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Structural Changes in the Elements of the Blood-Brain Barrier of the Hippocampus in Rats after Exposure to Perinatal Hypoxia and the Potential for Their Pharmacological Correction

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Objectives. To study the dynamics of structural changes in elements of the blood-brain barrier of the hippocampus during the early postnatal period and subsequent ontogeny after exposure to perinatal hypoxia and to investigate the potential for pharmacological correction of these changes. **Materials and methods.** Experiments were performed on Wistar laboratory rats with a model of the encephalopathy of premature human infants using electron microscopy. **Results.** Perinatal hypoxia was followed by detection of structural damage to capillary walls: there were increases in the size of endotheliocytes, increases in the numbers of cytoplasmic processes, changes in the diameter of capillary lumens, and delays in the formation of the basal membrane. A marked endothelium-protecting effect was seen with the formulation Salifen at all study time points. Salifen was found to decrease the intensity of the reactions of elements of the blood-brain barrier to the harmful actions of perinatal hypoxia. **Conclusions.** Perinatal hypoxia has marked damaging effects on all elements of the blood-brain barrier of the hippocampus, the induced lesions being detected at both the early stages of postnatal development and in animals reaching adulthood. Use of Salifen immediately after exposure to perinatal hypoxia eliminated much of the structural damage to endotheliocytes and the basal membrane by the juvenile period. This study provides grounds for believing that the use of Salifen in ischemic brain injury in neonates has potential and our series of further preclinical studies will be continued.

Keywords: hippocampus, perinatal hypoxia, blood-brain barrier.

Many experimental and clinical studies have provided evidence that perinatal hypoxia, which is frequently encountered in medical practice, can initiate the development of central nervous system pathology which becomes manifest in later ontogenesis in the form of neuropsychiatric diseases and disorders. It is believed that the hippocampus, with its extensive connections with many brain formations, may have an active role in these processes [2, 8].

Our previous studies [4] using a model of neonatal encephalopathy demonstrated that exposure to perinatal hypoxia was followed by marked (to different extents) death of nerve cells in all the hippocampal fields, along with thinning of the pyramidal neuron layers and decreases in pyramidal neuron size. The literature lacks systematic reports on the reactions of elements of the blood-brain barrier (BBB) of the hippocampus.

The aims of the present work were first to study the dynamics of structural changes to elements of the BBB of the hippocampus during the early postnatal period and subsequent ontogenesis after exposure to perinatal hypoxia and,

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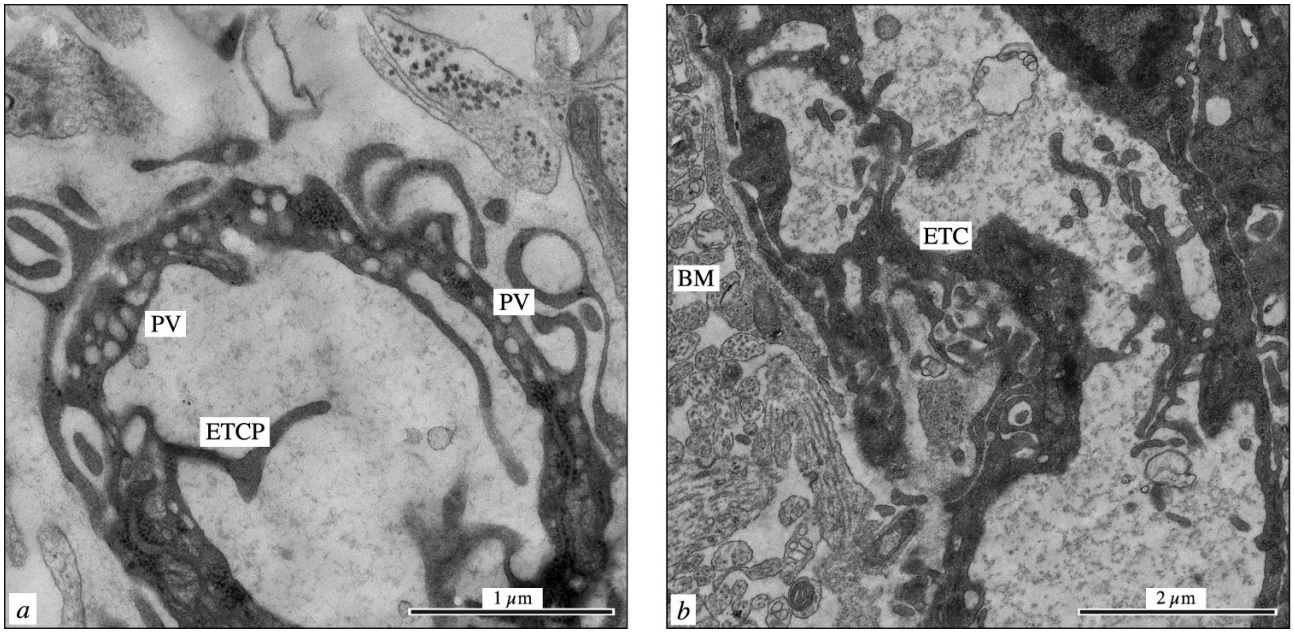

Fig. 1. Capillaries in hippocampal field CA4 during the early neonatal period after exposure to hypoxia. *a*) A capillary in a three-day-old rat pup one day after hypoxia. Abundant pinocytic vesicles in the cytoplasm of endothelial cells and their individual processes; *b*) a capillary in a five-day-old rat pup after hypoxia, with increased electron density of endothelial cells and their nuclei and cytoplasm, along with numerous processes in the vessel lumen (PV – pinocytic vesicles; ETCP – endothelial cell processes; BM – undifferentiated basal membrane.

second, to investigate the possibility of pharmacological correction of these changes.

Pharmacologists at the Volgograd Medical University have identified a novel class of pharmacological agents – endothelial protectors. Using a model of placental hypoxia, they demonstrated that the Russian synthetic compound Salifen has endothelium-protecting effects [1, 7]. Our subsequent studies using a model of the encephalopathy of premature human infants observed a neuroprotective effect in relation to GABAergic neurons and an endothelium-protective effect in the neocortex in rats [5, 6].

Salifen was found to have a wide spectrum of actions and chemically is a GABA derivative. Salifen has clear protective actions in cerebral ischemia (it decreases death among animals, improves retention of memory traces, and prevents reductions in movement and orientational-exploratory activity in animals after brain injury) and has positive influences on hemorheology (it decreases platelet and erythrocyte aggregation), and it improves cerebral circulation in cerebral ischemia (it prevents decreases in the blood flow rate in the middle cerebral artery in bilateral occlusion of the common carotid arteries). This agent improves the development of sensorimotor reflexes and muscle strength in the offspring of rats with experimental gestosis, promotes increases in locomotor and orientational-exploratory activity, decreases anxiety levels, and has positive mnemotropic influences [7]. Working from these data, we elected to study the protective effects of Salifen in relation to the elements of the BBB of the hippocampus in a model of neonatal encephalopathy and to investigate the possibility of using it therapeutically in the early postnatal period.

Materials and Methods. Studies used Wistar laboratory rats from the animal house of the Pavlov Institute of Physiology, Russian Academy of Sciences. All animal procedures were carried out in compliance with the "Regulations for Studies Using Experimental Animals" and the requirements of the Directives of the Council of the European Community (86/609/EEC) on the use of laboratory animals. The experimental protocols were approved by the Commission for the Humanitarian Treatment of Animals of the Pavlov Institute of Physiology. The effects of hypoxia on the brains of neonatal rats pups were studied by exposure in an individual chamber for 1 h breathing a mixture consisting of 7.6–7.8% oxygen, 0.15–0.21% carbon dioxide and 91.8% nitrogen at 21.3–23.0°C and normal general atmospheric pressure. Hypoxia was imposed on postnatal day 2. The study used three groups of animals: group 1 consisted of rat pups subjected to hypoxia in the barochamber and given s.c. injections of Salifen one day after hypoxia (15 mg/kg for 14 days); group 2 consisted of animals subjected to the same hypoxia and given injections of physiological saline at the same times as Salifen in group 1; group 3 consisted of control animals of the same age which were placed in the barochamber without hypoxia and given physiological saline for 14 days starting one day later. Each group included 8–10 rat pups collected from different litters.

Ultrastructural study of fragments of hippocampus (fields CA1–CA4) was performed after fixation in 2.5% glu-

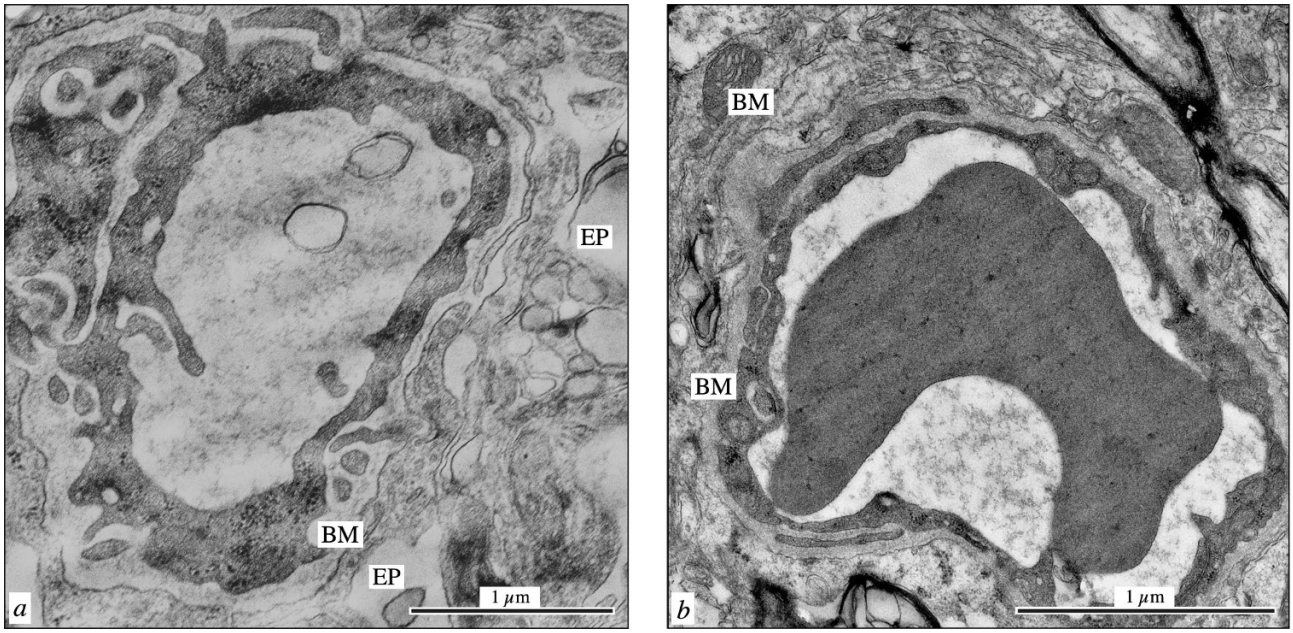

Fig. 2. Hippocampal capillaries in the juvenile period of ontogenesis. *a*) After perinatal hypoxia. Uneven distribution of filaments in undifferentiated basal membrane (BM) (EP – edematous processes, delayed maturation of neuropil; *b*) after perinatal hypoxia and use of Salifen; filling of BM with filaments without formation of plates, absence of astroglial reaction.

taraldehyde solution in 0.1 M phosphate buffer pH 7.4 containing sucrose followed by additional fixation in 2% osmium tetroxide solution and embedding in resin. Ultrathin sections cut on an LKB-III ultratome were contrasted with 1.5% uranyl acetate and lead citrate solution and examined under a Tecnai G2 Spirit electron microscope (FEI, Germany) with an accelerating voltage of 70 kV. Experimental specimens were studied in the early postnatal period (on days 5 and 10), at juvenile age (20 days), pubertal age (60 days), and in animals reaching adult age (80–90 days).

Results. The BBB is a complex multicomponent structure responsible for the selective bidirectional transport of substances between blood and the brain. The main barrier structures are believed to be the slowly renewing endothelial cells, which have specialized intercellular contacts, transport vesicles and vacuoles, and a basal layer (or basal membrane (BM) in the nomenclature we use) with an extracellular component – filaments containing mainly collagen type IV, laminin, and other filaments. Externally, the basal layer of the barrier function is carried out by pericapillary astrocyte processes or bodies, as well as pericytes.

These experiments showed that all these elements displayed different extents of reactions to the actions of acute hypoxia. Structural damage induced by hypoxia was seen on all the sections examined. The most significant changes in BBB structures were seen in the early postnatal period. At one day after hypoxia, there was a sharp increase in the electron density of the nuclei and cytoplasm of capillary endothelial cells. Tight junctions between endotheliocytes were open and BM were often not clearly outlined; the extracellular component was minimal and not spatially organized (Fig. 1, *a*).

Endotheliocytes showed different levels of differentiation and their nuclei had different sizes and electron densities and different chromatin concentrations. The endoplasmic reticulum consisted of dilated individual cisterns; there were few cytoplasmic processes and there were few of them within capillary lumens (see Fig. 1, *a*). The electron density of pericytes was elevated. The edematous neuropil had a sharply reduced number of cell process growth cones.

On day 5 after hypoxia, the nuclei of a large proportion of endothelial cells acquired a paddle shape and had marked chromatin condensation, with increased electron density, increased cytoplasm volume, and the appearance of multiple processes, leading to some narrowing of microvessel lumens (see Fig. 1, *b*). The width of the basal membrane was increased, though its extracellular material was present as reticular structures, though plates (laminae rarae et densae) were generally not seen.

By the end of postnatal day 10, there was an increase in the volume of the more differentiated cytoplasm of endothelial cells, filling and narrowing the lumens of a significant proportion of capillaries. The BM acquired a smoother outline and virtually the whole perimeter was embedded in fibrous extracellular material, some areas showing the very initial stages in the formation of the dense plate. Externally, the BM showed edematous terminal pedicles of perivascular astrocytes. Attention is drawn to the large number of mitochondria with high electron density and altered cristae, typical of mitochondrial disease.

During the juvenile period (20 days), the number of large vessels increased, though new capillaries continued to form. Endotheliocyte cytoplasm volume decreased as compared with the previous time point and it contained small numbers of organelles; the extent of tight junctions increased. Capillary lumens contained small cytoplasmic processes. BM width and the number of fibrous structures within it increased, though in terms of density the layer remained undeveloped. Cleavage and folding of the BM was seen. The sizes of the astrocyte processes adjacent to the outer surface of BM increased (Fig. 2, *a*). Degenerate pericytes were sometimes seen.

By the pubertal period (60 days), the microcirculatory bed and structural elements of the BBB were formed, and in some areas the BM was identified as a dense plate. The actions of perinatal hypoxia had uniform effects on the state of the elements of the BBB in the prepubertal and pubertal periods, differing only in intensity. The proliferative activity of endotheliocytes decreased, cytoplasmic volume decreased, and their processes did not fill capillary lumens, as they did at the earlier postnatal and juvenile periods.

Animals reaching adulthood (80–90 days) after perinatal hypoxia showed moderate dilation of capillary lumens, high pinocytic activity in the cytoplasm, and an increased number of fibrillary pericapillary astrocyte processes. Some capillaries showed increased cytoplasmic electron density in individual endothelial cells and irregularity of the BM (dilation and constriction, appearance of folds) and contained an abundance of loosely distributed, chaotically oriented filaments.

Studies of experimental material from animals subjected to perinatal hypoxia and given therapeutic doses of Salifen demonstrated its positive actions on the state of all elements of the BBB at all time points.

Animals at the juvenile, prepubertal, and pubertal periods and animals reaching adulthood showed decreases in endotheliocyte size, greater differentiation of their cytoplasm, and decreased numbers of cytoplasmic processes in the capillary lumens, so marked deformation of microvessel lumens was uncommon.

The BM consisted of extracellular material organized into plates all around the perimeter, though dense plate was present only in some segments of the perimeter. Protoplasmic astrocytes showed a high level of differentiation (Fig. 2, *b*).

Discussion. The present study provided the first observation of the dynamics of ultrastructural changes in the cellular and noncellular elements of the BBB in the hippocampus after perinatal hypoxia using a model of the encephalopathy of premature infants. The development of encephalopathy is believed to involve a significant role for various impairments to the structure of the walls of brain capillaries, which include unique elements (endotheliocytes, BM, pericytes) whose concordant operation is required not only for BBB permeability but also for the blood supply to the brain overall [8]. This latter circumstance is what attracts extensive research attention to studies of the role of the endothelium in the development of vascular diseases and the search for and development of new-generation agents with endothelium-protective properties. Some hold the view that the defense and protection of BBB elements constitute a defining strategy in the treatment of cerebral ischemia [11, 12].

The abnormalities in microvessel walls found in this study after hypoxia (increased endothelial size, elevated number of cytoplasmic processes, changes in capillary lumen diameter, etc.) have been assessed functionally in the review by Distefano and Pratico [9], which addressed studies of the molecular bases of the pathogenesis of encephalopathies and restorative processes in the brain after perinatal hypoxia-ischemia. This review presents data on impairments to the mechanisms of cerebral blood flow autoregulation, which in normal conditions maintain the stability of the volume blood flow in term neonates. In preterm individuals, autoregulatory processes are altered because of imbalance in humoral vasoconstrictor and vasodilator factors synthesized by endothelial cells, pericytes, and astro- and oligodendroglia. Impairments to the microcirculation become a quite long-lasting inducer of delayed differentiation of all the cellular and noncellular components of nervous tissue, as noted in our results.

Many authors currently take the view that the basal membrane has a significant role in the barrier function [10, 13]. This study confirmed the view that first, the BM undergoes final differentiation after birth and, second, perinatal hypoxia delays the establishment of this element of the BBB. The factual data obtained here suggest that the formation of BM plates, i.e., laminae densae et rarae, serves as a measure of the level of BM differentiation, the former appearing not around the whole perimeter of capillaries on postnatal day 5 in control animals but only on day 20 in animals subjected to perinatal hypoxia. It should be noted that plates are made up of filaments (mainly made of collagen type IV and laminin), which also have an important role in regulating BBB permeability.

Our studies demonstrated that Salifen had a marked endothelium-protecting effect at all time points. Administration of Salifen was shown to decrease the strengths of the reactions of BBB elements to hypoxia, and it probably promotes the development of adaptive responses, which is made possible by implementation of the histogenetic development program. Confirmation of this may come from physiological data evidencing improvements in a variety of behavioral tests in animals subjected to perinatal hypoxia and given Salifen [3].

These facts provide grounds for the view that after the harmful action of hypoxia, Salifen has a marked protective effect on elements of the BBB in the hippocampus, which probably initiates the development of adaptive processes, leading to normalization of the differentiation of both neurons and glial elements.

Thus, these studies using a model of encephalopathy of premature human infants showed that perinatal hypoxia has

significant harmful actions on all elements of the BBB of the hippocampus, induced damage being detected both at the early stages of postnatal development and in animals reaching adulthood. Use of Salifen immediately after exposure to perinatal hypoxia ameliorates many of the structural impairments to endotheliocytes and the BM by the juvenile period. These studies provide grounds for regarding the use of Salifen in ischemic brain damage in neonates as having potential, and our series of further preclinical studies will continue.

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