration of solutions, i.e. a doubling of the ethanol concentration by the number of particles is equivalent to half the NaCl concentration, because this salt ionizes into Na^+ and Cl^- in an aqueous solution. Therefore, the concentration of particles in the system cannot explain the membrane destruction by ethanol, and the interactions among the molecules in the environment must be considered.
Indeed, the bipolarity of the ethanol should generate a disruption of the cell membranes [35]. If one considers that the ethanol hydrocarbon could dissolve the membrane, at least, partially, the general membrane structure should be disorganized, and all cell metabolism could suffer these effects, with the addition that the polar head of the ethanol can link with

the membrane lipid heads, but it could have a lesser effect on the membrane stability that is generated by the molecular Van der Waals interactions. Despite the Van der Waals force being a weak one, in proximity, it becomes very strong because this interaction is proportional to the inverse of the distance raised to the power of 14. As the arrangement of the cell membranes is linked to the solvent properties, ethanol, because of its chaotropic effects, could change the solvent features and interfere with the Van der Waals forces.
Accordingly, the ethanol effects occur, on all cell membranes through the alteration of the cell environment (cell matrix) and disruption of membrane stability, according to the interaction force proportions. In this way, partial disruption of the blood-brain barrier could occur, as verified before [25,26] in neurons.

5. CONCLUSION

According to this work, the ethanol injection in the rats showed marked brain tissue destruction and a decrease in brain mass relative to the control, and ethanol's effects on erythrocytes indicated that membrane destruction could be one of the causes of brain cell disruption in neural migration.

On the other hand, the chaotropic ethanol properties could destroy the matrix clues for neuron migration and glia organization, thus altering the cell environment and membrane stability.

The histology data linked to theoretical concepts indicate that neuron migration involves cellular structures such as the cytoskeleton, cell membranes, and biomolecules of the extracellular matrix, which are together responsible for the space-time relationship that determines the specific location of neurons in the layers of the neocortex.

Chronic exposure to ethanol affects neuronal migration, producing severe changes in the structure of the neocortex, which comprise 1) decreased neuronal density in all lobes studied in animals treated with ethanol, 2) a change in the location of neuronal cells, such as the occurrence of pyramidal neurons in the molecular layer; and 3) an alteration in the specific migration path of cells in the formation of the cortex.

Acute ethanol treatment of rats on the twelfth day of intrauterine life determines a degree of severity in the neural system that is very similar to the chronic treatment with this drug throughout pregnancy in rats, including brain mass loss.

These results are an indication for controlled ethanol ingestion or no use of ethanol during known or suspected gestation in human females.

ETHICAL APPROVAL

A previous project about the immunohistochemistry and erythrocytes histology in rats were approved by the Federal University of Tocantins as the experimental protocols described here [ethics committee from the Federal University of Tocantins, number 23101.003220/2013-85] and all procedures were performed according to a Brazilian College of Animal Experimentation. The immunohistochemistry study was partially performed at the Federal University of Uberlândia, and at the University of Toyama-Japan, experiments were performed on brain and body masses and the stability of erythrocyte membranes in humans.

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