ABSTRACT

Doctoral coordinator:
Prof. univ. dr. Laurențiu Mogoantă

Doctoral student:
Pintea Irina Lavinia

CRAIOVA
2012
CONTENTS

Introduction

PART I - STATE OF KNOWLEDGE
I. Anatomy, histology and histophysiology of the central nervous system
II. Anatomical histological and functional features of the central nervous system
III. Cerebral ischaemia

PART II - PERSONAL CONTRIBUTIONS
IV. The purpose and objectives of the paper
V. Assessment of motor and cognitive performance in rats with cerebral ischemia produced by unilateral and bilateral clamping of the internal carotid artery
VI. Histological study of brain parenchyma in chronic cerebral ischemia
VII. Immunohistochemical study of brain parenchyma in chronic cerebral ischemia
VIII. Conclusions

REFERENCES

Keywords: stroke, chronic cerebral ischemia, neuronal death, apoptosis
Cerebral stroke is a severe neurological disorder of great severity, which may result either by blocking blood flow to an artery supplying a cerebral territory (ischemic vascular events) or by producing brain hemorrhage. Worldwide, stroke kills five million people every year and causes severe disabilities in another five million people (Lopez AD et al, 2006). Fatality through stroke is 11% for women and 8.4% for men. It is estimated that the risk of stroke during a person’s lifetime is between 8% and 10% (Seshadri S et al, 2006).

Multiple prospective studies have shown that both the incidence and the prevalence of this clinical syndrome is continuously increasing. Experts from the World Health Organization have suggested that by 2030, stroke will become the leading cause of mortality. It is important to note that stroke in developed countries is a major health and social problem, being the third cause of death after heart disease and cancer and the first cause of disability.

According to the American Stroke Association, approximately 87% of strokes are ischemic, and 13% are hemorrhagic (BM Kelly, PH Pangilinan Jr and Rodriguez GM, 2007; American Heart Association / American Stroke Association, 2008). Hemorrhagic stroke is associated with a higher mortality rate, suggesting that a higher proportion of recurrent attacks are ischemic (DG Jamieson et al, 2005).

Cerebral ischemia is caused by reduced cerebral blood flow, which is insufficient to meet the metabolic needs of the brain (Sullivan J, 2008). Reduced cerebral blood flow over a period of several seconds to several minutes results in a transient ischemia. This, depending on its intensity, can cause reversible or irreversible neuron damage. If the blood flow reduction lasts more than a few minutes, it leads to the onset of brain tissue infarction, characterized by the appearance of massive cell necrosis. A localized cerebral blood flow reduction leads to specific neurological deficits. The effects of hypoxia on neurons depend on the type of onset, the duration and the intensity of oxygen supply. The timeframe in which behavioral or cognitive disturbance can be identified is usually much shorter (seconds) than that required for the emergence of histopathological changes that usually require a few minutes (Bachevalier J and Meunier M, 1989; Lipton P, 1999).

If the hypoxia reaches a critical threshold, neuronal excitability and synaptic transmission disturbances occur, affecting multiple neural circuits. Interruption of neural circuits is usually attributed to intrinsic changes and postsynaptic membrane

Neurons and neuronal circuits present very different responses to hypoxia. Although in hypoxia, most neurons stop generating action potentials, some neuronal populations are more resistant to it. There are some mechanisms that allow the brain to function under low oxygen conditions, in a "dormant" state, but the amount of oxygen needed increases when activated. As a result the brain may naturally respond to moderate hypoxia through chronic and acute adaptation mechanisms (La Manna JC, 2007). Reactivity of brain tissue to hypoxia is directly proportional to hypoxic sensitivity, which changes with age. In general, newborn mammals are more resistant to hypoxia than adult mammals (Jiang C, Agulian S, Haddad GG, 1991; Singer D, 1999). Other studies have shown that various central nervous system structures such as the brainstem, hippocampus (Friedman JE, Haddad GG, 1993) and cerebral cortex (Bickler PE et al, 1993), contain neural components that are less sensitive to hypoxia (Ben-Ari Y, 1992).

Based on data extracted from medical literature, in this doctoral thesis we aimed to investigate by means of histology and immunohistochemistry, the influence of varying degrees of chronic cerebral ischemia of brain parenchyma in experimental animals. For this purpose we have formed groups of common Wistar adult rats, which had either on internal carotid artery or both clamped, causing varying degrees of cerebral hypoxia. Then, the animals were sacrificed at varying intervals to track the influence of chronic ischemia of brain parenchyma, depending on the time elapsed since ischemia on set. We hope that our study will bring new data on the microscopic mechanisms underlying neuronal injury behind the onset in chronic hypoxia, data that will be useful to neurologists, general practitioner and other specialties, which must treat different symptoms triggered by chronic cerebral ischemia.

**CHAPTER I**

**ANATOMY, HISTOLOGY AND HISTOPHYSIOLOGY**

**CENTRAL NERVOUS SYSTEM**

Considered to be the most complex system in the human body, the central nervous system (CNS) is comprised of: nervous tissue, blood vessels and connective
tissue. Structure with selective permeability, the blood-brain barrier is interposed between nervous tissue and vascular elements. The structure-function relationship is best reflected in the central nervous system, ensuring the functional unity and integration of the human body in its environment (Mogoantă L et al, 2004).

Therefore, in terms of neuroanatomy, the nervous system consists of all the structures which ensure the receiving, transmitting, processing, storing, integrating and comparing of information from the environment with previously stored experiences and with predetermined reflex answers in order to select, develop and initiate an appropriate response (Gartner LP and Hiatt IL, 1987).

The central nervous system (CNS) consists of centers which develop, integrate and coordinate nervous system commands. Together with the peripheral nervous system, it has a fundamental role in controlling behavior. Anatomical structures of the CNS are the encephalon and spinal cord, which are surrounded and protected by three connective tissue layers, forming the meninges, but also the bone structure: skull and vertebral column.

CHAPTER II
ANATOMICAL, HISTOLOGICAL AND FUNCTIONAL FEATURES OF CEREBRAL CIRCULATION

The central nervous system is one of the best vascularised systems, the brain receiving about 17% of blood flow, despite it representing only 2% of body weight; cerebral vascularization is intense and is comparable to that of the heart. The brain utilizes 20% of the amount of oxygen supplied to the lung, resulting in approximately 20% of its energy (JA Kiernan, 2009). The main blood flow to the brain is 50 ml/100g of tissue/minute in healthy individuals, depending on the region. The brain is similar to other tissues in the body, in the sense that blood flow varies depending on the functional activity of the tissue and its metabolism (Noback CR et al, 2005).

The brain is an organ, which requires a constant and permanent supply of glucose and oxygen for it to function. Nerve cells are very sensitive to changes of these elements, regardless of whether it is a deficit or excess. Therefore, during the phylo-ontogenetic development, the development of brain structures take place concurrent with the formation of the arterial network, which is able to provide an adequate blood supply in order to support brain function.
The CNS is one of the most active metabolic systems, brief interruptions of cerebral circulation being able to cause serious problems.

Neurons differ from the cells of other organs by the increased oxygen requirements. Deprived of oxygen, neurons almost always die within minutes, fact that has enormous medical implications (Noback CR et al, 2005). If the blood flow to the brain is completely stopped, unconsciousness occurs within 5 to 10 seconds. A rapid and complete reduction of cerebral blood flow leads to irreversible brain damage accompanied by brain tissue death. It is estimated that neuronal function stops after about 1 minute and irreversible changes begin to occur after about 4 minutes (Snell RS, 2010).

Lesions of vascular origin are responsible for more neurological disorders than any other category of disease. Stenosis or rupture of a single small artery or arteriole can quickly deprive of oxygen a region of the brain, causing cerebral ischemia (Squire LR, 2003). There are certain mechanisms that allow the brain to function in low oxygen conditions, when it is in "sleep", but the amount of oxygen increases when activated. Thus, the brain may naturally respond to moderate hypoxia through chronic and acute adaptation mechanisms (La Manna JC, 2007).

On the surface of the CNS, arteries form numerous anastomoses, but after entering the nervous mass, they become terminal blood vessels. There are also very thin anastomoses between the vessels inside the nervous mass, but they are insufficient in case of an acute obstructive accident of an artery. Blood capillary density is higher in gray matter than in white matter and more recent phylogenetic structures are richer irrigated (eg. cerebral cortex and cerebellum) (Afif AK and Bergman RA, 2005). Given the great sensitivity of the nervous tissue to hypoxia, cerebral vascular lesions are severe (stroke due to thrombosis, embolism, etc.) (Grigorescu Sido F, 2007).

CHAPTER III
CEREBRAL ISCHEMIA

Cerebral vascular disease, usually occurs in the second half of life, but their installation is insidious and slow, yet starting from a much younger age (30-40 years). The scale you have cerebrovascular disease is one of the most important health problems with major implications, medical and social (Arseni C, 1982).
Vascular accident (CVA) became in time a major public health problem, worldwide, accounting, together with myocardial infarction, the first complication of atheromatosis the first cause of disability in adults, the second leading cause of dementia, the third cause of mortality in economically advanced countries and by some authors (Popa C, et al, 2011), the first cause of mortality in Romania.

In terms of pathogeny, stroke involves a heterogeneous group of processes. Occlusion of blood vessels (ischemic stroke) are taken into account for 85% of all strokes, while primary intracerebral bleeding (hemorrhagic stroke) for the rest. Emboli cause approximately 75% of total occlusion of cerebral vessels and are the most common cause of obstruction of blood flow to the brain (Woodruff TM et al, 2011).

About 85% of all primary strokes are ischemic, 10% are due to primary intracerebral haemorrhage and about 5% are due to subarachnoid hemorrhage (Rothwell et al, 2004)

CHAPTER IV
THE PURPOSE AND OBJECTIVES OF THE PAPER

The large number of studies on ischemic strokes has led to the development of methods for provoking focal or global cerebral ischemia in experimental animals, methods that allowed the identification of mechanisms that contribute to brain tissue lesions.

Reduced blood supply to an area of the brain leads to a complex cascade of events that evolve in time and space. Understanding and identifying these events illustrates the therapeutic targets for the development of stroke therapy.

Currently, there are relatively few treatment options available to reduce brain tissue death following stroke. Consequently, there is an urgent need to explore the molecular and cellular mechanisms leading to damage and destruction of CNS tissue following an ischemic event in the brain.

In this context, the aim of this doctorate thesis and of the research behind it, is to highlight and analyze the range and location of brain parenchyma changes consecutive to chronic cerebral ischemia, depending on its duration and therefore its severity. A better understanding of this data and information will help early recognition of patients with high risk of stroke and contribute to the further development of
effective methods of prevention, in order to reduce the frequency of cerebral ischemia in people aged over 45.

CHAPTER V

ASSESSMENT OF MOTOR AND COGNITIVE PERFORMANCE IN RATS WITH CEREBRAL ISCHEMIA PRODUCED BY UNILATERAL AND BILATERAL CLAMPING OF THE INTERNAL CAROTID ARTERY

Experiments performed in this study were conducted during October 2009-September 2011 in the Biobase of the University of Medicine and Pharmacy of Craiova, in a special laboratory for behavioral tests and laboratory small animal surgery.

Our study was conducted using a group of 75 all male, Wistar, adult rats aged between 4 and 6 months, weighing between 300-450g.

Rats were divided into three large study groups, each group with 25 rats as follows:

- first group with permanent occlusion of the left internal carotid artery;
- second group with permanent occlusion of both internal carotid arteries;
- third control group (sham surgery) without occlusion.

Each large study group was divided into 5 subgroups according to survival period after stroke, each subgroup having 5 rats. (Distribution and survival of subgroups can be found in Table no.V.1.)

The methods used were as follows:

- Experimental model of unilateral and bilateral occlusion of the internal carotid artery
- Vestibulo-motor function assessment using rotarod test
- Assessment of spatial memory function, using T-maze test
- Monitoring of cerebral blood flow using a vascular Doppler

In our study, using models of chronic ischemia through unilateral or bilateral internal carotid artery clamping, we were able to identify certain differences in the development of motor and cognitive deficit and recovery of normal functions.

Rats in the control group, without any kind occlusion of the ICA, but which underwent surgery, have a decrease in these functions, which can be explained by disturbances caused by postoperative stress.
Regarding the two groups of rats with unilateral or bilateral ICA occlusion, more pronounced deficits can be attributed to secondary changes in total cerebral blood flow reduction, up to a value of 75% for unilateral occlusion or 55% for bilateral occlusion. Obviously, in the latter, the more consistent reduction resulted in a more pronounced decline of motor and cognitive functions, and also a slower recovery.

CHAPTER VI
HISTOLOGICAL STUDY OF BRAIN PARENCHYMA IN CHRONIC BRAIN ISCHEMIA

This study aims to evaluate changes in cerebral parenchyma following chronic cerebral ischemia induced by reducing cerebral blood flow mechanically through unilateral and bilateral clamping of the internal carotid artery (ICA).

For histological studies, we collected the entire brain from each group of animals in weeks 2, 4, 6, 8 and 10 after ischemia produced by ICA clamping.

Biological material collected was immediately placed in neutral formalin fixing solution 10% and then sent to the "Center for microscopic morphology and immunology studies" of UMF Craiova, where it was processed for examination under a microscope.

Histological methods used
Our technique involved the following procedures:
- dehydration;
- clarification;
- paraffin inclusion;
- paraffin block sectioning;
- adhesion of sections on slides and drying them;
- staining of sections;
- drying and storing of histological material.

Results
Analysis of changes in brain parenchyma after ischemia produced by unilateral and bilateral clamping of ICA

Decrease cerebral blood flow to the brain was followed by a reduction in number of neurons in the cerebral cortex due to processes of necrosis, autolysis and apoptosis.

Microscopic histological analysis performed in our study allowed us to identify and highlight significant changes in brain parenchyma, resulting from chronic
ischemia, achieved by unilateral or bilateral clamping of the internal carotid artery (ICA) in animal experimental model (Wistar rat).

In our study, we have shown that, while the animals in the control group presented a normal neuronal density, animals who underwent clamping of ICA uni or bilaterally presented decreased cerebral blood flow to the brain resulting in a reduced the number of neurons, particularly in the cerebral cortex, probably due to cell necrosis, autolysis and apoptosis.

Furthermore, our study pointed out that impaired brain parenchyma in chronic ischemia is heterogeneous, both in the cerebral cortex, as well as in the cerebellar cortex, the number of neurons varying greatly from one area to another of the cerebral cortex.

A particular issue identified in our study was the histological appearance of "ghost neurons", starting from the second week after chronic cerebral ischemia was produced through unilateral clamping of ICA. Molecular biology of the lesions induced by chronic cerebral ischemia is a rapidly growing area of research and may lead to identification of new therapeutic targets and possibilities. As a result, over time, several different models of ischemic stroke have been developed, in order to simulate as close as possible the changes that occur in the human body. The data collected in our study regarding the mechanisms implicated in damage to CNS cells induced experimentally by unilateral and bilateral clamping of the ICA on laboratory animals, reflect the results from one stage to another of ischemia. The results are of fundamental importance for establishing new therapeutic targets and medication administration window, which may lead to an increase in recovery after a stroke.

CHAPTER VII
Immunohistochemical study of brain parenchyma after cerebral ischemia

Immunohistochemistry (IHC) is a relatively new microscopy technique, used to identify cell and tissue antigens. This procedure is based on antigen-antibody interaction.

Histopathological material studied through immunohistochemistry technique was represented by encephalon fragments, collected from all animals included in the study.

In this study, we used the following IHC techniques:
technique for identifying neuron modifications, by using anti-NeuN antibodies;

- technique for identifying astrocyte modifications, by using anti-GFAP antibodies;

- technique for studying neuron apoptosis, by using anti-caspase 3 antibodies;

- technique for identifying changes in cerebral microcirculation, by using anti-CD34 antibodies;

- technique for identifying astrocytes, by using anti-GFAP antibodies

In our study, we have shown that some neurons surrounding the center of cellular necrosis appear enlarged and their cytoplasm (neuroplasm) has a weak staining, the nucleus is large and hypochromic, due to increased permeability of cell membranes to Na+ ions, Ca2+ ions and water.

In our immunohistochemical study we have noticed, as other authors before (Schaller B, Graf R, 2002), a process of ischemic cell tolerance in chronic cerebral hypoxia. We believe that chronic cerebral ischemia may determine some neurons to readjustment their cellular metabolism, allowing their survival, especially those in deeper layers. Through the immunohistochemistry techniques that we used, we were also able to identify the specific aspect of "ghost" cell, resulting from processes of autolysis and neuronal autophagy induced by cerebral ischemia.

Regarding the astrocyte population, in our study, by using anti-GFAP antibodies (an antibody directed against a protein filament present in astrocytes), we were able to identify their response to chronic cerebral ischemia, depending on the severity of ischemia and the time elapsed since occurrence.

In our study, a careful analysis of immunohistochemical reactions concluded that the process of apoptosis mainly affected the internal granular and pyramidal layers comprised of large neurons in the cerebral cortex. Superficial layers showed lower immunohistochemical reaction and at this level, the majority of neuronal loss occurred through a process autolysis. Analysis of immunohistochemical slides revealed a reduction in caspase-positive neurons, together with significant neuronal depletion, proving that the severity of neuron apoptosis is not dependent on the duration of ischemia. The changes we observed through immunohistochemistry techniques demonstrates that chronic cerebral ischemia involving complex mechanisms, both in terms of neuron alteration and modifications in glial cell population which, together
with the development of cerebral edema and inflammation, help shape the specific pathological picture of ischemia.

CONCLUSIONS

The results of our study have shown that after permanent unilateral clamping (chronic) of left internal carotid artery (ICA), followed by permanent bilateral clamping of both internal carotid arteries, irreversible or partially reversible changes develop both histologically and in terms of motor and cognitive performance in experimental animals.

The results of our research show that if cerebral blood flow is reduced to a value of about 55% of base level (bilateral occlusion of the ICA), there is a more pronounced decline of motor and cognitive functions, which recover more slowly, but not to their full capacity, in comparison to the other group with a cerebral blood flow reduction to a value of about 75% of baseline (unilateral occlusion) and of course to the control group.

We can also state that the differences between the two groups of rats and the control group and even between members of the same group, in terms of decline and recovery of motor function and cognitive architecture can be explained by the heterogeneity in cerebral circulation of each rat with various distributions of arterial border areas.

Regarding the analysis of brain parenchyma changes as a result of chronic ischemia by unilateral or bilateral ligature of internal carotid arteries, we demonstrated that the decrease in cerebral blood flow to the brain resulted in the reduction of neuron population in the cortex due to processes of necrosis, cell autolysis and apoptosis. Consequently, we have observed that in the contralateral hemisphere, cerebral parenchyma lesions were not as prominent compared to lesions occurring on the same side with the ligature, in the case of unilateral internal carotid artery occlusion.

Furthermore, in terms of ischemic lesions evolution, we demonstrated that, over time, due to a phenomenon of habituation similar to ischemic preconditioning, the differences in lesion intensity in the two groups, although still apparent, seems to decrease, as shown also by the assessment of motor and cognitive recovery.

Analysis of the effects of cerebral ischemia through bilateral occlusion of the internal carotid artery, after two weeks, allowed us to note that neuronal losses were
more severe than in unilateral ischemia; between 4-6 weeks, the differences were maximum and at the end of the experiment, after 10 weeks, differences are preserved, but the rate in which they occur seems to diminish.

In terms of immunohistochemistry, the changes we observed in neuron population coincided with the histological studies and in addition these changes were did not present the same intensity all over the cortex, there were areas of significant neuronal loss and areas with reduced neuron depletion.

Therefore we can conclude that chronic cerebral ischemia determined the neural cells to readapt their cell metabolism to the new levels of irrigation, which allowed the survival of certain groups of neurons, especially those in deeper layers.

After analyzing the glial cell population, we demonstrated that glial cells are sensitive to ischemia and suffer necrosis processes similar neurons. However, reactive astrocytes were found surrounding the center of cerebral necrosis, this histological aspect demonstrates the capacity of astrocytes to react to ischemia and products of cell necrosis.

Our research, in concordance with the proposed objectives, has obtained an experimental model for the study of chronic cerebral ischemia, which is useful in exploring phenomena caused by chronic ischemic cerebral blood flow reductions. Changes highlighted in this study may be useful in developing therapies to improve cognitive functions and motor recovery, altered due to chronic cerebral ischemia.