**UNIVERSITY OF MEDICINE
AND PHARMACY OF CRAIOVA**

DOCTORAL SCHOOL

PhD THESIS
ABSTRACT

PROTEINS AND GENES INVOLVED IN THE OXIDATIVE STRESS AND THE METABOLISM OF THE CENTRAL NERVOUS SYSTEM, AFTER FOCAL ISCHEMIA

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Introduction

Focal ischemic stroke (the stroke) is the cerebral equivalent of myocardial infarction. According to the latest data from the World Health Organization, approximately 15 million people worldwide suffer a stroke. Of these, one third die (3 million women and 2.5 million men) and one third remain with permanent disability, becoming a real burden for the family and society (WHO, The Atlas of Heart Disease and Stroke, 2011). Due to increased life expectancy and population's aging all these are the privilege of elderly, in fact, even old age is a risk factor for ischemic stroke (Barnett HJ, 2002). Thus, any model of stroke that uses old animals is beneficial both to highlight cellular mechanisms triggered by ischemia / reperfusion, and to see the response capacity and defence of the neuron in the elderly.

PART I - STATE OF KNOWLEDGE

1. Cerebral circulation - anatomical and functional aspects

The brain, a complex and heterogeneous organ is strictly dependent on its blood intake. Encephalon irrigation consists of an arterial circulation and a venous one, in the brain being no lymphatic vessels. Compared with other organs, the brain is the single beneficiary of vasculature provided by four major arteries that eventually merge, forming the polygon or arterial circle of Willis, located on the basal brain. These four primary blood vessels are: the two internal carotids (most important in terms of quantity, each providing about 40% of cranial perfusion) and the two vertebral arteries that join together, to form intracranial basilar artery.

Most of the blood is drained from the brain, in the two transverse sinuses, that along with the rough sine, form the internal jugular vein. A small part of the venous blood, especially in the occipital sinus, drains blood into internal vertebral plexus. As classification, there are two types of cerebral veins: superficial and deep. Superficial cerebral veins carry blood through the grooves of the cortical surface of the brain. Terminal and choroid veins form the internal cerebral veins, into which flows blood coming from the cerebellum (superior and inferior cerebellar veins). Through their union with basal veins arise great cerebral vein of Galen. The veins of the hemispheres are widely anastomosed together: some anastomosis connect superior longitudinal sinus with cavernous sinus or with lateral one; other connect the veins of the two cerebral hemispheres together (communicating veins from the base of the brain); deep veins and basal veins are anastomosed by their home venules.

Cerebral blood flow (CBF) is the amount of blood delivered to the brain in a certain period of time; in normal conditions, in an adult, it is found to be 750 ml/min or 15% of the cardiac output; it is about 45-50 ml/min/100g for an average blood pressure values in the aorta of 60 -130 mmHg (Orlando Regional Healthcare, 2004). When CBF falls below 20-25 ml/min/100g, serious disturbances of cerebral metabolism happens, such as hydro-ionic imbalance in different brain regions. Thus, at lower amount of 18-20 ml/min/100g cerebral ischemia occurs, and CBF of 8-10 ml/min/100g, spontaneous depolarization of the neurons is due to rapid loss of cell potassium and its passage in the extracellular space, eventually leading to cell death.

3. Cerebral metabolism - physiological and pathophysiological aspects

If in the '60s and '70s post- ischemic mechanisms researches were focused on cerebral perfusion and tracking control of energy metabolism, and the results obtained have established the concept of *core* and *penumbra* of the damaged area by ischemia-reperfusion (Astrup J et al., 1981), following decades were able to explain a number of internal and external cellular mechanisms. Thus, studies demonstrate that nervous tissue responds differently to injury: after a small period of ischemia (that do not causes an immediately cell death) transient and reversible disruption of cell signalling and calcium influx appears, free radicals are generated, and kinase and gene transcription are activated. Protein synthesis is transient affected and molecules are synthesized in order to repair or replace damaged or dysfunctional proteins, to ensure physiological neuronal protection after ischemic event - on a variable - days/weeks (Shimizu S et al., 2001). In case of severe ischemia, but non-lethal cellular damage, the disturbance of cellular components is more intense and toxic compounds are generated.

However, DNA repair is performed, protective protein synthesis is activated, cellular structures are rebuilt and endogenous enzyme systems neutralize newly formed toxic compounds (Li P et al., 2011). If ischemia is prolonged , irreversible changes occur in cell signalling, proteases and lipases are activated, oxidative lesions become more intense, aberrant gene expression appear and inflammatory cells are activated (Lee JM et al., 2000; Pellegrini L , et al., 2013). Massive penetration of calcium ion into the cell irreversibly redistributes regulatory proteins from the cytosolic part to the membrane, thus changing the redox state of the cell to an oxidized one (Zaidi, 2010). Into the *core* area of stroke, cell death occurs rapidly by breaking the neuronal membrane; in the *penumbra* part, transient depolarization causes progressive cytotoxicity due to calcium ions and oxidative damage that activate mitochondrial pores; they maintain the pathological chain. Neuronal death occurs after several hours.

3. Proteins and genes involved in general and nervous cellular metabolism and 4. Proteins and genes involved in oxidative stress, after focal ischemia

As during cerebral ischemia, and reperfusion also, lesion mechanisms exist and act synergistically: endoplasmic reticulum stress that becomes dysfunctional, proteolysis, oxidative stress, mitochondrial damage, inflammation, it is obvious the need to elucidate the mechanisms pro- survival and to identify those which promote neuronal apoptosis. Therefore, this paper tries to identify new genes and proteins of cellular metabolism or oxidative stress that can influence the brain tissue after ischemia - reperfusion by analysis of 15 genes involved in cellular metabolism and 19 in oxidative stress; they participate in various processes such as: *antiapoptotic/ neuronal protection, DNA changes, limitation of the inflammatory response, remodeling CNS protein, lipid, carbohydrate, and energy metabolism, membrane transport, intracellular transport and signaling.*

Chapters 3 and 4 present the activity of each analyzed gene, according to the latest data from the literature.

PART II - PERSONAL CONTRIBUTIONS

5. Goals and objectives

Ischemic stroke, the cerebral equivalent of myocardial infarction, with a mortality rate of 33% and also 33% rate of sequels, is a devastating disease. Unfortunately, up to now, no therapy is fully effective in neural rehabilitation for the older population, because the majority of investigations have been conducted on young animal models; only a few specialized studies provide some relevant comparisons regarding the difference response to ischemia between young/mature experimental animal and the old one (Markus et al., 2005; Popa-Wagner et al., 2006, 2011). Even preclinical studies on young animal models, with ischemic stroke, that initially proved certain therapeutic methods, on the transition to human clinical trials have become ineffective.

Therefore, the present study had the following objectives:

1. Demonstration of differentiated activation of oxidative stress (the path of O_2 reactive species) and cellular metabolism induced by their presence in young rats *versus* old rats.
2. Evaluation of oxidative stress signalling pathways and opportunities to use them as targets for therapies needed to increase the protective effects and cognitive functions of the cerebral cortex.
3. Selection of potential anti-oxidative stress drugs, for the modulation of pro-oxidants and antioxidants in the sense of achieving neuroprotection potentiation in order to maintain cognitive function after ischemic stroke.

6. Material and methods

The experiments for this study were conducted in the Laboratory of Molecular Neurobiology, Neurology Clinic of the Ernst- Moritz- Arndt University of Greifswald, Germany, from July 2007 - September 2010 (during which I benefited of an ERASMUS-PHARE mobility grant from UMF of Craiova and a governmental scholarship from doctoral Romanian Government) and under a Research Grant - Biochemistry Department Project PN- II -PT- PCCA -2011 -3.1 - 0222.

Approval to conduct all experiments was guaranteed by the Ethics Committee on Animal Experiments (Federal Animal Care Committee) of the university above mentioned, according to German and European law requirements regarding animal experiments and the Academic Commission of Scientific Ethics and Deontology of the University of Medicine and Pharmacy of Craiova (advisory report no 104/19.12.2012). Experiments were conducted under the close coordination of Professor PhD Aurel Popa -Wagner.

The study was conducted on a lot of 78 Sprague Dawley male rats, young (3 months) and old (aged 19-20 months), held in strict laboratory conditions (Table no. 1.). The experimental model of reversible middle cerebral artery occlusion, used in this study, involves a transient interruption of blood flow through it, by the occlusion of three vessels, after a preliminary trepanation. Both common carotid arteries were also involved, in addition to middle cerebral artery, and there were closed by two clamps that caused a decrease in blood flow in the irrigated region below 20 % of baseline, determining thus the occurrence of ischemia in the area. After 90 minutes,

blood flow through the three arteries was restarted, ensuring ischemic tissue reperfusion. Survival time was set to 3 days and 14 days respectively after surgery using one group at a time, to which ischemia- reperfusion procedure has been applied, for each age. The time after which the animals were sacrificed were chosen based on previous experience of the laboratory (Popa -Wagner et al., 1998, 1999).

After storage at -70 ° C, rat brains were cut in coronal sense with a criotom (CM3000 Cryostat Leica, Bensheim), at a temperature of -30 ° C.

Young rats	**3 days MCAO**	**14 days MCAO**	**Control**
Number of animals	**15**	**15**	**10**
Old rats	**3 days MCAO**	**14 days MCAO**	**Control**
Number of animals	**15**	**15**	8
TOTAL of ANIMALS = 78			

Table no. 1. Distribution of animals by age and survival time

To determine the volume of cerebral infarction consecutive to reversible middle cerebral artery occlusion, mature neurons were marked with a specific antibody, anti-NeuN (Mouse anti- NeuN, Millipore # MAB377) and stained with diaminobenzidine. The volume was measured by two different techniques in order to eliminate measurement errors, including post - lesion edema.

Genomic and protein analysis was carried out using samples of tissue from the cortex of the injured hemisphere and of the contralateral hemisphere, which were stored at -70 °C and then homogenized for isolation of protein and total RNA (using TRIzol reagent - Invitrogen , Germany).

After using microarray hybridization to a large number of genes, the qPCR technique ("polymerase chain reaction "/Polymerase chain reaction) was used to synthesize cDNA from higher funds of RNA (n = 19-22), by reverse transcription; the technique was also a way of control. The levels of gene expression were normalized to the requested media of expression of two "housekeeping" genes (genes involved in basic cellular functions maintenance): HPRT1 (hypoxanthine guanine phosphoribosyl transferase 1), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), of each sample.

We took equal amounts in micrograms per animal in both experimental and control groups and provide a pool of proteins whose concentration was determined by Bradford method. Detection, analysis and protein identification was made by Dot Blot, a technique similar to Western blotting, but more efficient as time. Protein identification was done using specific antibodies (in this case to identify proteins ID3 - rabbit anti ID3, Abcam).

The technique of immunohistochemistry and immunofluorescence respectively were used to detect apoptotic neurons by cleaved caspase 3 (from Cell Signalling) and also the neurons in the division and cell differentiation state by ID3; we also used a different antibody, DAPI (4', 6- diamidino -2 fenilindol dilactate a commonly used

fluorescent antibody labelling living cells, but also those established unaffected at harvest) to highlight viable neurons.

7. Results

A mean of 36.36 ± 5.731 mm3 for elderly rats, respectively 33.33 ± 4.084 mm3 for the young was found for the infarct volume. The difference between the two groups of animals was not statistically significant (p = 0.3374) (Figure no. 1.).

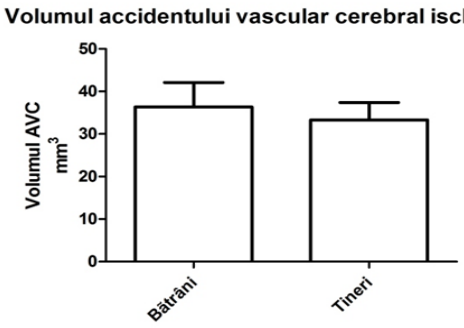

Figure no. 1.Volume of stroke with SEM (*standard error of the mean*).

There were observed several models of gene regulation detected by microarray and qPCR; there were over-expressed genes: persistent (at 3 days and at 14 days MCAO), transient, only in the acute phase (3 days) and late, at 14 days after focal ischemia; also under - expressed genes: persistent / transient / late.

- *Antiapoptotic / neuronal protection*

TPP1, Hmox1, Id3 genes showed the same behaviour: they were permanent overexpressed, but with a better protection in young rats in the acute phase and at 14 days for the old ones. The group of young animals received additional protection by rapid neuronal overexpression of *E2f5* and especially *Serpin 1* (6x), and the elderly through *Ddr1*.

- *Changes in DNA*

In the brain of experimental animals was observed protection exerted by *Xrcc6bp1, Brca1and Mapkapk,* genes overexpressed except youth group, a 14 days. DNA damage was extended by *SP100* and *Irf1* activity (in the group of aged rats, at 14 days, expressed 4x), and to the young *Ripk3* in the acute phase (5x).

- *Limiting the inflammatory response*

Inflammatory process seems to have been limited by *CD36* overexpression (especially in the acute phase up to 4 times for the young group of rats versus elderly group) and *TWIST1* (3 days - young, 14 days - old).

- *Remodelling SNC*

Remodelling capacity of the injured area was increased 5-fold in the group of young rats in the acute phase post-ischemia, by *Flna* overexpression; age differences are significant and correlated with the presence of other genes involved in determining cell lines and differentiation: *Twist1*. *Ube2C* presented a permanent gene overexpression, but more pronounced in the first 3 days after injury, in both groups of animals.

- *Protein metabolism*

Protein synthesis was observed by three genes (*Ap3b1, Necap2, Picalm*). We have found high levels of it, only to old rats, at 14 days (*Ap3b1 and Picalm*). Protein degradation showed a higher value for *Ube2t* for animals in the acute phase and for *Cndp1* in the elderly, at 14 days.

- *Carbohydrate metabolism*

It was followed by analysis of a single gene that was overexpressed *Pygl,* only in the acute phase, in young rats.

- *Lipid metabolism*

Because of experimentally induced lesions, the gene was down-regulated during the acute phase of all animals, and after 14 days, but only in young rats.

- *Energy Metabolism*

Overexpression of *Rab 32,* involved in mitochondrial fission and energy metabolism in all groups of animals studied, especially in the elderly, appears to be a protective mechanism against oxidative stress.

- *Intracellular transport and signalling*

Ehd4 and *Scamp2,* involved in intracellular protein transport, were overexpressed in all rats, especially in the elderly. Instead cellular signal transduction and regulation of microtubule dynamics was generally unchanged, only the group of young rats showed *Stmn3* down-regulated gene.

- *Membrane transport*

Rab27a intensified late, in the elderly group, and remained unchanged in the others groups of animals.

Dot Blot obtained results show an increased synthesis in both age groups for *ID3* protein to the blank (lysis buffer). However, the values were much better represented in the elderly, both in the acute phase and at 14 days.

Comparing this with the gene expression of *ID3* that was also overexpressed, but at the young group at 3 days, and old to 14 days, we conclude that elderly rats present a possible mechanism of protection by effective protein synthesis of *ID3*, especially late, in the day 14.

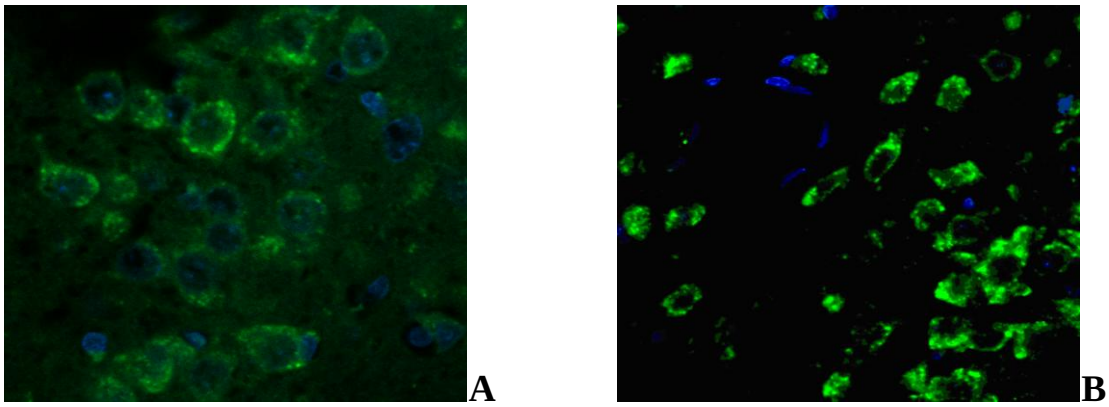

Figure no. 2. Young rats (A) and old (B) -14 days_**id3**_**dapi**_40x_focal ischemic zone

Double immunofluorescence was monitored by the appearance of the cell in the same area and found that cells showed co-localization of the two types of antibodies (both

ID3 and cleaved caspase 3). Also by double immunofluorescence were co-located *ID3* and *DAPI*; *ID3* presence is marked in green (Figure no. 2. -A) and viable neurons, labelled with *DAPI* - in blue. For rats sacrificed at 14 days, both young and old, *ID3* and *DAPI* expression is intense. In accordance with the results obtained by Dot Blot and qPCR, the increased expression of the protein *ID3* is observed in older animals at 14 days (Figure no. 2. -B).

8. Discussions

Recent data accepted by all researchers, believes that oxidative stress (OxS) is generated by the accumulation of reactive O2 species (ROS) and nitrogen ones (RNS), while reducing serum antioxidant capacity. ROS appearance is characteristic of places where develop inflammatory type reaction processes, ROS acting as signalling molecules and as intermediate regulators of cellular fundamental activity, such as growth, differentiation and cell division (when are at low concentrations); at high concentrations, SRO cause cell death.

Therefore the present study, by genomics and proteomics analyse of 34 genes involved in oxidative stress and cellular metabolism after focal cerebral ischemia, is trying to clarify certain aspects of the pathophysiological response to neuronal injury and especially for old neurological tissue.

9. Conclusions

1. Our results showed for the first time, the correlation between gene over-expression, determined by qPCR, protein synthesis appreciation by quantity Dot Blot and by double immunofluorescence labelling of ID3 as a possible mechanism of protection in older animals, especially in the late phase, after focal ischemia.
2. Also, we identified other possible protective mechanisms of cellularity and brain function in elderly rats, after focal ischemia via following gene overexpression: *TPP1, Hmox , Ddr1, Xrcc6bp1, BRCA,1 Mapkapk, Twist1, Ap3b1, Picalm Rab 32 and Rab27a.*
3. These results are important for sustaining future experimental studies, especially for ID3 in order to improve recovery and survival after ischemic stroke, in the elderly, after acute focal ischemia.
4. Genes involved in carbohydrate metabolism, expressed after ischemia, occur in young animals in the acute phase, while older animals express Rab32, a gene coordinator of mitochondrial fission and energy metabolism, a possible protective mechanism against oxidative stress.
5. Genes that support the proteins in intracellular signalling pathways are connecting to the immunological synapse, their overexpression being late, in aged animals.
6. For young animals, repair genes are overexpressed in acute phase, injured area being remodelled stronger to them, while for older animals are expressed in the late phase.
7. The death of altered cells (non-viable, necrotic, apoptotic ones) is essential for the production of other cells, in order to maintain cellular homeostasis; the emergence of imbalances for loss, shortages cause problems in the affected areas, especially in older animals in the zone of cerebral ischemia (although not statistically significant, p =

0.3374, old animals developed a larger volume of the infarct zone, compared to the young rats).
8. Permissiveness to detect cerebral infarct volume is given by the combination of two methods of investigation (Cavalieri method and the difference between contra - and ipsilateral cortical areas) that highlight also the destruction form: apoptosis or degeneration.
9. The phenomena of ischemia / reperfusion (I / R) and their consequence, various degrees of hypoxia, caused by interruption of blood flow for a long period of time (90 minutes), is the cause for neuronal death, more pronounced in older animals.
10. Activation of neuronal protective pathways in young rats might be a model for development of anti-oxidative stress therapies, which couples with the proteins in cell signalling pathways and in particular in the apoptosis path.

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