UNIVERSITY OF MEDICINE AND PHARMACY
CRAIOVA
FACULTY OF MEDICINE

SUMMARY OF DOCTORAL THESIS
EXPERIMENTAL APPLICATIONS OF SOME NEUROPROTECTIVE
EXOGENOUS AND ENDOGENOUS METHODS IN STROKE AND ITS
COMPLICATIONS

Scientific coordinator
MD PhD Iancau Maria

PhD Student
Vintilescu Raluca Elena

Craiova
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INTRODUCTION

Cerebrovascular diseases represent the third death cause after the cardiac diseases and neoplasia. The cerebrovascular accident represents one of the main causes of morbidity and long-term disability in Europe.

Epileptogenesis represents the process by which, after a cerebral injury, there might appear morphopathological and physiopathological changes in certain cerebral regions which might lead to the expression of epilepsy.

The understanding of the mechanisms of epileptogenesis is crucial for the development of therapeutic interventions which will prevent the manifestation of epilepsy after a cerebral lesion or which will reduce its severity.

The first part consists of three chapters:
- Morpho-physiological characteristics of cerebral circulation
- Current aspects regarding the answer of the central nervous system to the cerebral ischemia
- Exogenous and endogenous neuroprotective methods in stroke and its complications.

In the first part of the thesis we propose to outline the classical data and the most recent information in the reference literature, presenting the anatomic and functional parameters of the cerebral circulation and the modern notions regarding the answer reactions, insisting on the self-regulation characteristic for this special circulation as well as the modern notions regarding the answer reactions of the central nervous system to the cerebral ischemic injury, the neuroprotective methods – hypothermia through H2S and the interrelations between post-stroke neurogenesis and epilepsy.

AIMS OF THE RESEARCH

The aim of our study was to following the following objectives: monitoring of the physiological parameters (temperature) and the assessment of the electroencephalographic changes for the sample of animals exposed to H2S as well as for the witness sample; the analysis of the changes in the infarcted area, through RMN and immunohistochemistry – as compared to hypothermia – witness sample; pointing out some mechanisms, through which hypothermia is exerting is alleged neuroprotective, post-ischemic role, on the level of the central nervous system; elucidating the ways of appearance and aggravation of some of stroke complications.
– epilepsy, by inducing unique convulsion crisis or the epileptic status and their correlation with neurogenesis.

We also proposed the analysis of the gene expression, the analysis of the proteomic expression and of immunohistochemistry, after the administration of L-NAME factor involved in the stimulation of neurogenesis.

MATERIAL AND METHODS

For the first series of experiments we used male rats, belonging to the Sprague-Dawley breed (N=43), aged between 17-18 months, with a weight of almost 520-600 g, kept in standard laboratory conditions, with free access to food and water. Before the surgical procedure of reversible occlusion of the middle cerebral artery, in the purpose of minimizing the effects of the effects of the variations of the glycaemia levels on the ischemic area, the animals were deprived of food but they were not deprived of water.

The animals were divided into two groups: 1st group (N=17) with reversible middle cerebral artery occlusion (MCAO) and the 2nd group (N=15), with MCAO and hypothermia. From these two groups there were selected 7 rats from each group and used for the biochemical analysis and 8 rats were used for the histological analysis. Six animals had electroencephalographic recordings. A group of animals was used as a control sample (N=5). No animal died during the experiments, but two rats were eliminated from the study due to the failure of the surgical procedure.

The working method used in this experiment was the intra-luminal reversible occlusion of the right middle cerebral artery with a tungsten hook attached to a micromanipulator. The physiological parameters were monitored for the entire surgical intervention (arterial pressure and body temperature). The local changes of the arterial flow were also monitored by Doppler. At a certain period of time from the occlusion of the middle cerebral artery the animals were sacrificed.

For the second series of experiments we used a sample of 212 adult Sprague-Dawley rats, male (2 months old), with a weight between 320-400 g.

The convulsive crisis was induced by the intra-peritoneal administration of a single dosage of PTZ (50mg/Kgc, Sigma). Bromodeoxyuridine was administrated post-seizure, in the days 1-3 (BrdU 50mg/Kg body, Roche, Germany, intraperitoneal). The animals were sacrificed in the 24th day after the seizure.
The induction of the epileptic status was accomplished by the initial administration of a first convulsive dosage of PTZ (50mg/Kgc, Sigma), followed by four sub-convulsive dosage of PTZ (30mg/Kgc, Sigma). The dosage was administrated at an interval of 25 days ± 1. For the evaluation of the formation of new cells, as well as for their phenotype, BrdU was administrated in the days 1, 2 and 3 after each dosage of PTZ. The animals were sacrificed in the 125 after the seizure.

In order to check the hypothesis that neurogenesis would have a role in the induction of the epileptic role, the rats were treated with a sub-convulsive dosage of PTZ (30mg/Kgc, Sigma) in day 0. We began the treatment with L-NAME (90 mg/kg a day, intra-peritoneal) in the day after the administration of PTZ (day 1) until day 23.

For the quantification of the newly formed cells and the analysis of their cellular phenotype we administrated BrdU (50mg/kgc, intra-peritoneal, Sigma), in the day 1, 2 after each dosage PTZ. The animals from the witness sample were injected intra-peritoneal with physiological serum.

For both series of experiments there were carried out electroencephalographic recordings, the quantitative analysis of the electroencephalographic paths were carried out with the help of the software analysis programs DSI and EEG-Neuroscore.

According to the experiment, the animals were sacrificed at certain time intervals, according to the brain prelevation. The brain frozen at -70 °C, was sectioned coronary, in thick slices of 25 and respectively 50μm, for the first series of experiments and midsagittal for epilepsy using a critrom at a temperature of – 30 °C.

In order to be able to determine the volume of the cerebral infarction, resulted after the occlusion of the middle cerebral artery, each 20th section was marked by a specific anti-body, a mature neuronal anti-nucleus (anti-NeuN, Millipore, Germany) and coloured with diaminobenzidine.

In the first series of experiments, the expression Annexin a 1 was also followed through immunohistochemistry techniques.

The immunohistochemistry analysis was used in both experiments, its objectives for the first experiment being the determination of the volume of infarct and the study of the Annexin a1 expression, and in the epilepsy experiment, the emphasis of the BrdU positive cells and of the immature neurons, the quantification of the BrdU positive cells as well as setting the neuronal phenotype.
The genomic analysis was carried out in both series of experiments using tissue samples taken from the shaded area for the first series of experiments, and from the level of hippocampus and temporal cortex for the second series of experiments which were homogenized in order to isolate the total RNA. In order to accomplish this goal we used microarray hybridization techniques and RT-PCR.

The proteomic analysis was used in both experiments, being carried out through the extraction of the proteins obtained from micro-dissections from the level of the shaded area and the corresponding counter-lateral region, respectively the level of the hippocampus for the epileptic experimental model, using the TRIzol reactive. The quantification of the proteins was made with the help of the Bradford method, then there was a pool of proteins proportional with the concentration, containing an equal quantity of proteins from each animals which represent an experimental group. For the Western Blot analysis we used 20 μg from the protein pools of each group.

**RESULTS**

The exposure of old animals, post stroke, to an atmosphere containing a low dosage of H2S, determined a gradual decrease of the temperature of the entire body, which was set at the value of 31±0.5°C, after an interval of 8 hours.

The electroencephalographic recordings showed a significant reduction of the cerebral activity, without epileptic time discharges, in the case of the animal group under Hypothermia as compared to the control group.

The data resulted from RMN pointed out a decrease of the volume of infarction by almost 57% as compared to the control group. The infarcted region was determined with the help of a marker for neuronal nuclei NeuN. Using the immunohistochemistry analysis the volume of infarction was reduced by ~67%.

With the help of the semi-quantitative RT-PCR we discovered a triple size of the ARNm expression of ANXA1, in the case of the animals with untreated infarction. This was annulled by the exposure to H2S.

The quantification of the expression of the protein ANXA1 through western blot, points out the fact that hypothermia caused a significant decrease of ANXA1.

The western blots results were obtained on the cellular level. Using the antibody rabbit anti-Annexin I, we discovered through immunohistochemistry, that in the peri-infarcted region there are immune-like cells.
Two days after hypothermia, their number is reduced significantly. The counter-lateral hemisphere did not register any detectable signal with the help of immunohistochemistry. The quantification of the positive cells ANXA1 points out the fact that hypothermia reduced the number of positive cells ANXA1 by 42%.

We pointed out this aspect by using the double immune-marking with the rabbit antibodies anti PMN (green) and goat anti Annexin 1 (red), ANXA1 co-localised with positive cells PMN. In order to exclude other main phenotypes of cells, we carried out additional experiments based on immunofluorescent techniques, using microglia marking elements ED1 and Iba1, as well as CD11b. No one of these markers are co-localised in ANXA-1.

Two days after hypothermia, the number of co-localised cells PMN-ANXA1 was significantly reduced. In fact, hypothermia was associated with a different morphology of the cells, meaning that the PMN-like cells had a prolonged morphology and sometimes were divided into groups. Occasionally, ANXA 1 was co-localized with the blood vessels.

A unique episode of convulsive crisis induced by Pentylenthetrazol (PTZ), determined the appearance in the 3rd day of a high number of BrdU positive cells which have a non-preferential distribution in the entorhinal and temporal cortex, as well as in the hippocampus.

After 25 days, most of the cells did not survive, but unexpectedly there was a selective survival of the positive BrdU cells, especially of those localised on the level of the hippocampus and temporal neocortex.

In the 3rd day, the number of positive BrdU cells (BrdU+) increased 16 times on the level of the hippocampus and 10 times on the level of the temporal neocortex for the sample of animals treated with PTZ.

After 25 days, the number of BrdU+ cells remained high in the hippocampus and the temporal neocortex, but there were registered much lower level as compared to day 3.

After two administrations of subconvulsive dosages of PTZ, up to 86% of the animals had an epileptic status (score 4, respectively 5 of the Racine scale). Then the proportion of the animals presenting an epileptic status remained constant.

Each treatment with PTZ has lead to an accumulation of BrdU+ cells which were in a large number on the level of the dentate gyrus of the animals under repeated stimulation.
The 3D projections of the confocal images obtained through double immunofluorescence BrdU(red)/NeuN(green) of the animals treated with PTZ showed that a number of 25 days was enough to allow certain positive BrdU cells to be differentiated in neurons especially in the granular cell layer from the hippocampus.

The number of double immune-marked cells BrdU(red)/NeuN(green) raised along with the number of PTZ injections and reached a maximal value for the sample of animals which were repeatedly stimulated.

The 3D projections of the double immunofluorescent marked images DCX (green)/NeuN(red), belonging to the animals treated with 1X and 2X PTZ, point out a clear, unexpected co-localization of DCX and of the positive NeuN cells along the hilux/cell border from the dorsal hippocampus.

In time the marked BrdU nucleus became fragmented and the number of positive DCX cells increased significantly, in the case of the animals with an epileptic status. Unexpectedly, the double market immunofluorescent cells had a clone appearance.

A quantitative estimation through RT-PCR, of the ARNm of DCX indicated as compared to the control group a significant growth of the relative DCX transcriptions quantity in day 25, after the last convulsive crisis, in the case of the animals with epileptic status.

A premature event, after the convulsive crisis was a transitory growth of the expression of the stem cells marker, the nestin and the protein level on the level of the hippocampus.

The L-NAME treatment did not change the level of the expression of stem cells marker, the nestin induced by PTZ, in the 3rd post-critical day but the group of animals treated with L-NAME had a higher significant level that that (2.7-times) than the animals treated with PTZ, in day 25.

On the tissue level, the immune reaction to nestin for the animals treated with PTZ in the 3rd day was limited to the radial, glial cells in the inner molecular layer of the dentate gyrus, the polymorph layer and interestingly on the level of the CA2 region.

In the case of the animals treated with PTZ, the level of the quantified DCX with the help of the Western Blot method increased, phenomenon which was accentuated by the combined treatment PTZ + L-NAME in day 50 after the epileptic crisis.
DISCUSSIONS

In this study we tried to carry out a complete biochemical, immunohistochemistry analysis of the gene expression, of the answer of the cerebral tissue to gas hypothermia applied for 48 hours and we identified ANXA1 as a prominent target of the anti-inflammatory actions of hypothermia induced by H₂S in the peri-infarcted region on the level of the brain of old animals.

The long exposure to hypothermia induced by sulphide hydrogen reduces the volume of the infarction after the vascular cerebral accident.

In this study we showed that the hypothermia induced by H₂S reduces the volume of the infarction after the cerebral vascular accident, as well as the number of PMN cells which express ANXA1.

In this study we demonstrated that in the day 25 ± 1, after the repeated stimulation of the brain through pharmacological factors, the Sprague Dawley rats are extremely susceptible to the development of the epileptic status if they are administrated two subconvulsive dosages of pentilene tetrazol (PTZ) for 25 days. Each stimulation in the 25th day, but not in the 30th day with PTZ increases the number of new neurons on the level of subgranular region from the dentate gyrus of the hippocampus.

Due to the strong resemblance with the time window of one month necessary for the neuronal development and the maturity of the newly born neurons in the adult hippocampus, our hypothesis is that the susceptibility to the convulsive crisis in the 25th day is in a certain extent connected to the newly formed neurons.

CONCLUSIONS

The results of our study pointed out the fact that from all neuro-protective methods, the hypothermia induced by H₂S, has proved to be an efficient method for old animals, by reducing the inflammatory answer after the cerebral ischemia contributing in this way to the improvement of the recovery after the cerebral vascular accident.

Our results suggest that increasing the susceptibility to epileptic crisis in the 25 ± 1 day after treatment coincides with the critical time required for the new neurons to be differentiated and integrated in the existent hippocampus network.

Therefore the experimental model can be used for the in vivo demonstration of the interrelations between the neurogenesis and the mechanisms contributing to the development of epilepsy.