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Dynamics of Histological Changes in the Frontal Cortex of the Brain in Rats Subjected to Antenatal Exposure to Alcohol

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The aim of the present work was to undertake a comparative study of the effects of prenatal alcoholization of the histological characteristics of neurons in the frontal cortex of the brains of rats of different ages. Experiments were performed on 175 mongrel white rats – the offspring of 25 females given 15% ethanol as drinking solution throughout pregnancy. The cerebral cortex was studied on postnatal days 2–90 using histological, histochemical, and morphometric methods. An increase (on days 2 and 5) followed by a decrease (on days 10 and 90) was seen in cortical thickness, with a decrease in neuron body size (on days 20–90), a decrease in the number of neurons in cortical layer V, a decrease in the number of normochromic cells, and increases in the numbers of hyperchromic shrunken neurons and ghost cells throughout the study period. Antenatal alcoholization in rats induced various histological changes in the frontal cortex, which were long-lasting and progressive during postnatal ontogeny.

Keywords: frontal cortex, neurons, antenatal alcoholization.

Alcohol consumption during pregnancy leads to the development of a number of specific injuries to the fetal body, which are combined in the concept of fetal alcohol syndrome, which is among the fetal alcohol spectrum disorders (FASD) [2, 14]. Published data indicate that the cerebral cortex is particularly sensitive to prenatal alcohol [4, 5], which in rodents induces neuron apoptosis and neurodegenerative changes [4]. Prenatal alcohol decreases the numbers and sizes of pyramidal neurons in the cerebral cortex in animals, decreasing their protein content and producing underdevelopment of the cytoplasm [7]. The sensorimotor cortex in rat pups shows signs of delayed development of neurons and their dendrites, with destructive and dystrophic changes to cells (karyocytolysis, chromatolysis, appearance of "ghost cells"), with significant ultrastructural damage [4, 14]. However, the dynamics of histological changes during postnatal ontogeny in these animals has received insufficient study.

The aim of the present work was to undertake a comparative study of the effects of prenatal alcoholization on the histological characteristics of neurons in the frontal cortex in rats of different ages.

Materials and Methods

Experiments were performed on 25 female mongrel white rats with a starting weight of 230 ± 20 g and their offspring (175 rat pups). All experiments were performed in compliance with the "Regulations for Studies Using Experimental Animals" [6]. The study was approved by the Biomedical Ethics Committee (Protocol No. 7 of December 23, 2013) of Grodno State Medical University. Animals were kept on a standard animal house diet. Rats of the experimental group received 15% ethanol solution as the sole drinking fluid throughout pregnancy (from the day on which spermatozoids were detected in vaginal smears until parturition), while control animals received the same quantity of water. Mean alcohol consumption by pregnant females was 4 ± 2 g/kg/day. On days 2, 5, 10, 20, 45, and 90 after birth, rat pups were weighed and decapitated, and brain weight was determined, along with the brain:body weight ratio. Fragments of the anterior part of the cerebral cortex were rapidly fixed in Carnoy's solution. Serial paraffin sections were stained with 0.1% toluidine blue by the Nissl method; ribonucleoproteins (RNP) were detected using the Einarsson method.

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TABLE 1. Characteristics of the Frontal Cortex in Rat Pups in Controls and after Prenatal Alcoholization, Me (LQ; UQ).

Study parameter	Group of rat pups	Time point, days					
		2	5	10	20	45	90
Cortical thickness, μm	Control	538 (532;567)	695 (679;711)	1156 (1131;1182)	1277 (1251;1307)	1465 (1444;1473)	1645 (1584;1654)
	Experimental	809* (802;823)	799* (796;880)	1020* (1016;1024)	1257 (1256;1262)	1445 (1379;1488)	1249* (1225;1262)
Neuron density, number per 1 mm^2 of section area	Control	16819 (16684;17223)	9553 (9553;9688)	7804 (7669;7804)	6122 (6055;6189)	5180 (4978;5247)	4575 (4306;4575)
	Experimental	13993* (13724;14263)	8880* (8880;9015)	6122* (6055;6728)	5382* (5247;5382)	3767* (3767;3902)	3229* (3095;3229)
Hyperchromic shrunken neurons, number per 1 mm^2 of section area	Control	0 (0;0)	0 (0;0)	0 (0;0)	134 (134;134)	134 (134;134)	0 (0;0)
	Experimental	0 (0;0)	0 (0;0)	0 (0;0)	336* (269;404)	404* (404;538)	404* (404;404)
Layer V neuron ribonucleoprotein content, OD units	Control	0.23 (0.22;0.23)	0.20 (0.18;0.22)	0.20 (0.18;0.22)	0.14 (0.13;0.15)	0.19 (0.18;0.2)	0.125 (0.12;0.13)
	Experimental	0.23 (0.2;0.24)	0.26* (0.2;0.28)	0.22 (0.19;0.26)	0.16 (0.145;0.165)	0.20 (0.19;0.21)	0.18* (0.17;0.19)

*Significant difference compared with control, $p < 0.05$. Me – median; numbers in parentheses are quartile boundaries (from 25 to 75).

Histological studies, microphotography, morphometry, and densitometry of chromogen deposits in histological preparations were performed using an Axioscop 2 plus microscope (Zeiss, Germany) with a Leica DFC 320 digital video camera (Leica, Germany) and the ImageWarp image analysis program (Bitflow, USA). The position of the frontal cortex on histological sections of the rat brain was identified using a stereotaxic atlas [13]. Preparations stained by the Nissl method were used to measure the thickness of the frontal cortex (five measurements in each animal) and neuron density was measured in layer V (five microscope fields for each animal), and among these the numbers of cells with different levels of cytoplasmic chromatophilia (hypo-, hyperchromic, hyperchromic shrunken, and ghost cells) were counted. The small and large diameters of neuron bodies were measured, and the cross-sectional areas, elongation factors, and form factors were calculated. At least 30 neurons from each animal and at least 150 from each group were examined, ensuring a sufficiently large sample set for subsequent analysis.

Mean values determined for numerical data from each animal were analyzed using nonparametric statistics in Statistica 6.0 for Windows (StatSoft Inc., USA). Descriptive statistics for each parameter were the median (Me), percentile boundaries (from 25 to 75), and the interquartile range (IQR). Numerical results are presented as Me (median), LQ (upper boundary of lower quartile), and UQ (lower boundary of upper quartile). Differences between parameters in the control and experimental group were regarded as significant at $p < 0.05$ (Mann and Whitney U test) [1].

Results

There were no significant differences in body weight, brain weight, or the brain:body weight ratio between control and experimental rat pups. Control pups displayed thickening of the frontal cortex throughout the observation period, while the frontal cortex of pups exposed to antenatal alcoholization was significantly thicker, by 33% on postnatal day 2 and by 13% on day 5, than in controls, while thickness on day 10 showed a statistically significant (by 12%) decrease. These differences were no longer present on days 20 or 45, though on day 90 there was a further decrease in cortex thickness, by 23%, compared with controls (Table 1).

Cortical layer V of alcoholized rat pups showed a significant decrease (by 10–25%) in the number of neurons per unit section area (density) at all time points studied (see Table 1).

In control rats, Nissl-stained preparations showed that the ratio of neurons with different levels of cytoplasmic chromatophilia differed significantly at different times of postnatal development; however, normochromic neurons were always dominant (Fig. 1, *a*) (the proportion was 60–70% of all neurons).

In the experimental animals, layer V of the cerebral cortex showed a decreased number of normochromic neurons and increased numbers of hyperchromic shrunken neurons and ghost cells at all time points, the intensity of these changes differing at different periods of postnatal development (see Fig. 1, *b*). The smallest changes were seen on day 2, where the increases in the numbers of hyper- and hypochromic neurons were statistically insignificant. The largest

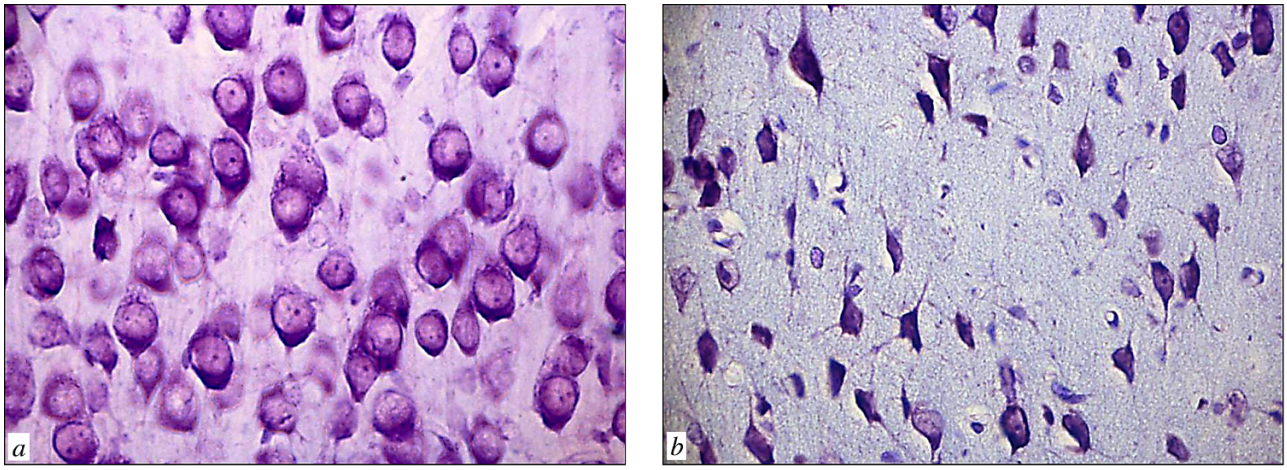

Fig. 1. Neurons in frontal cortex layer V in 45-day-old rat pups of the control group (*a*) and after antenatal alcoholization (*b*). *a*) Dominance of normochromic neurons; *b*) dominance of hyperchromic and hyperchromic shrunken neurons. Stained with thionine, Nissl method. Magnification ×400

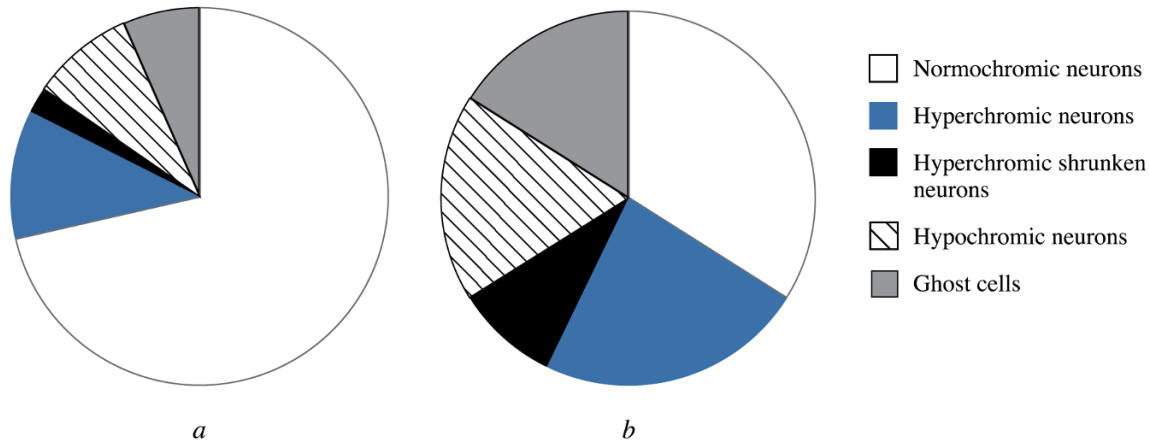

Fig. 2. Ratios of types of neuron with different chromatophilias in the frontal cortex in 45-day-old rat pups: control (*a*) and after antenatal alcoholization (*b*).

changes in the frontal cortex were seen on days 20–90 of postnatal development. Thus, the number of hyperchromic shrunken neurons was increased by 66% on day 45, while the numbers of hypochromic neurons and ghost cells increased by 20% and 40%, respectively, compared with controls (Fig. 2). Hyperchromic shrunken neurons in control animals were almost absent on days 45 and 90, while the numbers of these cells, conversely, increased sharply in animals subjected to antenatal alcoholization (Fig. 3).

Studies of the sizes and shapes of neuron bodies revealed a statistically significant transient increase in the cross-sectional area of neuron perikarya in layer V on day 2. However, on days 20–90 of postnatal development, neuron body size in experimental rat pups became significantly smaller than that in controls (see Fig. 3), while neuron size progressively increased in control animals. In rats subjected to antenatal alcoholization, the growth of neuron bodies ceased after 10–20 days of postnatal development (see Fig. 3). There was a negative correlation between neuron cross-sectional area and the number of hyperchromic shrunken neurons ($r = -(0.87–0.98)$; $p < 0.05$).

The RNP content in the cytoplasm of frontal cortex layer V neurons in alcoholized rats was significantly greater on days 5 and 90 (see Table 1) than in controls, which correlated with increases in the number of hyperchromic neurons at these time points ($r = 0.86–0.96$, $p < 0.05$).

Discussion

The thickening of the cortex seen here in antenatal alcoholized rat pups on postnatal day 5 and especially day 2 may be linked with the perivascular edema and neuron swelling seen on histological preparations. The strongest correlation between cortical thickness and neuron body cross-sectional area ($r = 0.98$, $p < 0.01$) was seen on day 2. The subsequent decrease in cortical thickness on day 10

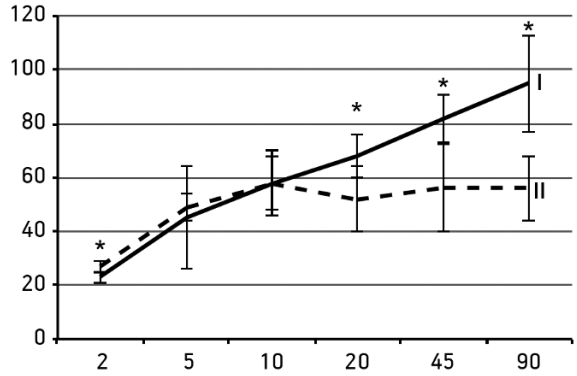

Fig. 3. Cross-sectional areas of frontal cortex layer V neuron bodies in control rat pups (I) and rat pups subjected to antenatal alcoholization (II). The abscissa shows age (days) and the ordinate shows study parameters (μm^2). *Significant differences compared with controls, $p < 0.05$. Vertical bars show interquartile ranges.

may be associated with the disappearance of edema, while recurrent thinning on day 90 may be due to total shrinkage of neurons, which is supported by the strong correlation between cortical thickness and the number of shrunken neurons ($r = –0.94$, $p < 0.01$). At the same time, cortical thickness in control animals showed a consistent growth.

Size reductions and shrinkage of neurons have been reported previously in adult rats subjected to antenatal alcoholization [7, 9, 10].

In both study groups, postnatal ontogeny was associated with a decrease in the density of neuron bodies, which may be linked with more intense growth of the neuropil as compared with neuron bodies. The decrease in neuron density seen here in the frontal cortex of experimental rats at all postnatal time points may be associated with the death of a proportion of neurons due to alcohol exposure during embryogenesis. This neuron deficit persists lifelong. Decreases in cortical thickness and the numbers of neurons within it and increases in the numbers of hyperchromic shrunken neurons and ghost cells at different postnatal time points in the offspring of rats consuming alcohol during pregnancy have been reported by other authors [12].

The cessation of growth and the shrinkage of neurons seen in antenatally alcoholized rats from postnatal day 20 may be linked with impairment to water-salt metabolism and the cell cytoskeleton, as well as with oxidative stress, activation of lipid peroxidation, and oxidation of proteins. Free radicals interact with DNA and produce structural modifications. In addition, free radicals damage cell membranes and also the membranes of organelles in neurons, particularly mitochondria, while the level of the endogenous antioxidant glutathione decreases [9]. Alcohol impairs transcription and translation processes in the developing brain and induces derangements of gene expression [11]. Electron microscopic studies have demonstrated increased cytoplasmic density in hyperchromic neurons, with cleft-like lightened areas and damaged organelles, deep invaginations into the nuclear envelope, breakup of Golgi complex cisterns into vacuoles, and severe swelling of mitochondria [7]. Published data also indicate that ethanol inhibits the expression of endogenous insulin and insulin-like growth factor (IGF) polypeptides, as well as IGF1 and IGF2 receptors in the brain. The results demonstrate that the main impairments in the brain in FASD are due to deranged insulin/IGF signaling [10].

These structural changes may underlie the known neurological and behavioral impairments in animals after antenatal alcoholization. Behavioral impairments include cognitive [3], sensorimotor, and emotional disorders [4], Neurological dysfunctions evoked by antenatal alcoholization include auditory dysfunction, speech delay, and inability to communicate and learn [3]. In particular, antenatal alcoholization leads to decreased motor activity in the open field test in rat pups at age 17 days and has negative effects on their ability to acquire (but not to reproduce) a conditioned food-related reflex. In addition, animals develop tolerance to alcohol [8].

Thus, antenatal alcoholization produces profound and varied histological changes in the frontal cortex in rats, which during postnatal ontogeny are wavelike, long-lasting, and sometimes progressive. Thus, there was an increase (days 2 and 5) and then a decrease (days 10 and 90) in cortical thickness and neuron size (days 20–90), a decrease in the number of neurons in cortical layer V, and decreases in the numbers of hyperchromic shrunken neurons and ghost cells at all time points. Of particular interest are the cessation of growth and the progressive shrinkage of neurons in the frontal cortex from day 20 of postnatal development.

REFERENCES

1. N. V. Batin, *Computerized Statistical Data Analysis: Textbook*, Institute of Scientific Training, Belarus Academy of Sciences, Minsk (2008).
2. S. M. Zimatkin and E. I. Bon', "Antenatal alcoholization: damage to the internal organs," *Nov. Med. Biol. Nauk.*, No. 2, 168–174 (2013).
3. S. M. Zimatkin and E. I. Bon', "Fetal alcohol syndrome: behavioral and neurological impairments," *Zh. Grodn. Med. Univ.*, No. 2, 14–17 (2013).
4. S. M. Zimatkin and E. I. Bon', *Fetal Alcohol Syndrome: Monograph*, Novoe Znanie, Minsk (2014).
5. S. M. Zimatkin and E. I. Bon', "Effects of alcohol on the developing brain," *Morfologiya*, **145**, No. 2, 79–88 (2014).
6. N. N. Karkishchenko and S. V. Gracheva, *Handbook for Laboratory Animals and Alternative Models in Biomedical Studies*, Profil-2S, Moscow (2010).
7. E. N. Popova, *Ultrastructure of the Brain, Alcohol, and Offspring*, Nauchnyi Mir, Moscow (2010).
8. E. L. Abel, "In utero alcohol exposure and development delay of response inhibition." *Alcoholism*, **6**, 369–376 (1982).
9. P. S. Brocardo, "Anxiety- and depression-like behaviors are accompanied by an increase in oxidative stress in a rat model of fetal alcohol spectrum disorders: Protective effects of voluntary physical exercise," *Neuropharmacology*, **62**, 16–18 (2012).
10. S. M. de la Monte and J. R. Wands, "Role of central nervous system insulin resistance in fetal alcohol spectrum disorders," *J. Popul. Ther. Clin. Pharmacol.*, **17**, No. 3, 390–404 (2010).

11. M. L. Kleiber and E. Wright, "Maternal voluntary drinking in C57BL/6J mice: advancing a model for fetal alcohol spectrum disorders," *Behav. Brain Res.*, **223**, 376–387 (2011).
12. D. Lopez-Tejero, "Effects of prenatal ethanol exposure on physical growth sensory reflex maturation and brain development in the rat," *Neuropathol. Appl. Neurobiol.*, **12**, No. 3, 251–260 (1986).
13. G. Paxinos and C. Watson, *The Rat Brain in Stereotaxic Coordinates*, Academic Press, London (1998).
14. E. P. Riley, M. A. Infante, and K. R. Warren, "Fetal alcohol spectrum disorders: an overview," *Neuropsychol. Rev.*, **21**, 73–80 (2011).