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Experimental and Theoretical Problems

Structural Changes in Human Brain Tissue in Prenatal Alcoholization at Different Periods of Intrauterine Development

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Objectives. To assess the influences of prenatal alcoholization on the formation of various structural components of the brain in human embryos. **Materials and methods**. The study examined 26 specimens of embryonic material at 8–11 weeks of intrauterine development. Specimens were divided into four subgroups depending on developmental period (Control 1 at 8–9 weeks of development and Control 2 at 10–11 weeks of development) and maternal medical history (presence or absence of a diagnosis of "alcoholism stages I–II" in the medical history). Morphometry was run on Nissl-stained semithin sections. Diameters and areas of each individual tissue element (neuroblasts, glioblasts, vessels of the micricirculatory bed (MCB)) were determined, along with specific areas (the ratio of the total area of the structure under study to the area of the entire section); mean numbers of these structures per unit area of sections were also calculated. Morphometric analysis was run in AxioVision 4.8 (Carl Zeiss, Germany) and statistical analysis of differences between study cohorts used the Mann–Whitney test (differences taken as significant at $p < 0.05$). **Results**: As compared with the intact groups, the alcohol groups showed insufficient increases in the area of MCB vessels, combined with compensatory increases in the numbers of vessels per unit area on sections (48.5 and 83.3 μm^2, respectively, $p < 0.05$). Comparison of glioblast sizes in the control and alcohol subgroups at different stages of development (average area 21.3 and 32.1 μm^2; 12.9 and 13.3 μm^2, respectively) revealed a delay in the increase in size of cellular structures in alcoholized groups at the initial stages, while comparison of data at later periods revealed no significant difference, though there was an increase in the specific number of cells in subgroup A2 ($p < 0.05$). Neuroblasts also showed a decrease in cell size with increasing development time in both the control and the alcohol subgroups. However, cell size in group A2 was greater than that in group C2, while the number of these cells was smaller ($p < 0.05$). **Conclusions**. Alcohol led to changes in the sizes and numbers of neuroblasts, glioblasts, and MCB vessels and, as a consequence, to disproportionality in the development of all brain tissue. Changes progressed with increasing development time.

Keywords: alcohol, brain, neuroblasts, glioblasts, microvasculature.

The challenge of the development of the human brain is currently one of the most pressing but sadly far from finally resolved questions. Many aspects of the formation of the most important brain structures in normal and pathological conditions still remain unexplored. Given that brain development processes are extremely sensitive to the effects of exogenous factors, including ethanol, study of this issue is a priority.

Alcohol is one of the most extensively used psychoactive substances worldwide [1]. Consumers include all groups within the population, regardless of sex and age, and women during the period of fertility are no exception. Alcohol consumption during pregnancy or breastfeeding is extremely dangerous. Ethanol is one of the most common and easily accessible teratogens [2] and can have a variety of adverse effects on the development of the central nervous system (CNS) of the embryo and fetus [3].

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The effects of alcohol on the development of the central nervous system are characterized by both macro- and microscopic changes. Macroscopic changes include the development of a number of different forms of dysgenesis [4], as well as decreases in total brain weight in general [5] and cortical weight due to a decrease in the gyrification index in particular [6]. Characterization of a tissue's response at the microscopic level requires characterization of the responses of all its major components individually.

There is as yet no consensus on what changes in the microvascular bed (MCB) are produced by chronic prenatal alcoholization. It is clear that this effect is mediated by a change in angiogenesis. However, the nature of these impairments is a matter of debate. Some studies indicate that changes such as a decrease in the density of blood vessels in brain tissue occur only after 30 weeks of development [7]. However, our previous studies indicate that there is an increase in the density of the microvascular bed of the cerebral cortex as early as 8–12 weeks of development [8].

The most adverse effects of alcohol are those on radial glial cells, astrocytes, oligodendrocytes, and microglia, as these lead to disruption of migration and subsequently to decreases in the numbers of cells in neuron populations, inducing abnormal brain plasticity [9]; this has been confirmed in other investigations [10, 11].

A number of studies have confirmed decreases in oligodendrocyte numbers [12], which in turn lead to impaired myelination [13]. In addition, there is a tendency for the number of microglial cells to decrease, as demonstrated in a rodent model [14]. As regards astrocyte responses, there is currently no consensus in the literature about the changes which occur, because alcohol does not affect astrocytes directly, but secondarily, for example via neuron damage, impaired maturation of progenitor cells, release of proinflammatory cytokines by microglia, or damage to the vascular bed of the brain [9].

The effects of alcohol directly on nerve cells are heterogeneous; study results depend on the protocols used and the cell populations studied. Common impairments include abnormalities of migration [15, 16], which results in anomalies in the location of neuronal populations in the layers of the cerebral cortex [17]. In addition, there is a general tendency towards apoptosis. Most authors agree that prenatal alcoholization leads to a decrease in the number of cellular elements in tissue [18].

The aim of the present work was to assess the extent of the influence of prenatal alcoholization on the formation of neuroblasts, glioblasts, and the vessels of the MCB in human embryos and fetuses at different stages of intrauterine development.

Materials and Methods. Women aged 25–41 years (mean 37 years) took part in the study and were divided into two groups. Group 1 consisted of participants without somatic or mental pathology, who had had no contact with toxic substances, radioactive radiation, or other teratogenic factors at work or at home, and did not drink alcohol before (during the period of one month before conception) or during pregnancy. Group 2 consisted of women with stage I–II alcoholism (for 3–13 years). All study participants had previously undergone courses of treatment for alcoholism (1–10 times), though none took disulfiram during the period preceding collection of material for the study.

During operations, 26 samples of embryonic material were obtained at 8–11 weeks of intrauterine development. Two groups were formed and divided into subgroups on the basis of the developmental period and the fact of alcohol consumption by the patients. Group A (Alcohol) consisted of specimens from women who suffered from alcohol addiction and was divided into two subgroups: A1 – embryos at 8–9 weeks of development and A2 – embryos at 10–11 weeks of development. Each subgroup included six specimens. Group C (Control) had a similar composition, but consisted of mentally healthy women: C1 at nine weeks and C2 at 10–11 weeks, each group consisting of seven specimens.

Specimens were obtained during surgery for artificial termination of pregnancy in maternity hospitals and gynecological departments of hospitals in Tomsk. All experimental participants were given detailed information and confirmed their consent in writing. All procedures were carried out in compliance with the requirements and recommendations of the Ethics Committee and were consistent with the Declaration of Helsinki of 1975 and the provisions of the 2000 revision.

Specimens were examined using an AxioScope A1 light microscope (Carl Zeiss, Germany) with material prepared as follows: initial fixation in 0.5% glutaraldehyde solution in 0.1 M sodium phosphate buffer pH 7.3–7.4, with additional fixation in 1% osmium oxide solution. Subsequent processing consisted of dehydration in alcohols of increasing concentrations and embedding in epoxy resin (Araldite). Semi-thin sections (0.5–1 μm) were prepared from the specimens using an Ultracut-E ultratome (Reichert, Austria) and stained with toluidine blue (Nissl dye) by the generally accepted standard technique. Images were made using a Canon G10 digital camera.

Neuroblasts in processed specimens were identified as round cells with a centrally located nucleus. Nuclei had a light matrix, occupying most of the volume of the perikaryon, and included 1–3 nucleoli. The cells were located in groups of different sizes. Glioblasts were identified as round cells with a high nucleus:cytoplasm ratio located in groups between nerve cells. A characteristic feature of nuclei and glioblasts in general was a more intense coloration than seen with neuroblasts. Nuclei contained intensely stained nucleoli, occupying a near-central or central position. Glioblast/neuroblast cellular contacts were detected, along with groups of glioblasts surrounding nerve cells (Figs. 1–4).

AxioVision 4.8 software was used for morphometric analysis. The aim of the study was to identify quantitative changes in neuroblasts, glioblasts, and MCB vessels in the

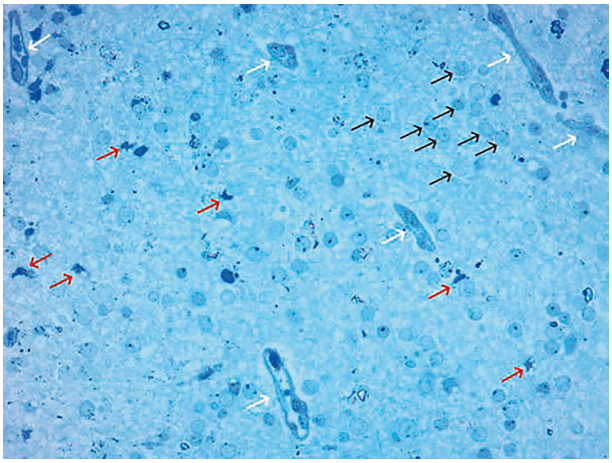

Fig. 1. Brain of a human embryo at 8–9 weeks of development, normal (group C1). Here and in Figs. 2–4: staining with toluidine blue. The photograph shows MCB vessels (white arrows), glioblasts (red arrows), and neuroblasts (black arrows). Magnification ×400.

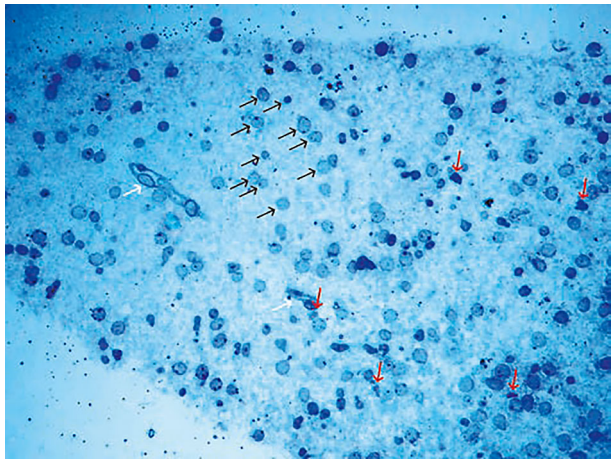

Fig. 2. Brain of a human embryo at 8–9 weeks of development, prenatal alcohol intoxication (group A1).

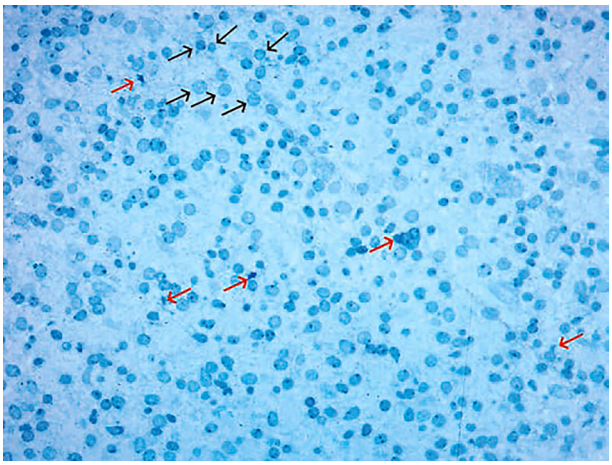

Fig. 3. Human fetal brain at 10–11 weeks of development, normal (group C2).

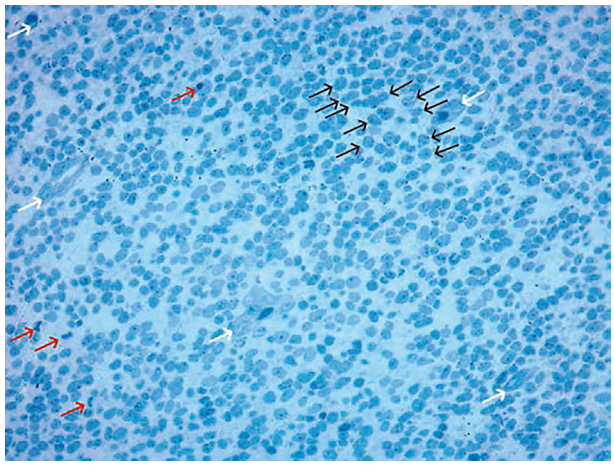

Fig. 4. Human fetal brain at 10–11 weeks of development, prenatal alcohol intoxication (group A2).

human sensorimotor cortex at different stages of intrauterine development. The diameters and areas of each individual tissue element were determined, along with their specific areas (the ratio of the total area of the structure under study to the area of the entire section) and calculation of the average number of each structure per unit area of sections.

Statistically significant differences between specimens were identified using the Mann–Whitney test run in Statistica 10; differences were taken as significant at $p < 0.05$.

Results. Analysis of brain tissue from human embryos at 8–12 weeks of development led to the conclusion that the cellular and vascular elements were sufficiently differentiated.

The MCB at this stage of development consisted of differentiated venules and arterioles, along with an extensive network of capillaries. The brain was characterized by the presence of nonmuscular venules, whose walls consisted of endothelial cells with a loose connective tissue membrane. Arteriole walls, conversely, contained circular smooth muscle cells. Capillaries consisted of somatic-type vessels and had formed walls consisting of endothelial cells and a basement membrane, along with pericytes located within its structure and adventitial cells located outside. Mean vessel diameter was 6–11 μm. Blood cells were seen in the lumens of some vessels.

Morphometric analysis showed that increases in developmental period were linked with significant decrease in the mean and specific areas of MCB vessels, as well as increases in their average and specific numbers as compared with the control subgroups (Table 1). Brain tissue developing in conditions of chronic alcoholism was characterized at the later stages by smaller vessels with greater density on sections. This situation might cause ischemia, leading to functional disorders during subsequent development.

These results showing changes in MCB vessel size correlate with published data [19, 20]. The current view is that decreases in vessel diameter may be the result of decreases

TABLE 1. Dynamics of Quantitative Indicators of Blood Vessels in Study Groups

Parameter	Mean area, μm^2	Specific area of vessels, %	Vessels per mm^2, *n*
C1	78.0	0.42	52
C2	83.3	0.77	91
A1	75.7	0.40	52
A2	48.5*,**	0.37*,**	136

*Significant difference compared with C2 ($p < 0.05$); **significant difference compared with A1 ($p < 0.05$).

TABLE 2. Dynamics of Quantitative Indicators of Glioblasts in Study Groups

Parameter	Mean area, μm^2	Mean perimeter, μm	Cells per mm^2, *n*
C1	32.1	23.9	75
C2	13.3*	15.0*	160
A1	21.3*	19.1*	121
A2	12.9*	14.8**	263

*Significant difference compared with C1 ($p < 0.05$); *significant difference compared with A1 ($p < 0.05$).

TABLE 3. Dynamics of Quantitative Indicators of Neuroblasts in Study Groups

Parameter	Mean area, μm^2	Mean diameter, μm	Neuroblasts per mm^2, *n*
C1	39.3	7.0	945
C2	19.2*	5.1*	8295
A1	35.2*	6.5*	1574
A2	25.4**,***	5.6**,***	7790

*Significant difference compared with C1 ($p < 0.05$); **significant difference compared with C2 ($p < 0.05$); ***significant difference compared with A1 ($p < 0.05$).

in pericyte energy levels. This condition leads to a persistent increase in intracellular calcium and resultant narrowing of vessels [20].

As noted earlier, the effects of alcohol on changes in the density of MCB vessels on sections are subject to debate. Despite the fact that the present study confirmed the data obtained earlier by other authors [8] indicating a significant increase in tissue vessel density, the literature contains studies generating the opposite results, i.e., decreases in the overall vessel distribution density in brain tissue [4]. This discrepancy in results may arise from dose dependence of the changes studied, differences in the sensitivity of the areas studied to the effects of ethanol, and differences in experimental protocols.

Analysis of the morphometric parameters of glioblasts established a significant difference in cell size at the earlier stages, when glioblast size in subgroup C1 was greater than that in subgroup A1. Parameters of specimens at 10–11 weeks of development did not show significant differences. There were also increases in the mean numbers of glioblasts in the alcoholic subgroups at all periods studied (Table 2). Although the study did not look at individual glial cell populations, a general increase in the number of glioblasts was detected.

The world literature contains many studies showing that prenatal exposure to ethanol leads to decreases in cell pools in almost all glial cell populations. However, most authors note that the reactions of the same cell populations may differ in different areas of the brain [12]. This phenomenon may be associated with the indirect effect of ethanol on cells and disruption of their migration and maturation. In addition, the reason for the increase in the number of cellular elements may be the development of astrogliosis resulting from accelerated transformation of radial glial cells [21].

The study noted reactive changes in both glioblasts and neuroblasts, with the appearance of hyperchromic wrinkled and non-wrinkled cells. A characteristic feature of these was a marked expansion of the perivascular and pericellular spaces.

Morphometric analysis of neuroblasts revealed adverse changes in the rate of differentiation as cells developed in conditions of chronic alcoholism, which was apparent as mean neuroblast area being larger in subgroup A2 than subgroup C2. Changes in cell distribution density on sections were as follows: in the early stages of intrauterine development, the number of neuroblasts in tissue exposed to ethanol was significantly greater. The number of cells per mm^2 in subgroup A1 was 40% greater than that in sub-

group C1. However, as development proceeded over time, this parameter became larger in tissue developing in normal conditions: the mean number of neuroblasts in subgroup C2 was 6% greater than that in subgroup A2 (Table 3).

Despite the fact that the literature currently lacks data describing changes in neuroblast size during normal development and in conditions of prenatal exposure to ethanol, we confirmed data reported from studies [8] indicating that intrauterine alcoholization leads to a decrease in neuroblast size. Our results on changes in the quantitative measure of neuroblasts are comparable with published data. Most authors have noted trends to both impaired migration and apoptosis [12, 18]. The greater number of cells in subgroup A1 and the subsequent lower level of this indicator in subgroup A2 may result from the fact that subcortical structures are affected first and only then various parts of the cerebral cortex [18].

Conclusions. Thus, this study found that alcohol has significant effects on the formation of brain tissue in embryos. This influence is apparent as changes in the sizes and numbers of neuroblasts, glioblasts, and MCB vessels, leading to disproportionality in the development of all brain tissue, which progresses with development.

The authors declare no conflict of interest.

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