

Study of cellular changes induced by moderate cerebral ischemia achieved through internal carotid artery ligation

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Abstract

Reduced cerebral blood flow beyond the compensatory mechanisms leads to cerebral hypoxia. Hypoxia causes various lesions of neurons, glial cells and cerebral blood vessels, depending on its duration and intensity. In our study, we reduced cerebral blood flow in the experience animal on average by 30%, by right internal carotid artery ligation. Fifteen days after the onset of hypoxia, by histology and immunohistochemical studies, we identified neuronal, glial and vascular damage. Lesions of nerve and glial cells ranged from changes of cytoplasmic tinting with the development of "red neurons", to neuronal and glial cytolysis with areas of focal necrosis. Vascular lesions were represented by the collapse, fragmentation and discontinuity of capillaries, always associated with a marked perivascular edema.

Keywords: cerebral ischemia, perivascular edema, focal necrosis, cytolysis, blood–brain barrier.

Introduction

Cerebral ischemia occurs when cerebral blood flow is reduced for a few seconds or minutes. If this reduction lasts more than a few minutes, cerebral infarction occurs, the brain being the most vulnerable organ to ischemia [1]. The key factor leading to neuronal lesions is the impairment of cerebral circulation.

Cerebral ischemia can occur at any age, from the newborn to the elders. During labor, it can cause ischemic encephalopathy, which is the most important cause of brain damage in the newborn, and of long-term neurological sequels [2]. In adults, the most common causes of ischemia are atherosclerosis and cardiogenic embolism. Atherosclerosis usually affects the internal carotid artery origin and the origin of major arterial branches of the brain. All these lead to reduced cerebral blood flow and less oxygen, and fewer nutrients.

In our study, we aimed to reduce the cerebral blood flow by 25–30% in the experiment animal and see if brain injuries occur, as well as their intensity.

Materials and Methods

We used a batch of 10 mature common Wistar rats, weighing 280–320 g. To compare the results of the histological and immunohistochemical studies, we also had a control group consisting of two adult Wistar rats, which were kept in the same environmental conditions as the studied rats. To achieve cerebral ischemia, animals were anesthetized using Sevoflurane in a 4% concentration for induction, and 1.5–2% for

maintenance, diluted in a mixture of 70% nitrous oxide and 30% oxygen. The gases were administered using a gas-tight Plexiglass cage to induce anesthesia and through a special mask to maintain anesthesia during surgery.

The animal's body temperature was continuously monitored using a rectal probe, and maintained at a value as close as possible to 37°C (Figure 1) by using a thermostatic blanket connected to the rectal thermometer through a special system (Homeothermic Blanket System – Harvard Apparatus).

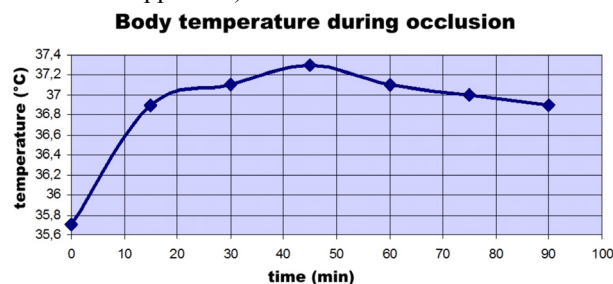


Figure 1 – Temperature curve for the 90 minutes of occlusion, for one rat, but characteristic for the whole batch.

After anesthetic induction, animals were immobilized on a thermostatic blanket, face up, and the cephalic extremity was fixed in a special anesthetic mask. The central artery of the tail was catheterized to allow continuous measurement of blood pressure and blood sampling for pH and blood gases (oxygen, carbon dioxide) measurements using a dedicated device

(Blutgassystem IL 1620, Instrumentation Laboratory, Germany), as well as and serum glucose levels (Omnican7 Balance, B. Braun, Germany).

The hair was removed and the scalp of the right half was disinfected with an alcoholic solution of iodine. A reversed “L” incision was performed starting halfway between the right orbit and the right external ear canal opening, towards the superior and rostral aspect, until the supraorbital region. Temporal muscle fascia was incised and the temporal muscle bundles were separated longitudinally, thereby exposing the temporal bone. A small (1 mm diameter) circular craniotomy was performed in the temporal bone reaching the internal lamina of the skull bone, using a micro drilling machine, 2–3 mm anterior and superior to the zygomatic process of the temporal bone, corresponding to the projection of the middle cerebral artery. In this small hole, the probe (optical receptor) of a special Doppler device for small animals (Periflux System 5000, Perimed, Sweden) (Figure 2) was fixed (using dental cement), which monitored the blood flow in the middle cerebral artery during surgery.



Figure 2 – The “Periflux System 5000” device (Perimed, Sweden).

This was followed by surgery on the anterolateral cervical region. Thus, after local hair removal and disinfection, a “T”-shaped incision on the ventral cervical region was performed and, using a $\times 5$ stereomicroscope and specific surgical instruments, the right common carotid artery was exposed as well as its bifurcation in the two carotid arteries (internal and external).

The vagus nerve filaments were removed using a forceps, and the internal and external carotid arteries were exposed. The right internal carotid artery blood flow was permanently blocked using a textile yarn.

The animals from the control group did not undergo internal carotid artery occlusion.

The animals were then kept in optimal temperature and light conditions and were given free access to water and food. Fifteen days after the permanent occlusion of the right internal carotid artery, the animals were euthanized. The brain from each animal was excised and immediately placed in 10% neutral formalin solution for three days for fixation of the biological material, after which the brain tissue was embedded in paraffin wax.

Using a microtome (Microm HM350) equipped with a special section transfer system (Section Transfer

System, STS, Microm), 4 μm thick serial sections were cut and collected on special slides covered with a layer of positively charged amino acid residues (poly-L-lysine) (Sigma), in order to increase adhesion of the sections. The sections were transferred to an incubator at 37°C to dry, and kept overnight, during which the biological material adhered perfectly to the surface of the histological slide.

For the histologic studies, we used the Hematoxylin–Eosin staining technique and for the immunohistochemical study, we used the following antibodies: anti-CD34, anti-GFAP, and anti-NeuN (Table 1).

Table 1 – Antibodies used for the immunohistochemical study

Antibody	Manufacturer	Clone	Host/Target/Clonality	Dilution
Anti-CD34	Epitomics	EP373Y	Rb/Hu/Monoclonal	1:100
Anti-GFAP	Dako	–	Rb/Hu/Polyclonal	1:30 000
Anti-NeuN	Millipore	A60	Ms/Hu/Monoclonal	1:400

For single immunohistochemistry, after antigen retrieval, sections were allowed to cool down to room temperature and were incubated for 30 minutes in a 1% hydrogen peroxide solution. The sections were next washed in PBS, followed by a blocking step of 30 minutes in 1% skim milk. Next, the slides were incubated with the primary antibodies overnight at 4°C, and the next day, the signal was amplified for 30 minutes using a peroxidase polymer-based secondary detection system (EnVision, Dako). Finally, the signal was detected with 3,3'-diaminobenzidine (DAB) (Dako) and the slides were coverslipped in DPX (Fluka) after Hematoxylin staining.

The sections were imaged with a Nikon Eclipse 55i microscope (Nikon, Apidrag, Romania) equipped with a 5-megapixel cooled CCD camera. Images were captured and archived using a Nikon frame grabber and the Image ProPlus 7 AMS software (Media Cybernetics Inc, Buckinghamshire, UK).

The Ethics Committee of the University of Medicine and Pharmacy of Craiova, in accordance with the European Council Directive of 11.24.1986 (86/609/EEC), the European Convention on the Protection of Vertebrate Animals (2005), and the Government Ordinance No. 37/2.02.2002, approved the experimental protocol.

Results

Following the permanent right internal carotid artery obstruction, the blood flow through the right middle cerebral artery (RMCA) immediately decreased on average by 30% (Figure 3), compared to the value measured before performing the occlusion, and remained at this value throughout the entire experiment. The blood flow reduction of only 30% in the RMCA is due to anastomoses from the Willis polygon that allowed a redistribution of the blood through the vertebral artery and left internal carotid artery.

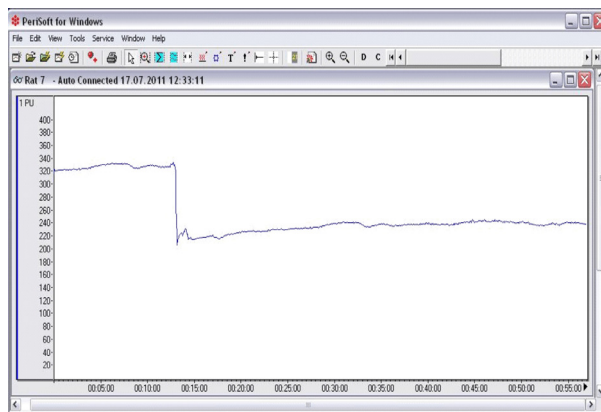


Figure 3 – Blood flow measurement through the right middle cerebral artery, before and after the permanent occlusion of the right internal carotid artery (data recorded for one rat, but characteristic for the whole batch).

Histological changes occurring after 15 days of moderate cerebral hypoxia were recorded (observed) in both neurons and glial cells as well as cerebral microcirculation. The neurons that were most affected were those on the same side as the lesion, i.e. the right cerebral hemisphere, evidence that the blood flow

remained unbalanced because of complete obstruction of an artery. Neuronal damage was uneven also within the same cerebral hemisphere. The most affected neurons were those from the molecular layer and those from the internal and external pyramidal layers. Most often neurons showed an intense acidophilic cytoplasm (“red neurons”), characteristic for cerebral ischemia (Figure 4). Hypoxic neurons showed irregular cell boundaries, due to damage of cytoskeletal proteins. The nucleus was either hyperchromatic, condensed, with irregular edges, or hypochromatic, with finely granular chromatin. A more or less extensive clear space of perineuronal edema was identified around neurons (Figure 5).

We frequently observed neuronal debris resulting from a process of increased hypoxic cytolysis, and even large areas of focal necrosis with multiple cellular and fibrillar debris (Figures 6 and 7), but without immune cells. Just like neural lesions, focal areas of necrosis had a patchy distribution in the cortex, being more numerous in the right hemisphere. We believe that this distribution is closely related to vascular network of the cortex, and the amount of oxygen it provides to nerve and glial cells.

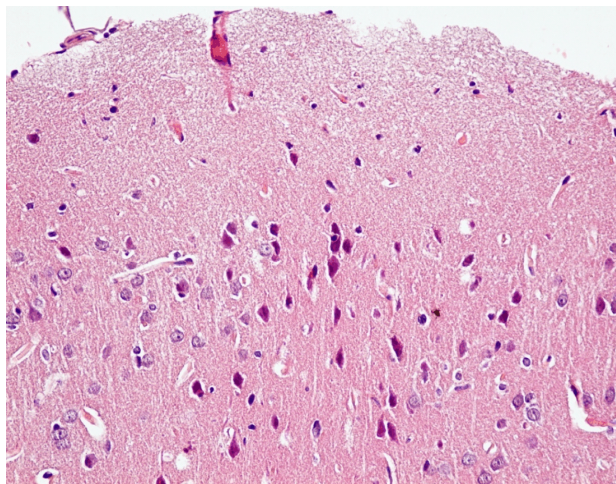


Figure 4 – Cerebral cortex with hypoxic neurons (“red neurons”) (HE stain, ×200).

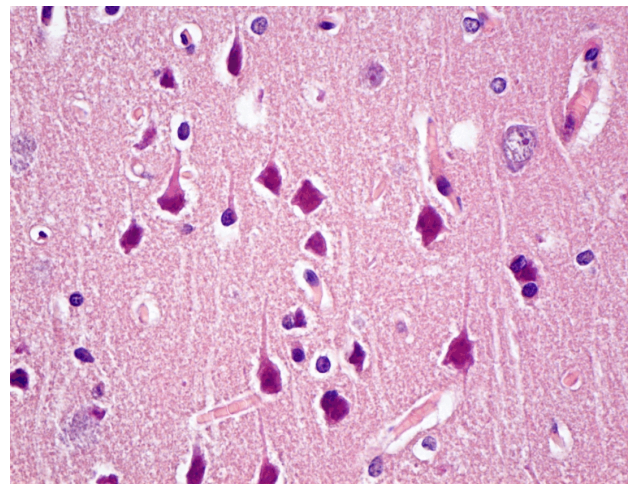


Figure 5 – Slightly irregular hypoxic neurons and perineuronal edema (HE stain, ×400).

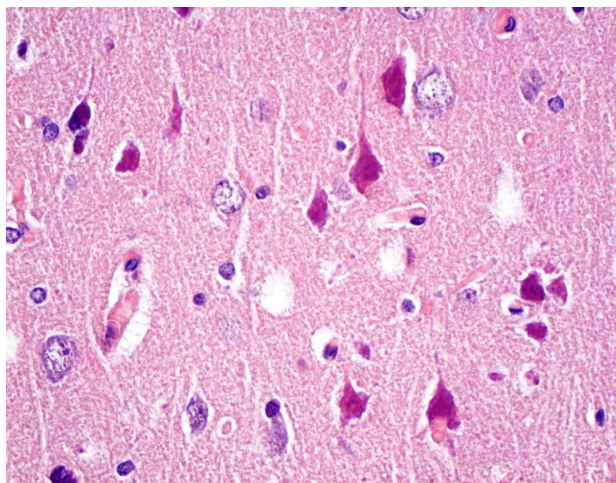


Figure 6 – Hypoxic neurons and neuronal debris resulted from hypoxic cytolysis (HE stain, ×400).

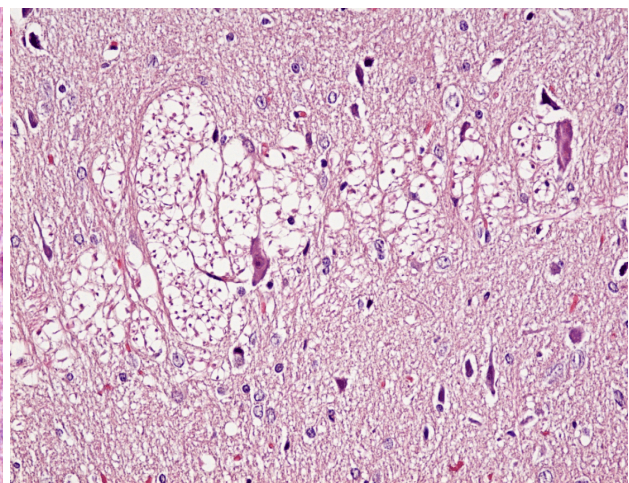


Figure 7 – Area of ischemic necrosis (HE stain, ×100).

At the periphery of focal necrosis areas neurons with increased size, distorted, with acidophilic cytoplasm, and with the nuclear material unevenly dispersed within the cytoplasm were noticed (Figure 8).

In order to assess neuronal viability we used the anti-NeuN monoclonal antibody (neuronal nuclear protein). The comparative study was performed using images obtained from similar cerebral regions, from the control group. In areas with ischemic necrosis, the number of NeuN-positive neurons is much smaller than in the control animals. It was also noticed that the positivity is both nuclear and cytoplasmic, implying that nuclear membrane integrity was compromised

and the neuron specific protein passed in large quantities in the cytoplasm. In ballonized neurons, at the periphery of the ischemic necrotic area, the NeuN labeling was weak, sometimes absent, with the same intensity in the nucleus and cytoplasm (Figures 9 and 10).

The astrocyte reaction as highlighted by anti-GFAP immunolabeling was more intense in the group with cerebral ischemia than in controls. Numerous astrocytes with increased size, large hypochromatic nucleus, and multiple long branched extensions were identified. We can say that cerebral ischemia induces the activation of astrocytes (Figure 11).

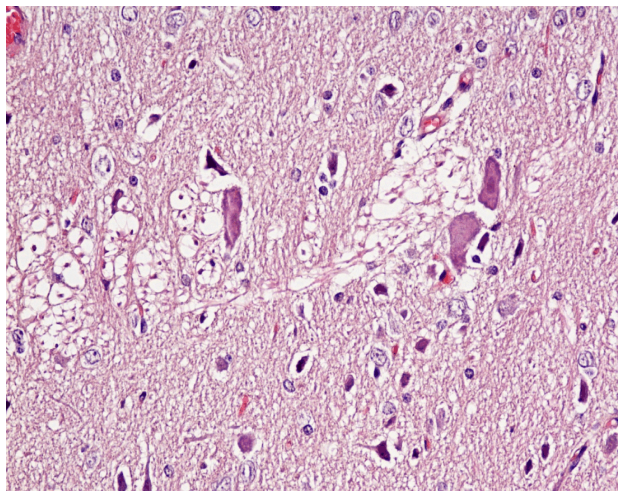


Figure 8 – Irregular, ballonized ischemic neurons at the periphery of focal necrotic areas (HE stain, $\times 200$).

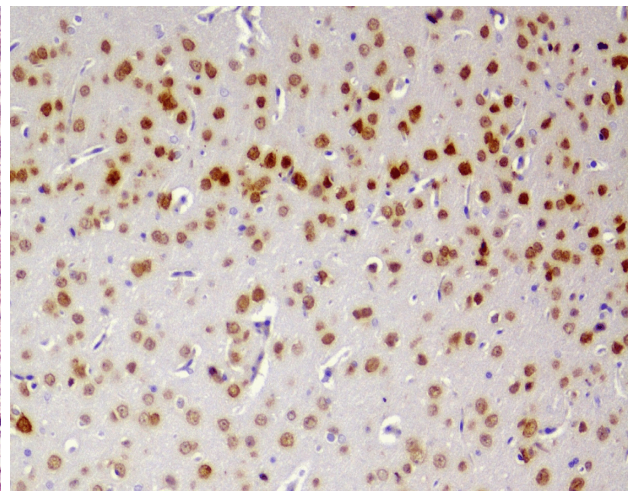


Figure 9 – Normal cerebral cortex (anti-NeuN immunolabeling, $\times 100$).

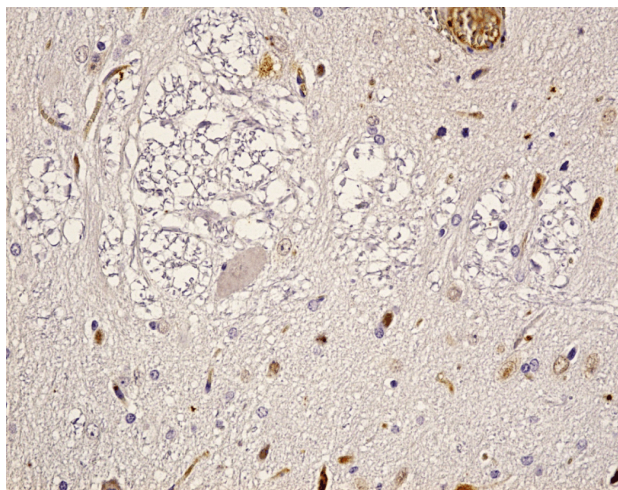


Figure 10 – Area of focal necrosis with ballonized neurons at the periphery, with inconstant NeuN immunolabeling (anti-NeuN immunolabeling, $\times 200$).

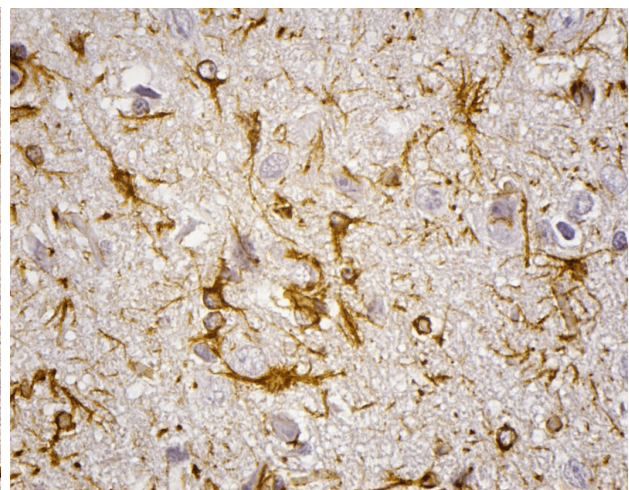


Figure 11 – Activated astrocytes (anti-GFAP immunolabeling, $\times 400$).

The highest number of activated astrocytes was observed around blood vessels. In areas of focal necrosis, astrocytes were absent, which suggests that they die together with the neurons when ischemia reaches a certain threshold (Figure 12).

Cerebral microvascularization showed significant changes indicative of blood–brain barrier damage

induced by cerebral ischemia. Most capillaries showed a discontinuous wall and marked perivascular edema (Figure 13). CD34 immunolabeling demonstrated that the vascular wall may collapse, may show discontinuities and fragmentations, which lead to the development of perivascular edema (Figures 14 and 15).

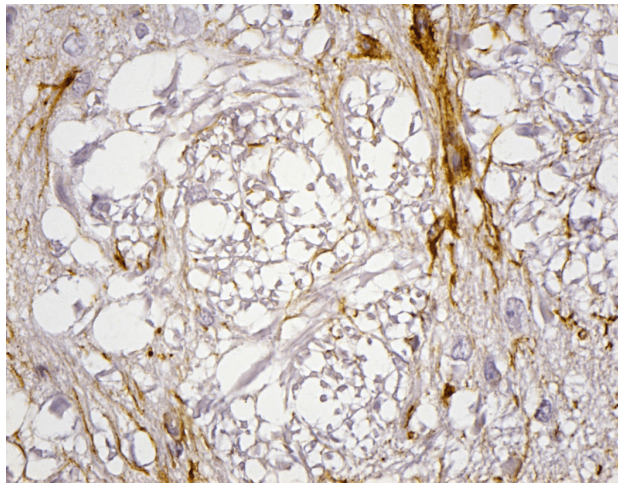


Figure 12 – Area of focal necrosis without GFAP immunolabeling (anti-GFAP immunolabeling, ×400).

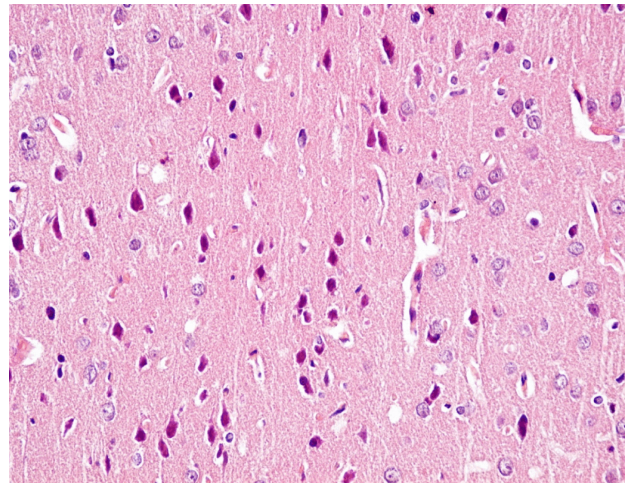


Figure 13 – Blood capillaries with perivascular edema (HE stain, ×200).

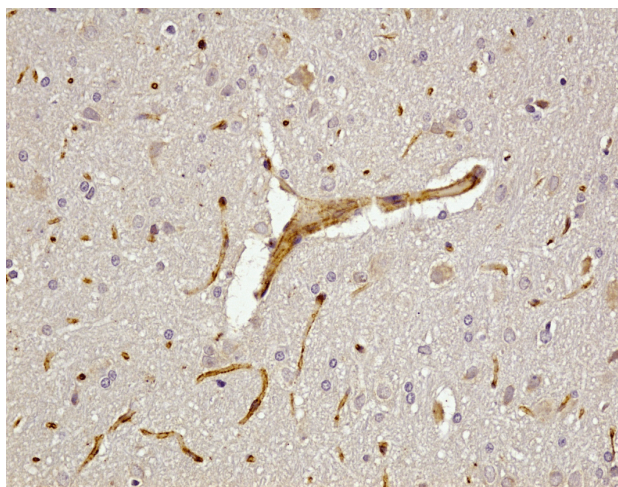


Figure 14 – Perivascular edema (anti-CD34 immunolabeling, ×200).

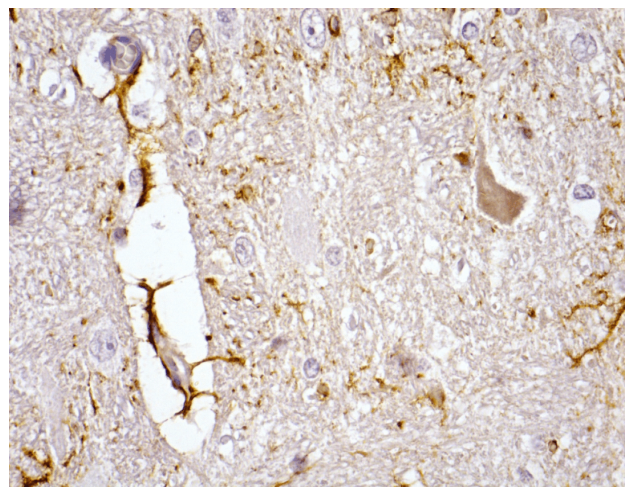


Figure 15 – Blood capillary from the ischemic area with fragmented wall (anti-CD34 immunolabeling, ×400).

Discussion

The general decrease in cerebral blood flow quickly determines a series of biochemical changes, which, in the absence of quick action to restore normal parameters of cerebral circulation, produces increasingly severe histological lesions. In physiological condition, cerebral blood flow is maintained constant by a mechanism of autoregulation, which maintains a constant flow velocity and cerebral blood volume, regardless of variations in systemic blood pressure. This mechanism is expressed by changes in cerebral arteriolar tone reflexes induced by the secretion of humoral factors. If systemic blood pressure increases, vasoconstrictor substances (such as endothelin and thromboxane) are released, leading to increased arteriolar resistance and a normal blood flow, while if systemic blood pressure decreases, vasodilators are released.

In our study, cerebral ischemia achieved by right carotid artery occlusion (ligation) resulted in a 30% reduction in cerebral blood flow. Under these conditions neurons, glial cells and cells in vessel walls received less oxygen and less glucose, which resulted in damage to neuronal and glial cells as well as blood vessels.

According to some authors [3], brain lesions are mainly necrotic in cases with severe hypoxia, and apoptotic in moderate hypoxia. It is widely accepted that the severity and extension of brain damage are strictly related to the intensity, timing and duration of hypoxia. The more intense hypoxia/ischemia is and the longer it lasts, the greater the number of dying neuronal and glial cells [4].

Molecular events triggered by cerebral ischemia in nerve cells and astrocytes are incompletely known. According to some authors [5], the crucial element induced by hypoxia, which triggers a cascade of chain reactions, is the depletion of ATP because of anaerobic glycolysis. Reduced availability of ATP causes dysfunction of ATP-ase systems, especially neuronal and glial Na^+/K^+ ATP-ase. This dysfunction causes depolarization of neurons that causes intracellular accumulation of sodium and water, with cytotoxic edema and/or cell lysis. At the same time, neuronal depolarization induces the accumulation of glutamate, a neuronal stimulating amino acid that tends to accumulate in intercellular spaces as well as inter-synaptic clefts because of glial ATP-ase dysfunction [4]. Glutamate causes a massive intracellular calcium intake

and activates certain enzymes, including endocellular protease and phospholipase. The protease degrades neurofilaments with cytoskeleton breaking and cell body disintegration, while phospholipase hydrolyzes phospholipids inducing cell membrane damage. In addition, excessive amounts of glutamate can result in excitotoxicity, directly causing the death of neurons and glial cells [5].

Excessive production of free radicals added to the decrease of protein synthesis and possible activation of phospholipase-A2 by Ca^{2+} , can also cause membrane dysfunction and disruption of microtubules by the dissociation of microtubule stabilizing protein [6].

Activation of enzymes like xanthine oxidase and cyclooxygenase generates reactive oxygen species (ROS) responsible for cellular oxidative damage [7]. Formation of free radicals such as hydroxyl radical is extremely harmful to neurons and glial cells as they may induce significant changes in cell ultrastructure by membrane lipid peroxidation and dysfunction of ion channels [8].

Other biochemical lesions caused by free radicals are related to the development of apoptosis. This may be due to the activation of the caspase enzyme system resulting from mitochondrial damage, and the activation of the proapoptotic gene, i.e. Bax-gene [9].

Reduced cerebral blood flow by 30% for 15 days resulted in significant neuronal changes, ranging from increased cytoplasmic eosinophilia, to enlarged, pale, edematous cells, with hypochromatic nuclei and powdery chromatin, to extended cellular necrosis. These histological aspects are the result of intense biochemical changes that affected the entire nerve cell. Some authors [10] showed that hypoxia itself and elevated intracellular Ca^{2+} ions and free radicals affect the normal functioning of the mitochondrial membrane. In addition to blocking the production of ATP, which results in loss of mitochondrial potential, the transient increase of mitochondrial pore permeability leads to mitochondrial swelling, an “explosion” of oxygen radicals and the release of pro-apoptotic molecules. The cytoplasm becomes almost transparent indicating cell swelling. Persisting ischemia causes both the nuclear and plasma membrane to become highly irregular and the development of multiple large cytoplasmic vacuoles appear, some of them being extremely enlarged mitochondria with broken mitochondrial cristae [11].

We believe that neuron and glial necrosis resulted from the combined effect of energy substrate loss, and intracellular organelles alteration resulting in the membrane disintegration, fragmentation and disruption of DNA and cell autolysis.

By damaging the junctions between endothelial cells, free oxygen radicals disrupt the integrity of the blood–brain barrier (BBB) causing interstitial edema, which aggravates vasogenic brain lesions. Disruption of blood–brain barrier and perivascular edema formation dramatically deteriorate clinical symptoms in patients with ischemic stroke [12].

Blood–brain barrier is a complex structure consisting of endothelial cells, pericytes, glial cells and neurons [13, 14]. Among these structures, vascular endothelium

seems to be the most important because it controls the fluid transition between blood vessels and cerebral parenchyma [15]. However, the efficient operation of the barrier requires stable intercellular and cell–extracellular matrix interrelations, since cellular permeability is regulated by complex adhesion interactions of transmembrane molecules, intercellular tight junctions and cytoskeletal proteins [16, 17]. Tight junctions are crucial for vascular barrier sealing [18]. Impairment of any of these interactions may increase endothelial permeability and lead to excessive edema formation [17].

While initial studies focused on transmembrane adhesion molecules, the function of tight junctions and the control of vascular permeability, recently it has been shown that endothelial cell cytoskeletal proteins are involved in modulating blood–brain barrier permeability [19, 20].

Our study demonstrated that vascular changes are complex, that besides the ubiquitous perivascular edema, blood vessels showed fragmentation and collapsing, with changes in size. We believe that chronic cerebral ischemia affected all structural elements of the blood–brain barrier, not only the vascular endothelium.

Lately, more and more studies show the importance of pericytes in blood–brain barrier function. In the brain, pericytes modulate the diameter of capillaries by constricting the vascular wall [21], a process, which, during ischemia, may further obstruct capillary blood flow [22]. It is also known that pericyte degeneration can influence neuronal phenotype, evidence of the close relationship between these cells within the functional units of the blood–brain barrier.

✉ Conclusions

Internal carotid artery occlusion reduced cerebral blood flow by around 30% of the baseline level. Under these conditions of chronic cerebral ischemia, nerve and glial cell changes ranged from changing cytoplasmic tinting with the appearance of “red neurons”, to cytolysis and focal necrosis, which involved both nerve and glial cells. Chronic hypoxia-induced vascular lesions were represented by the collapse, fragmentation and discontinuity of blood capillaries, always associated with a marked perivascular edema.

Acknowledgments

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