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Study of vascular microdensity in areas of cerebral ischemia on experimental model

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Abstract

Stroke is the third leading cause of death and the first cause of disability with an increasing incidence, especially of the ischemic type. There is no effective curative treatment for stroke and therefore the therapy for this disease currently relies on identifying patients at risk and instituting preventive measures. Since ischemic stroke in middle cerebral artery territory (and with preferential localization in the striated nuclei) is the main type of stroke in humans, animal models obtained by surgical ligation of the middle cerebral artery (MCAO) are valuable tools for the fundamental study of this disease. In this study, we investigated the morphological and immunohistochemical remodeling of the tissue after stroke and in particular the vascular component in a MCAO murine model. The analysis included the sequential sacrificing of animals at 1, 14, 28 and 60 days post-stroke and brain processing for paraffin embedding and sectioning. Our results show a gradual revascularization of the lesion as we move away from the time of the surgical intervention. This effect is accompanied by the development of an increasingly dense glial scar at the periphery of the lesion. The perilesional area itself, the penumbra, is characterized by minimal histological changes (such as eosinophilic neurons), but also by an increased expression of activated caspase 3 as a sign of apoptotic neurons and glial cells. Our study confirms the potential of the organism in its attempt to revascularize the injured area, and raises questions on the role of the glial scar in limiting the process of neovascularization.

Keywords: stroke, penumbra, cerebral infarction, angiogenesis.

Introduction

Stroke is a severe, acute neurological disorder, resulting either from a decrease or even complete blocking of the blood flow towards an area of the brain, or from cerebral hemorrhage, leading to irreversible damage to the brain tissue with major motor, sensory and cognitive impairment.

Worldwide, stroke is one of the leading causes of morbidity and mortality [1] as it "kills" five million people annually and causes five million other severe disabilities. According to *World Health Organization*, in 2001, there were 5.5 million deaths from strokes and annually, about 15 million people survive a stroke. Lethality of stroke is 11% for women and 8.4% for men.

Stroke is the main etiological factor for the installation of long-term disabilities constituting the third leading cause of death after heart disease and cancer [2]. In addition, stroke is the second cause of dementia and the most common cause of epilepsy in the elderly and a frequent cause of depression [3, 4].

The cerebral vascular element plays a leading role in terms of the extent and severity of brain damage, but also in neurological recovery after stroke.

The efforts of the scientific community to understand cellular, molecular and biochemical changes that occur during the development of ischemia-induced brain damage has increased during recent years in hopes of finding and implementing new therapies with beneficial effects in stroke patients. Most studies have been conducted and are conducted on laboratory animals to understand the complexity of vascular and neural lesions.

Our study aimed to evaluate the cerebral vascular microdensity in the experimentally induced necrotic core in the laboratory animal, at different time points.

Material and Methods

In a group of 20 adult common Wistar rats with an average weight of 250–275 g, after sedation with 0.1 mg/kg of Dormicum, limited craniotomy (2–3 mm) was performed at the junction between the zygomatic arch and the squamous portion of the temporal bone. Then the dura was opened with a sharp angled hook, followed by the identification and isolation of the middle cerebral artery (MCA) and its ligation as close as possible to its origin. The skin was then rebuilt by suture with surgical thread. Thus, severe cerebral ischemia developed within an area supplied by a middle cerebral artery.

Animals subjected to experiment were divided into

four groups of five animals each and kept in the same microclimate conditions, with free access to food and water.

In a fifth group (four animals), only craniotomy was performed followed by the suture of the skin, without damaging ACM.

The first group of animals was sacrificed one day after cerebral ischemia, the second one after 14 days, the third group at 28 days, and the last one at 60 days after.

From each animal, the whole brain was collected, with extreme caution to avoid inducing new lesions resulting in artifacts. The brain of each rat was placed in 10% neutral buffered formalin for one week, and then was processed routinely for paraffin inclusion. Serial sections were obtained using a Microm rotary microtome equipped with a Peltier cooling element and a waterflow section transfer system; selected sections were then stained with Hematoxylin and Eosin, the rest being available for the immunohistochemical study.

For the immunohistochemistry study sections were attached to poly-Lysine slides to increase the adhesivity. After rehydration, sections were boiled in a microwave oven in citrate buffer solution of pH 6 for 20 minutes, for antigen retrieval. Endogenous peroxidase was then inactivated with 1% hydrogen peroxide and nonspecific antigenic sites were blocked by incubation with 1% skimmed milk for 30 minutes. The sections were then incubated overnight with the primary antibodies in optimized dilutions (rabbit, polyclonal) (Table 1).

Table 1 – ***Rabbit antibodies used in this study***

Antibody	Clone	Target	Dilution	Manufacturer
Anti von Willebrand factor	Polyclonal	Mature vascular endothelium	1:200	Millipore
Anti-GFAP	Polyclonal	Astrocytes	1:20 000	Dako
Anti-cleaved Caspase 3	Polyclonal	Apoptotic cells	1:100	Cell Signaling

The next day, after washing, sections were incubated with goat anti-rabbit biotinilated specific antibody, 1:500 (Dako) for 30 minutes. Then Avidin-Biotin-peroxidase complex was added followed by signal detection with 3,3'-diaminobenzidine (DAB, Roche). The sections were then stained with Hematoxylin and covered with DPX (Fluka).

In order to make an accurate assessment of vascular microdensity in areas with cerebral ischemia as opposed to areas free of cerebral ischemia, we performed serial sections in the frontal plane having both the area with cerebral ischemia and the normal cerebral hemisphere.

Complete sectioning of the brain was performed, and finally this has enabled us to compare the equivalent levels relative to the bregma.

The evaluation of vascular microdensity was performed manually on images taken with a 20× objective in areas of infarction and the contralateral hemispheres, respectively. The count included both mature vessels (marked with anti-VW) and neoformation vessels (recognized as branching lumens sometimes with RBC, lined by a turgid discontinuous endothelium). For the areas of lesions, infarct areas were delineated on the monitor screen, and then their number was normalized according to the lesional areas themselves, extrapolating the values corresponding to complete areas measured with the 20× objective. These values were then exported to Excel tables and compared between groups. For comparing two groups, the Student *t*-test was used; and for multiple comparisons we used the ANOVA test. Central tendencies were expressed as mean ± standard deviation (SD).

Results

In all tested animals, the complete obstruction of the middle cerebral artery led to bigger or smaller infarct areas in the temporal lobe, depending on the vascular anastomoses present in the brain of each animal. The infarct area showed liquefactive necrosis of all tissue structures of the brain, namely neurons, glial cells, nerve fibers and blood vessels (Figure 1A). It included areas from both the gray and white matter. From the morphological point of view, several areas were observed in the structure of the ischemic zone, depending on the intensity of the lesion. First, the infarct core was characterized by total necrosis of cellular and vascular elements. At the periphery of the infarct core there was an area showing hypoxic changes and perineuronal glial satelitosis, which is the so-called penumbra with potentially recoverable neurons following stroke therapy. This was the transition area between the actual infarct core and the normal cerebral tissue. The extent of the penumbra was also variable from one animal to another, depending on the time elapsed from the occurrence of stroke until the death of the animal, because the morphological reshaping started within the first 24 hours.

The initial stages of blood flow interruption, as well as those following the development of liquefactive necrosis showed ischemia of neurons located in the middle cortical layers of lesional and perilesional areas (hypoxic and eosinophilic neurons with pyknotic nuclei) (Figure 1, B and C), which suggests that neuronal impairment is not limited only to the area of cerebral infarction and penumbra, but is much more extensive within the cerebral parenchyma. In addition, this microscopic appearance represents indirect evidence that collateral circulation is not sufficient to fully compensate the lack of oxygen caused by the middle cerebral artery occlusion.

The process of cortical necrosis of the area served by the MCA started the first day after ischemia was triggered. The presence of a few foamy macrophages filled with cellular debris and hemosiderin pigment was also reported during this period.

The onset of the angiogenesis process has been reported as early as the first 24 hours after stroke, and in our case, we also identified a few vessels with small diameter and turgid endothelium.

These vessels resulted from the angiogenesis process beginning in the necrotic foci and originating from endothelial cells of cerebral parenchymal vessels, as well as from leptomeningeal vessels from the periphery of the infarct zone. The potential of endothelial cells to transform into angioblasts under the influence of potentially angiogenic factors is well established.

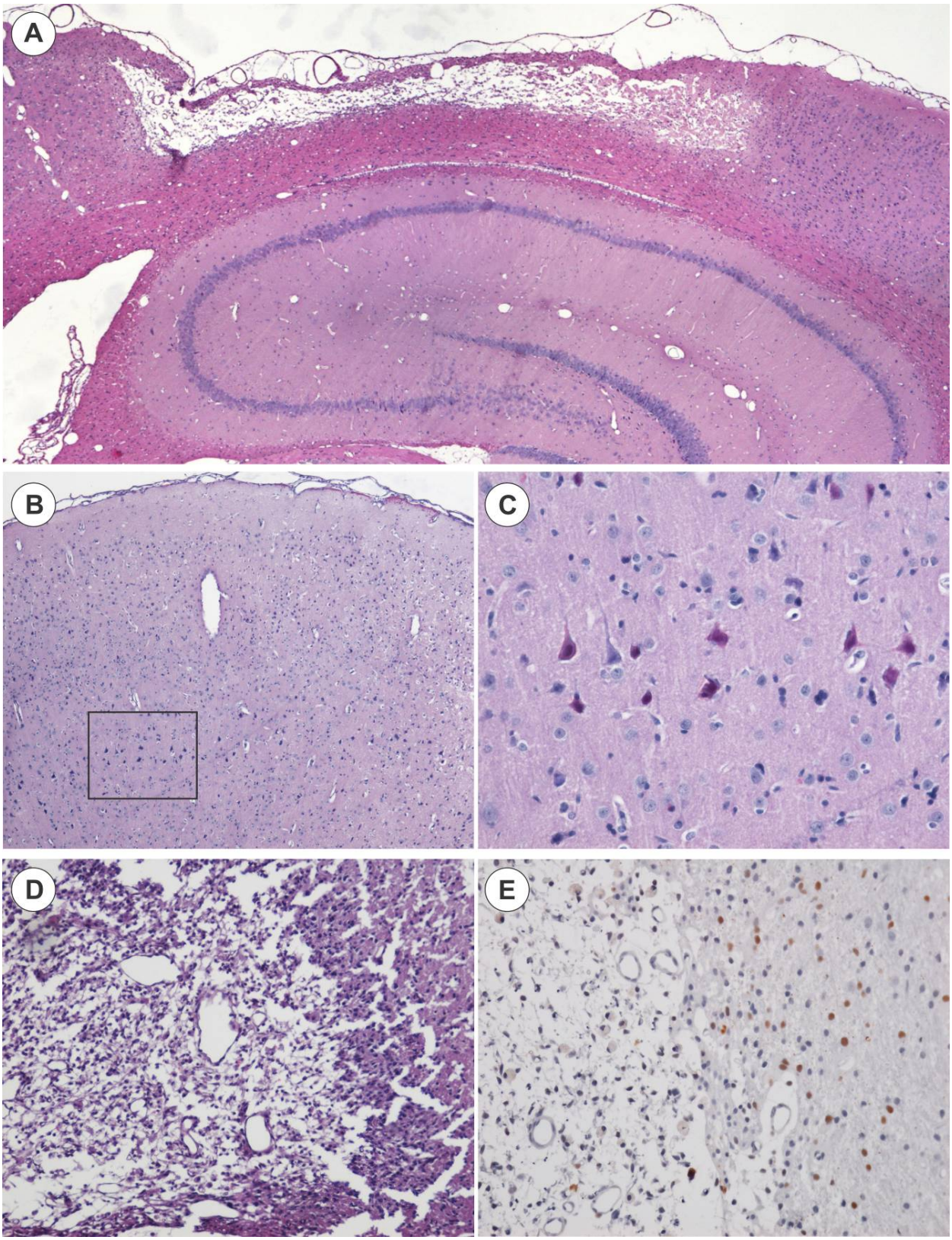

Figure 1 – ***(A) The first areas affected by liquefactive necrosis are the middle cortical layers (in a rat sacrificed at 14 days after MCA occlusion) (ob. 2×); (B) and (C) Hypoxic neurons in the internal pyramidal layer in a rats sacrificed at one day after MCA occlusion (ob. 4× and 40×); (D) The liquefaction area bounded by a reactive inflammatory front (microglia and activated glial cells) (ob. 10×); (E) Caspase 3-positive nuclei in the penumbra (perilesional) (ob. 20×).***

Proportionately with time, the animals sacrificed at 14, 28 and 60 days showed an increasing density of blood vessels in the area of infarction, and the size, course and histological appearance changed. Thus, neoformation vessels varied in size showing irregular branching with less turgid endothelial cells. This microscopic appearance may be due to the space created by the necrotic lesion allowing them to dilate, but also

possibly to lack of pericytes which would have a contractile effect on the vascular wall. Direct measurement of their diameters showed a progressive increase from those developing in the scar tissue after 14 days (14.21±4.25 mm), to those developing at 28 days (21.5±4.45 mm) and 60 days (30.15±6.28 mm) respectively. The differences between the three groups were significant: ANOVA ($F(2.12) = 3.21$, $p<0.05$) (Figure 2, A, C and E).

Imunostaining for cleaved caspase 3 identified an increased number of apoptotic cells in the perilesional area (penumbra).

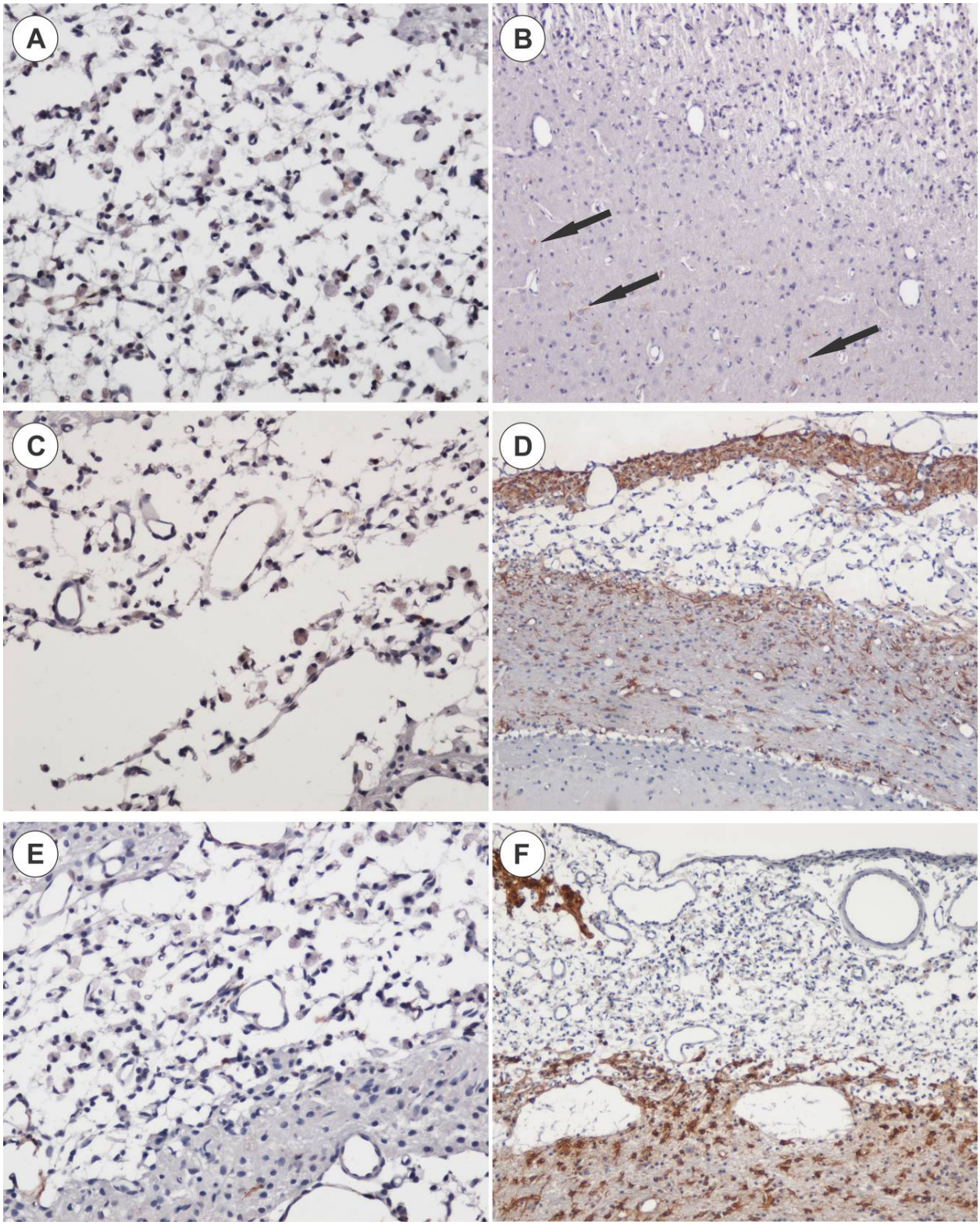

Figure 2 – ***(A), (C) and (E) Progressive revascularization of the lesion at 14, 28 and 60 days respectively (ob. 20×); (B), (D) and (F) Progressive gliosis of the lesion at 14, 28 and 60 days respectively (GFAP-positive cells) (ob. 10×).***

The angiogenesis process was accompanied by glial cell reaction that appeared in large numbers around the lesion tending to form the so-called glial scar, otherwise specific for the CNS reaction to injury. The response of

the activated perilesional glial cells resulted in GFAP overexpression in the penumbra, particularly in older lesions (Figure 2, B, D and F).

In one case, we observed a hemorrhagic transformation of ischemic stroke, characterized by parenchymal flooding with erythrocytes and macrophages with hemosiderin pigment. As a peculiar feature, glial reaction was much more reduced compared with areas of ischemic stroke.

The reaction of the macrophage system increased with the age of the lesion, the highest number of macrophages being observed in animals sacrificed after 14 and 28 days. To highlight the process of neuronal apoptosis induced by vascular ischemia, we used the immunolabeling for activated caspase 3. As it can be seen in our images (Figure 1E), we identified an increased number of apoptotic cells in the perilesional area (penumbra) compared with the contralateral unaffected cerebral parenchyma.

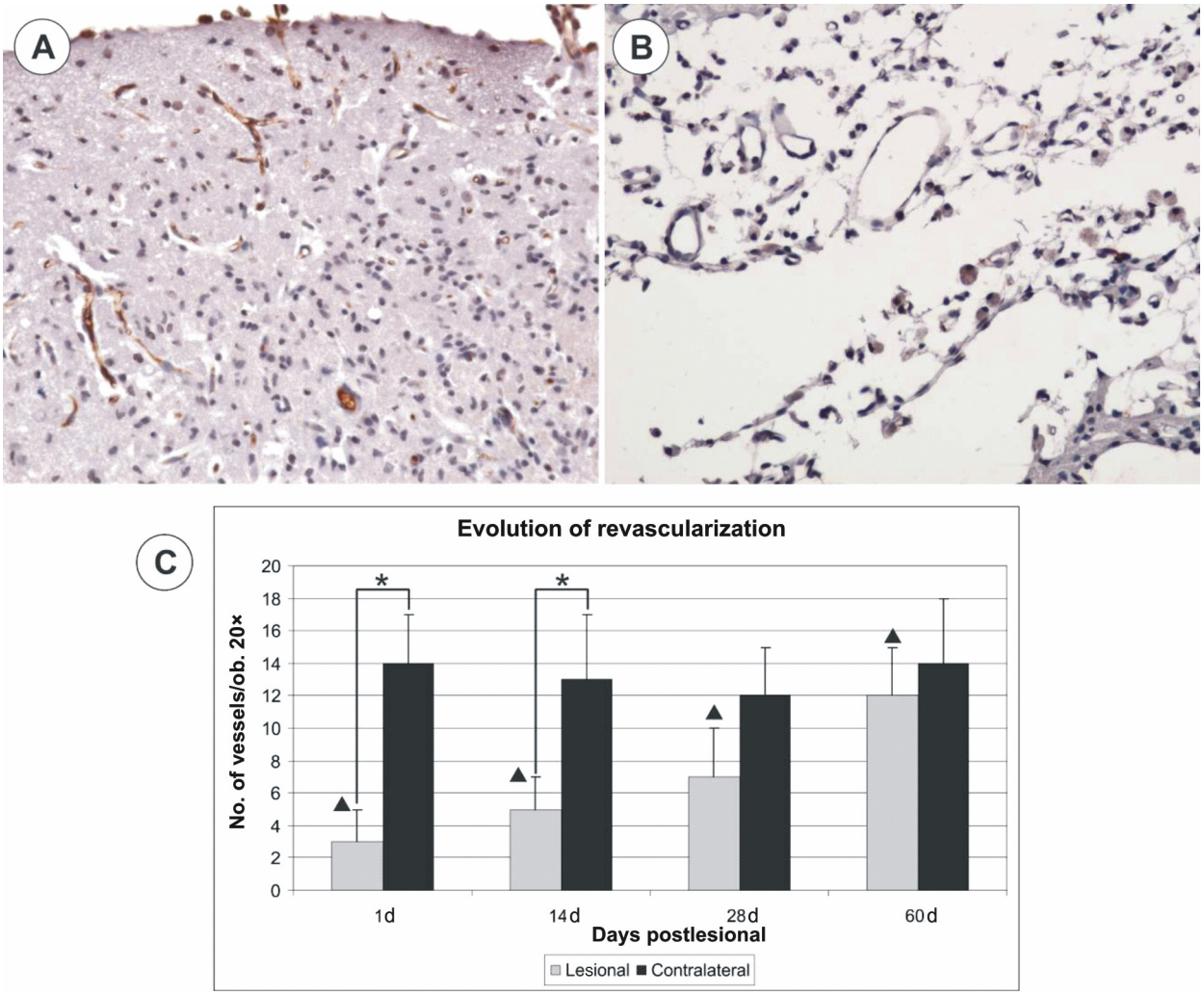

Figure 3 – ***(A) Mature cortical vessels, positive for von Willebrand factor (ob. 20×); (B) Immature vessels in the area of infarction, negative for von Willebrand factor (ob. 20×); (C) With evolution, there is a gradual increase in the number of vessels in the lesion. The total number of vessels (mostly mature ones) in the contralateral hemispheres showed no significant changes. Error bars represent SD, * represents statistically significant differences using the Student t-test ($p<0.05$), and the triangles are the significance for global comparison using the ANOVA test ($F(3.18) = 4.32$, $p<0.05$).***

Discussion

Compared to any tissue or organ in the body, the central nervous system (CNS) shows an increased vulnerability to discontinuous and inappropriate oxygen and glucose supply. The low cerebral tolerance to ischemia correlates with some of its structural, metabolic and functional features. Thus, brain artery anastomoses are not significant and therefore, their partial or complete occlusion cause cerebral hypoperfusion or ischemia, followed by abnormal cellular function and ultimately neuronal death.

This phenomenon is more pronounced in the terminal segment of the arterial component, which in clinical imaging is called watershed. From the metabolic point of view we must remember that neurons have a high metabolic activity, the rate of oxygen consumption by the entire brain in normal conditions being around 49 mL O_2/min. and intrinsic energy reserves are extremely low, hence the high dependency of neurons on the oxygen and glucose continuously supplied by the cerebral circulation.

Cerebral ischemia initiates a complex cascade of serious cellular, molecular and metabolic events because

of the irreversible alteration of the extremely sensitive cerebral homeostasis.

The onset of ischemic process occurs through inhibition of mitochondrial respiration and pronounced decrease in the amount of intracellular glucose and hence a profound collapse of the ATP level. Tissue ATP reserves are completely depleted locally in cerebral blood flow values of 13–14 mL/100 mg/min., which causes cell death (complete ischemia). The "penumbra" of an injury occurring because of focal ischemia often shows less significant lesions due to residual irrigation from collateral blood vessels [5].

Energy depletion temporary inhibits ATP-dependent ion pump activity and induces the influx of extracellular Na^+ and Cl^- and the efflux of intracellular K^+. In addition, passive diffusion of water through these impaired pumps causes cellular swelling. The increased amount of extracellular K^+ induces depolarization of surrounding cells, which may contribute to lesion expansion [6].

The development of tissue necrosis is extremely rapid after complete suppression of blood flow in a certain area. Within hours of hypoxia, hypoxic neurons can already be identified and at approximately 12 hours after the infarction, liquefactive necrosis occurs.

However, complete infarction accompanied by necrosis of all structures occurs in the area of maximum ischemia and defines the infarct core. At the periphery of the infarct, there is an area where there is less intense ischemia, area called the penumbra, where the tissue retains its texture, but with an impairment of cellular metabolism that allows a full recovery in case of early vascular repermeabilization [7]. In hemodynamic terms, the "penumbra" is defined as the region where local cerebral blood flow is reduced to intermediate values (i.e. from 20% to 40% of normal values), while in the infarction area local blood flow is severely reduced, with maximum values ranging from 0 to 20% of normal values [8].

Cerebral ischemia is associated with increased blood–brain barrier permeability and even its destruction by affecting the tight junctions. This may explain the hemorrhagic transformation of an ischemic stroke, phenol-menon that occurs especially in the first 24 hours after an ischemic episode. Also, the extension of the cellular necrosis foci depends heavily on the size of the damaged vessel. The area of necrosis may affect both the gray and the white matter, and some authors [9] showed that the remarkable sensitivity of the white matter in various animal models of ischemia has proven to have the same relevance in humans.

Just as the other researchers [10, 11] showed, we also noted the heterogeneity of the microscopic aspects of cerebral infarctions on experimental animal models due to the time-related evolution of the reaction involving the intrinsic cellular elements within the nervous tissue and inflammatory cells. As far as the occurrence of angiogenetic processes is concerned, our observations confirm the data obtained by Mena H *et al.* [12] showing that on animal models neo-vascularization can be observed during the first 24 hours after stroke.

Some authors consider that the process of angiogenesis is correlated with that of neurogenesis [13, 14]. They have shown that pharmacological blockade of angiogenesis after stroke significantly reduces the number of immature neurons that are present in the cortex near the infarct, by approximately 90%.

We also believe that stimulation of post-stroke angiogenesis and the reduction of perilesional glial reaction may represent a useful therapeutic target that would foster more rapid revascularization and recovery of damaged nervous parenchyma.

Conclusions

Angiogenesis was an early event in this murine stroke model, as we observed immature newly formed vessels even after 24 hours from the ischemic event. Blood vessel density increased with time in both the core and the penumbra of the lesion, with the parallel formation of a denser GFAP-rich scar tissue. A direct measurement of the newly formed vessels also showed a progressive increase of the average inner diameters of blood vessels from the first days after MCAO, and up to 60 days post-stroke.

Acknowledgements

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