UNIVERSITY OF MEDICINE AND PHARMACY FROM CRAIOVA

PHAMACOLOGICAL MODULATION OF THE ANGIOGENETIC AND MICROGLIAL RESPONSE AFTER STROKE IN ANIMAL MODEL

- **SUMMARY** -

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1. INTRODUCTION

In 1970, WHO defined the stroke as a rapid installation of signs and symptoms of focal or global perturbation of cerebral function, which last for more than 24h or lead to death, without any obvious reason other than vascular origin. This definition is still used nowadays [1].

Although this wide definition encloses many entities like stroke, cerebral and subarachnoid bleeding, without keeping track of the whole progress of science and the new discoveries in the field, which allow us to separate this entities using imagistic and histopathological criteria. In 2013 American Heart Association redefines: stroke as cellular death secondary to focal ischemia at the lever of the cerebral tissue, spine or retinal cells, based on pathological, imagistic or objective evidences of ischemic cerebral, spinal or retinal lesions in a territory tributary to a known artery or clinical evidence of focal ischemia signs or symptoms that lasts longer than 24h or death, excluding other ethiology [2].

This thesis is structured in 10 chapters presenting the novelties in the field, risk factors and general findings of stroke, the role of inflammation on neuronal system, before and after stroke, materials and methods that were used, results and discussions.

The first part follows epidemiological data regarding stroke, risk factors and global burden of stroke. The following chapters focus on cellular and molecular changes secondary to stroke, penumbra angiogenesis and neurogenesis.

Moving forward we describe the animal lot, treatment and methods used, immunohistochemistry and immunochemistry protocols, ARN extractions, PCR, optical and confocal microscopy and statistical analyses.

The last part concentrates on the results obtained by this study and discussions.

Key words: inflammation, angiogenesis, stroke, BrdU, 2-photon.

2. EPIDEMIOLOGY AND ETHIOLOGY

Triggers of stroke

A risk factor is any characteristic of a person that increases the risk o develop of stroke, compared to the individual not presenting this characteristic [3]. Age, gender, race and family history of cardiovascular diseases are uncontrollable risk factors, whereas others can be modified leading of the risk of developing the disease. Most of the times, there is factors coexist in the general population adding to approximately 60% to 80% of the risk of stroke [3].

Scientist are yet to identify genes that are associated to the stroke, despite some complex genes studies conducted in the past years [4].

Risk factors for stroke

One of the main effects of the risk factors identified is the change in the structure and function of the blood vessels: a proatherosclotic effect that leads to stiffening, narrowing and twisting of the arteries [5].

Moreover, a cerebrovascular regulatory mechanism is affected by this morphological changes associated with cerebral blood flow reduction. Hypoperfusion alters vital adaptive mechanism and for many patients is caused by ageing, diabetes, hypertension and hypercholesterolemia [6]. By increasing the blood flow secondary to neuronal activity and the inability of the endothelial cells to maintain the microvascular regulation the tissue is under stress without being able to manage the needs and energetic intake [7].

Protective vascular mechanisms in charge of maintaining the blood flow to the brain in times of hypotension are altered by diabetes and hypertension, increasing the number of ischemic lesions of those patients [8].

On the other hand, the inability to develop collateral vessels increases the cerebral vulnerability to ischemia, making it even harder to limit the infarct area [9].

Diabetes and ageing adds to the vascular risk the susceptibility of brain cells to injury amplifying the effects of ischemia [10].

3. STROKE

In the first minutes from the beginning of the stroke, irreversible neurological damage is produced, with the start of the pathological ischemic cascade. The magnitude of the lesion is depending on the period of ischemia exposure and the ability of the brain to recover [11].

In the first staged of cellular ischemic death calcium ions excess and excitotoxicity plays the major role. Glutamate, one of the most abundant neurotransmitters accumulates in the extracellular space as the consequence of ion pump dysfunction and reuptake mechanism [12].

As a consequence, overstimulation of NMDA and AMP receptors increases sodium, calcium and water intake of neurons. Massive calcium intake affects catabolic processes mediated by nuclease, lipases and proteases [13]. Moreover, nitric oxide and arachidonic acids metabolites are produced by calcium depending enzymes acting as triggering factors for cellular death. Inefficient phosphorylation is followed by depletion of ATP and ROS production, accelerating the events that lead to cellular death [14].

Taking into account all of the above increased calcium concentration is not enough to compensated the cellular deposits of calcium as a result of excitotoxic stimulation. Other channels and ion pumps activated during ischemia need to be involved including sodium/calcium pump, hemichannels, volume regulated anion channels and TRP channels [15-18].

Both ischemia and inflammation are responsible for stroke pathogenesis with many involved mechanisms [19]. The penumbra area that surrounds the ischemic tissue is targeted by most therapies involved in the recovery after stroke, being the area easily restored. Thus we can define this area as the surrounding cerebral tissue affected by the low blood flow, but without cellular death speculating that once the blood flow is restored the tissue can be recovered limiting the infarct area and the deficits secondary to a stroke [20].

4. ROLE OF THE INFLAMMATION IN STROKE.

A proinflammatory systemic and cerebral state is encountered in many of the risk factors for cardiovascular diseases that increase the production of ROS [21]. The main source that generated ROS are mitochondrial enzymes, xantin-oxidase, NADPH oxidase and nitric oxide synthetase [22].

Nitric oxide biological inactivation is correlated with many of the toxic effects of oxidative stress on the blood vessels. The regulatory effect on the blood vessels of nitric oxide leads to vasoconstriction with a negative effect on microvascular regulation [22].

Platelet aggregation, leukocyte addition to the endothelium cells, smooth muscles proliferation are key steps of vascular inflammation [23]. Once the ischemic cascade starts, astrocyte, microglia, endothelial cells and neurons express a series of cytokines like interleukin 1, TNF alpha facilitate activated leucocytes to adhere the endothelium, resulting the obstruction of the blood vessels. The cerebral lesion can be limited by the mediating post ischemic inflammation. On the other hand, inflammation promotes some of the events that are needed for the recovery of the tissue in the ischemic process [24].

Targeting the inflammatory response as a potential therapy for stroke needs to take into account both the destructive potential of the inflammation in the acute phase and the benefits obtained in the late states of the stroke in the tissue recovery [25].

5. MATERIALS AND METHODS

The experiments in this study were performed in the Centre of Clinical and Experimental Medicine, within the Biochemistry Discipline of the University of Medicine and Pharmacy of Craiova, with the approval of the University Ethics Commission on Animal Experiments, meeting the ethical requirements of the National Law on the Use of Experimental Animals.

All experiments were supervised by Prof. univ. Dr. Aurel Popa-Wagner.

The present study is structured in two stages.

For the first experiment we used a group of 65 animals, rats of the Sprague-Dawley breed, ranging in age from 3 to 5 months and weighing between 290 and 360 grams. The animals were randomly divided into three post-stroke survival groups: animals sacrificed on days 3, 14 and 28, each lot being composed of 20 animals, subdivided into groups of 10 animals, each group receiving daily BrdU for one week before or after stroke. The control group consisted of 5 rats.

The second experiment used 30 male C57BL6 / 6J mice, aged between 6 and 9 months, and weights between 27 and 40 grams, divided into 3 groups: the group named "1/2 x Treatment" (N = 10), the "1/3 x Treatment" group (N = 10) and the "Control" group (N = 10). Consecutively, for the in vivo evaluation of the effect of the treatment we used 21 transgenic mice, male CX3CR1 divided properly into 4 treatment groups: the group "1xTreatment" (N = 4), "1 / 2xTreatment" (N = 4), "1/3 x Treatment" (N = 4) and the control group (N = 5).

The animals in the first experiment received intraperitoneal injections with Bromodeoxyuridine (BrdU) at a dose of 50mg / bw, daily, for one week prior to the stroke induction to highlight the proliferating cell phenotype before the stroke induction. To identify the cells that proliferate after the onset of the infarction, the BrdU was administered daily, in the first week after stroke.

The second experiment consisted of administration of a selective C3a receptor antagonist of the complement (SB 290157 trifluoroacetate salt - N2- [2- (2,2-diphenylethoxy) acetyl] -L-arginine trifluoroacetate salt): 1mg / ml in 1xPBS / 0 1% DMSO. In vivo, a Hamilton syringe mounted in a stereotaxic instrument was used for injection, thus injecting 1 µl of solution, single intracortical injection. For groups "1 / 2xTreatment" and "1 / 3xTreatment" we used the following dilutions: 1:1 and 1:2 from the initial concentration, and the "Control" group received a solution of PBS / 0.1% DMSO.

After stroke, all animals in the "1 / 2xTreatment" and "1 / 3xTreatment" group received three injections of 1 µl of the appropriate dilutions, while the "Control" group received PBS / 0.1% DMSO solution.

The technique of stroke induction was the same for both experiments: middle cerebral artery occlusion. The bifurcation of the middle cerebral artery was cauterized at three points with an electrical cauter, without damaging the adjacent tissue, for the induction of anesthesia a mixture of 3-5% Sevoflouran in 75% nitric oxide and 25% oxygen was used, and the anaesthetic effect was maintained with 1–1.5% Sevofluran.

In the first experiment the animals were sacrificed at 3, 14 and 28 after middle cerebral artery occlusion, using the procedure of injectable anaesthesia by intraperitoneal administration of a mixture of xylazine and ketamine. The animals from the second experiment were sacrificed 7 days after stroke, and the in vivo study aimed to sacrifice the animals 3 days after following the changes induced by the injury. After deep anaesthesia of the animals, verified by the abolition of the nociceptive reflexes (Pinch Reflex), the brain tissue was harvested, and then specific steps of preserving the cerebral tissue were followed according to the processing techniques. Thus, the tissue that was to undergo the stages of genetic analysis was subjected to a freezing procedure at -70grC, and the tissue to be processed by sectioning was fixed in paraformaldehyde solution 4% in 5xPB at a temperature of 4grC for 24 for hours.

Tissue homogenization was performed in solution D (Guanidinum 4M thiocyanate / 1M sodium citrate / 10% Sarcosyl / 2-mercaptoethanol in water without RNA), and RNA was extracted using TRIzol reagent (Invitrogen life technology, Karlsruhe, Germany). Genomic DNA removal was performed using the Rneasy Plus kit (Qiagen). The reagent works by destroying cells and cellular components, but by maintaining RNA integrity, producing an efficient inhibition of RNA during lysis and homogenization.

For real-time quantitative PCR (qPCR), cDNA was synthesized from purified RNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, USA). Quantitative PCR was performed on a plate with 96 walls (Applied Biosystems) using the One Step Plus system. Each well was contained a volume of 20 µl obtained from: 10 µl iQ SYBER Green Master Mix (BioRad Laboratories, Hercules, CA), 2 µl primer sense and anti-sense specific gene quantified (Qiagen, Alameda, CA) and 8 µl prediluted cDNA. The control probes contained water-NF as a substitute for the primer.

The data wes analyzed using the ΔΔCt method [208]

The volume of the infarction after the average cerebral occlusion was obtained by the immunohistochemical determination of NeuN - nuclear neuronal marker, its absence indicating the loss of neurons at that level. Each tenth section was marked, the images obtained under the optical microscope were processed using Image J software, and the volume was obtained as the sum of the partial areas of the infarction areas.

Immunohistochemistry and immunofluorescence techniques were represented by:

a) triple detection Laminin, SMA, BRDU: SMA 1: 1000 (mouse anti-gamma smooth muscle actin, Sigma-Aldrich, Munich, Germany), Laminin 1: 2000 (rabbit anti-laminin, Sigma, Munich, Germany), rat anti-BrdU 1: 2000 (AbD Serotec, Puchheim, Germany).

b) triple detection Collagen IV, P4Hbeta, BrdU: anti-collagen IV 1: 1000 (polyclonal rabbit anti-collagen IV, abcam, UK) and anti-P4Hbeta 1: 1000 (mouse anti-P4Hbeta monoclonal antibody, Novus Biological, UK) , rat anti-BrdU 1: 2000 (AbD Serotec, Puchheim, Germany).

c) triple detection of RECA, SMA and BrdU: anti-actin 1: 2000 (rabbit polyclonal anti-actin, Sigma, Munich, Germany) and anti-RECA 1: 200 (mouse anti-rat endothelial cell antigen, abcam, UK), rat anti-BrdU 1: 2000 (AbD Serotec, Puchheim, Germany).

d) Dual detection DCX and BrdU: anti-DCX (guinea pig anti-doublecortin pigment - DCX, Millpore) and rat anti-BrdU 1: 2000 (AbD Serotec, Puchheim, Germany).

e) IBA1: Rb anti IBA1 1: 1000 detection (Wako Chemicals USA Inc., Richmond, VA, USA, 019-19741).

f) ED1 detection: Rb anti ED1 1: 1000 (Rb pAb to CD68 - ab 125212).

Cell quantification was performed using the 40x objective from sections in the coronal series. The results are presented as cell numbers per 100µm2, using Fiji software (National Institute of Health) [209].

The quantification of BrdU-labeled blood vessels was performed following approximately 30% of the infarction area, with emphasis on areas where the density of RECA / BrdU-positive cells was higher. Thus, the cells were identified with the objective of 40, and for counting we used the objective of 20, covering a microscopic field of 0.7386 mm2. The count was performed by two independent observers, and the results are expressed as a percentage ± DS (standard deviation).

The high-resolution real-time image was obtained using a Zeiss two-photon 7MP laser (2P-LSM).

Fluorescence was visualized using a fs-pulsed titanium sapphire laser (Chameleon Vision II, Coerent, Glasgow, United Kingdom) having a peak power greater than 3.5W set at 910 nm.

The lesion was performed with the aid of a Hamilton syringe that produced 500x500x1000µm injuries and was administered SB 290157 (in the doses described above: the full dose, half and one third, the animals in the control group receiving only the vehicle). The injection was performed slowly, over a period of 60 seconds. After the injury over the craniotomy area a window was fixed with cyanoacrylate and dental cement, so that repetitive images could be obtained. The same area was scanned for twenty minutes, every 24 hours, for 3 consecutive days. Further computer analysis, such as tree branching of microglia, was done using Fiji software and its plugins, as well as Adobe InDesign (Adobe, USA).

Confocal microscopy was performed with a Zeiss LSM710 confocal microscope (Zeiss, Germany, Germany) and Zen 2010 software version 6.0 software (Carl Zeiss Microscopy GmbH, Jena, Germany). The fluorescence emission used was 500-530 nm for FITC (green), 550-600 nm for rhodamine (red) and 650-710 nm for Cy5 (blue).

3D reconstruction was performed using software with maximum projection algorithm.

Statistical analysis was performed using GraphPad 6 and Microsoft Excel. Immunohistochemistry results were evaluated using two-way ANOVA. The Mann-Whitney test was used for all morphological analyses. Unless otherwise stated, all figures show the mean value and standard error of the mean (SEM) and the statistical significance is displayed as follows: $*$ for $p < 0.05$ and $< p < 0.01$ and $***$ $p < 0.001$.

6. RESULTS

1st Experiment

BrdU administration prior to the induction of stroke does not affect the volume of the infarct.

Infarct volume was quantified by immunohistochemistry using NeuN as a sensitive marker for viable neurons. The volume of infarction was higher in the group of animals perfused 3 days after the induction of stroke, probably due to the presence of cerebral edema (129 +/- 39 mm3), stabilizing at 116 +/- 29 mm3 at day 28. Thus, the volume of infarction between groups was similar and independent of BrdU administration.

Cerebral vascularization is constantly remodelling in the adult brain without injury.

By injecting BrdU before stroke, we were able to identify proliferating endothelial and neuronal cells in the adult brain without any injury.

Thus, 3 days after the induction of stroke, BrdU was preferentially incorporated into the nuclei of cells close to the dentate gyrus. By double labelling with BrdU and DCX we identified the precursor neuronal cells. BrdU-positive nuclei and co-localization of DCX-positive cells were identified both in the subventricular area of the lateral ventricle and in the contra-lateral cortex. Furthermore, BrdU-positive nuclei co-localized the RECA endothelial cell marker in areas distant from the infarct area, thus describing the lumen of the blood vessels. Triple staining BrdU / RECA / SMA identified endothelial and actin-positive cells from smooth muscle tissue, most likely pericytes, and 3D image reconstruction identified an uneven distribution of newly formed cells, suggestive of a new endothelial cell incorporation pattern formed in pre-existing blood vessels. An uneven distribution of BrdU-positive cells was also detected in the blood vessels branched in the infarct area. However, BrdU-positive cells were not identified in the microglia in the perilesional zone. However, at 28 days after the infarction, we identified a co-localization of BrdU / RECA and BrdU / NeuN in the region behind the glial scar, called regeneration islands, by the presence of numerous blood vessels and endothelial cells.

After stroke, BrdU-positive nuclei are mostly incorporated into proliferating endothelial cells

Daily administration of BrdU after focal ischemia led to the identification of endothelial cells aligned to blood vessels in areas distant from brain injury 3 days after stroke.

At the same time, cells expressing the p4Hbeta endothelial marker that appear to cross large blood vessels labelled with IV-collagen antibodies have been identified. At 14 days after the ischemic event, proliferating endothelial cells were still detected using anti-P4Hbeta and anti-BrdU antibodies. Using these markers, we were able to visualize the endothelial cells that detach from the disintegrating blood vessels whose basement membrane was labelled with anti-collagen IV antibodies.

Increased P4Hbeta expression is confirmed by mRNA in RT-PCR using specific primers in groups of animals perfused at 3 and 14 days, when an increase in response to hypoxia was expected.

On day 28 after stroke, new blood vessels appeared in the perilesional area, most BrdU + and were, most likely endothelial cells embedded in a laminin matrix. BrdU + / RECA + double staining identifies large blood vessels in the glial scar. BrdU + / RECA + blood vessels also appeared in areas beyond the glial scar, areas called "regeneration islands". Even at 28 days after the ischemic event, BrdU + cells left the vascular wall marked with laminin antibodies in the vicinity of the infarct. BrdU / RECA + blood vessels are still present in areas far from infarction and in the contralateral cortex. At 3 days post-injury, most neuroepithelial cells in the glial scar and the perileional region present antigen for nystatin.

2nd Experiment

C3aR antagonist has effects on post-stroke inflammation, but not on stroke volume

The volume of infarction at 7 days after occlusion of the middle cerebral artery was similar between the control group and the treated animals, regardless of the dose. However, when we analysed the cellular response of microglia, we found that the animals treated with the C3Ra antagonist, SB290157, showed a 50% decrease in the number of phagocytic microglial cells in the group of animals that received half the treatment dose and a 75% decrease in the number of activated microglial cells in the group of animals that received one third of the dose.

Prevention of inflammatory morphological transformation by C3aR administration

Local administration of SB290157 attenuated the inflammatory changes in microglial morphology, which were easily visible in the penumbra. Indeed, we found a reduction in the number of cells expressing Iba1 by 40% at a third dose. However, the dose-dependent effect

was not pronounced. By manually tracking microglial processes, we were able to identify that there was a decrease in the number of first, two, and three branches in the control group, showing that microglia in these animals shortened their processes in preparing an amoeboid transformation. Also, there was a clear increase in the number of terminal processes in the animals in the control group compared to the treated animals, a clear sign of a decrease in microglial activation in the treated group compared to the control.

Intercortical administration of SB290157 has an impact on both microglia migration and phagocytosis

Two-photon laser scanning microscopy has become a gold standard for in vivo cell behaviour analysis. By injecting the C3aR antagonist locally, we induced a small cortical lesion that was directly influenced by diffusion of the antagonist. Although microglial migration occurred, at all doses of SB290157, after 48 hours, there was a decrease in the number of microglial cells around the lesion. At the same time, we could observe a dose-dependent decrease in the phagocytic capacity of the microglia, with fewer phagocytic phenotypes observed in the high-dose group compared to the lower ones or the controls.

7. CONCLUSIONS

Firstly, the administration of BrdU to stroke-free animals showed an uneven distribution of endothelial cells newly incorporated into the mature blood vessels of the adult rat brain.

Secondly, by injecting BrdU before stroke, neural precursor cells specifically labelled in the region beyond the glial inhibitory scar that appears permissive in regenerative events.

Thirdly, BrdU injection after stroke resulted in labelling of endothelial cells that intersect or detach from disintegrated blood vessels and incorporate them into new blood vessels in the middle of the infarct, scar tissue, and the region behind the glial scar.

Fourthly, administration of BrdU after stroke resulted in the specific incorporation of BrdU + nuclei into the "pinwheel" architecture of the ventricular epithelium.

In the second experiment, we showed that the C3aR antagonist, SB290157 administered intracortically, may be used in the future to limit neuroinflammation and therefore neuronal death after ischemic injury by modulating microglia transition to phagocytic type and secondary phagocytosis.

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