The influence of acelysin and nimotop on the cellular response of the hippocampus during the dynamics of experimental subarachnoid hemorrhage

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Aim. Immunohistochemical study of rat hippocampal neurons and astrocytes at different time-points of experimental brain hemorrhage treated with acelysin and nimotop.

Materials and methods. Subarachnoid hemorrhage (SAH) was modeled according to R. V. Dudhani et al. using 35 Wistar rats which were divided into 7 groups. Animals of the 1st and 2nd groups, for 4 and 7 days respectively received acelysin at a dose of 15 mg/kg once a day; animals of the 3rd and 4th groups received nimodipine at a dose of 0.3 mg/kg every 8 hours for 4 and 7 days. In groups 5th and 6th (control), SAH was modeled without treatment with observation periods of 4 and 7 days, respectively. The 7th group consisted of intact animals. Hippocampal CA1 fields were studied immunohistochemically evaluating the expression of CASP3, NeuN, GFAP by the relative area of immunostaining in ImageJ.

Results. CASP3 expression in hippocampal neurons increases by 2.3 and 5.7 times on day 4 and by 1.8 and 3.9 times on day 7 of experimental SAH (groups 1–4) compared to intact group. GFAP expression increases in all observation groups compared to intact animals with the maximum values in the 5th group – by 8.14 times. The dynamics of NeuN expression in hippocampal neurons when using acelysin and nimotop corresponds to the inverse dynamics of CASP3 expression. The NeuN expression increases maximally on the 7th day of acelysin use and is equal to 91.76 % of the intact group values.

Conclusions. The experimental SAH is accompanied by a change in the expression of CASP3, NeuN in neurons and GFAP in astrocytes. The use of acelysin and nimotop leads to decrease in CASP3 expression in hippocampal neurons on the 4th day by 3.82 and 1.54 times compared to control group, and on the 7th day by 4.00 and 1.84 times, respectively, which reflects the positive effect of the therapy on the prevention of apoptotic death of hippocampal neurons. Compared with nimotop, the use of acelicine significantly increases the expression of NeuN on day 4 and 7 – by 1.84 and 1.95 times, respectively, which indicates a more pronounced neuroprotective effect of acelysin on hippocampal neurons in SAH. Increased GFAP expression reflects the dynamic reactive remodeling of astrocytes, while the use of acelysin and nimotop does not affect the reduction of GFAP levels on days 4 and 7 of SAH and their return to baseline values. The results of the present experimental study provide a theoretical justification of the feasibility of including metabolitotropic drugs in the basic therapy of SAH.

Key words: subarachnoid hemorrhage, cerebral aneurysm rupture, GFAP, NeuN, CASP3.

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

Vплив ацелізину та німотопу на кількінну відповідь гіпокампа в динаміці експериментального субаракноїдального крововиливу

О. Ю. Полковніков, С. І. Тертишний

Мета роботи – імуногістохімічне дослідження нейронів та астроцитів гіпокампа щурок у різні терміни експериментального крововиливу в умовах застосування ацелізину та німотопу.

Матеріали та методи. Субаракноїдальний крововилив (САК) моделювали на 35 щурах лінії Вістар, яких поділили на 7 груп. Тварини 1 і 2 груп протягом 4 і 7 діб відповідно отримували ацелізин у дозі 15 мг/кг один раз на добу; тварини 3 і 4 груп упродовж 4 і 7 діб одержували німотоп у дозі 0,3 мг/кг кожні 8 годин. У 5 і 6 групах (контрольних) моделювали САК без лікування, терміни спостереження – 4 і 7 діб відповідно. Інтактні тварини сформували 7 групу. Модель САК відтворена з урахуванням методики R. V. Dudhani et al. У гістологічних зразках поля CA1 гіпокамма за допомогою програми ImageJ оцінювали відносну площу імуногістохімічної експресії CASP3 та NeuN у нейронах, GFAP в астроцитах тварин усіх груп.

Результати. Експресія CASP3 у нейронах гіпокампа зростає у 2,3 та 5,7 раза на 4 добу, в 1,8 та 3,9 раза – на 7 добу експериментального САК (групи 1–4) щодо показника інтактної групи. Експресія GFAP зустрічається у сім групах спостереження щодо параметрів інтактних тварин, максимальні показники зафіксовано в 5 групі – збільшення експресії в 8,14 раза. Динаміка експресії NeuN в нейронах гіпокампа при використанні ацелізину та німотопу відповідає зворотній динаміці експресії CASP3. Експресія NeuN максимально підвищується на 7 добу використання ацелізину і становить 91,76 % від показника інтактної групи.

Висновки. Розвиток САК в експерименті спроводжується зміною експресії CASP3, NeuN у нейронах і GFAP в астроцитах. Застосування ацелізину та німотопу сприяє зниженню експресії CASP3 у нейронах гіпокампа на 4 добу в 3,82 та 1,54 рази порівняно з контрольною групою, на 7 добу – в 4,00 та 1,84 раза відповідно. Це відображає позитивний вплив призначення терапії щодо запобігання апоптотичного виклинання нейронів гіпокампа. Ацелізин порівняно з німотопом підвищує експресію NeuN на 4 і 7 дні – в 1,84 та 1,95 раза відповідно. Це свідчить про більш виражений нейропротекторний вплив ацелізину порівняно з німотопом.

Ключові слова: САК, розрив церебральної аневризми, GFAP, NeuN, CASP3.

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Rupture of cerebral arterial aneurysms in the majority of cases manifests by subarachnoid hemorrhage (SAH) of varying severity. The latter is complicated by the formation of parenchymal hematoma in 39.8 % and intraventricular hemorrhage in 44.3 % of cases [1].

The early complications of SAH at the first 72 hours are represented by the early brain injury, while during next 4–30 days delayed complications develop in form of delayed brain injury. Aneurysm rupture triggers pathophysiological processes that lead to brain damage and impairment of the latter’s function. Neuronal death and damage to the endothelium leads to cytotoxic edema and destruction of the blood-brain barrier, followed by the development of vasogenic edema [2]. In addition, cell death is caused by insufficiency of microcirculation, microthrombosis, changes in ion homeostasis, excitotoxicity, oxidative stress, and neuronal edema [3]. Historically, it was believed that delayed cerebral ischemia (DCI) is a frequent complication of SAH and is one of the main causes of poor functional outcome. It was supposed that DCI is caused by cerebral vasospasm, but recent clinical trials failed to confirm this hypothesis. Since then, human and animal studies have evidenced the multifactorial pathophysiology of DCI [4].

In order to study the molecular mechanisms of SAH, as well as the action of potential neuroprotectors, the expression and synthesis of proteins that regulate the cell cycle and modulate compensatory cellular reactions during ischemia are currently being actively studied. Among these proteins, caspases play a special role being the main companions of cellular apoptosis / necrosis as well as strong instigators of the pro-inflammatory response [5]. GFAP (glial fibrillary acidic protein), the marker of damage and reactive responses of astrocytes [6], as well as NeuN – loss of the immunoreactivity is indicative for neuronal damage.

Currently, both surgical and therapeutic methods are used to reduce the complications of aneurysmal subarachnoid hemorrhage, in particular delayed cerebral ischemia. According to previous studies, nimodipine treatment of aneurysmal subarachnoid hemorrhage reduces secondary ischemia and shows a favorable trend in mortality, and although nimodipine is a component of the most current guidelines for the drug therapy of SAH, there is no reliable confirmation of its effectiveness [7].

The search for pharmacological agents which can reduce complications of cerebral aneurysm rupture continues. One of such agents is aspirin. Regular use of aspirin can positively affect the risk of DCI and the outcomes of patients with SAH, without increasing the risk of clinically significant bleeding [8]. The injectable form of acetylsalicylic acid is acelysin, which properties increase its bioavailability and analgesic effect. The drug penetrates most body tissues, including synovial, cerebrospinal, and peritoneal fluids. It is hydrolyzed in the blood, forming acetylsalicylic acid and lysine. Lysine, in turn, is able to transform into pipicolic acid and increase the affinity of GABA receptors, which leads to a decrease in anxiety and fear and providing anxiolytic effect. In addition, L-lysine reduces NMDA hyperexcitability, excitotoxicity, and preserves the viability of neurons in the hippocampus and sensorimotor cortex [9].

Thus, nimodipine and acelysin can be considered important elements of drug therapy of subarachnoid hemorrhage, necessary for the prevention of secondary complications of a cerebral aneurysm rupture. Despite some positive experience of using these drugs in the treatment of subarachnoid hemorrhages [10,11,12], their influence on the mechanisms of brain cellular damage, reactivity and death still remains insufficiently studied.

**Aim**

Immunohistochemical study of rat hippocampal neurons and astrocytes at different time-points of experimental brain hemorrhage treated with acelysin and nimotop.

**Materials and methods**

The experimental study was performed using 35 Wistar rats of both sexes weighing 170–230 g (10–12 weeks old), which underwent preliminary acclimatization for 14 days.

Care, maintenance and feeding of animals was carried out in standard conditions of a stable microclimate of the educational and scientific medical and laboratory center of Zaporizhzhia State Medical and Pharmaceutical University under conditions of a 12-hour daylight. Animals were fed by standardized ration feed “Rezon-1” KP-120-1 with free access to food and water and exclusion of stress factors. Rats were housed in standard conventional polycarbonate cages (Tecniplast S.p.A., Italy) measuring 610 × 435 × 215 mm or 335 × 235 × 190 mm, 5 animals each.

Animals were divided into 7 experimental groups: animals receiving acelysin in a standard therapeutic dose (15 mg/kg once a day), animals receiving nimodipine in a standard therapeutic dose (0.3 mg/kg every 8 hours (8:00 a.m., 4:00 p.m. and 12:00 p.m.), two control and one intact group:

- Group 1 – SAH treated with acelysin for 4 days (n = 5);
- Group 2 – SAH treated with acelysin for 7 days (n = 5);
- Group 3 – SAH treated with nimotop for 4 days (n = 5);
- Group 4 – SAH treated with nimotop for 7 days (n = 5);
- Group 5 (control) – SAH untreated, 4 days (n = 5);
- Group 6 (control) – SAH untreated, 7 days (n = 5);
- Group 7 – intact animals (n = 5).

Only healthy animals were included in the study. Before the beginning of the study, the commission on bioethics of the Zaporizhzhia State Medical and Pharmaceutical University checked and approved the study protocol, as well as all procedures related to the maintenance of animals, their humane treatment and their use in the experiment. All procedures were conducted according to...
the European convention for the protection of vertebrate animals (Strasbourg, 18 March 1986; ETS No. 123) and the Directive 2010/63/EU.

Experiment design: The SAH was modeled according to R. V. Dudhani et al. [13], but without aspiration of cisternal cerebrospinal fluid and with a single injection of autoblood. Anesthesia was performed by slow intravenous administration of sodium thiopental 40 mg/kg through the tail vein using a 26 G needle. Rats were prewarmed under heat lamp for 5 minutes to dilate the tail vein before sodium thiopental administration. Surgical intervention was run under aseptic conditions on a heated table (37 °C) to maintain body temperature during the procedure. For disinfection, the tail and suboccipital puncture sites were treated with chlorhexidine in 70 % ethanol. Blood aspirated by incision of the tail vein in a volume of 0.20 ml was drawn into a syringe with heparin immediately before injection. A puncture of the large occipital cistern was performed, after which 0.20 ml of collected autologous blood was injected. SAH as a manifestation of hemorrhagic stroke developed immediately after blood injection into the subarachnoid space. After the operation, a 6 % glucose solution was given to the drinker next to the water bowl. Food was also placed on the bottom of the cage to facilitate its intake. Nimodipine was administered intraperitoneally at a dose of 0.3 mg/kg every 8 hours (8:00 a. m., 4:00 p. m. and 12:00 p. m.), acelysin 15 mg/kg once a day at 10.00 a. m. Animals were withdrawn from the experiment on days 4th and 7th.

The cerebral hemispheres were fixed in a 10 % neutral formalin and dehydrated in a battery of ethyl alcohol. For general histopathology, sections were stained with hematoxylin and eosin. Paraffin sections of 3 μm thickness were used for immunohistochemical studies. De-paraffinization, rehydration, and antigen unmasking were performed by heating in a citrate buffer of pH 6.0 using the PT Thermo Fisher Scientific module. Endogenous peroxidase was blocked with a 3 % hydrogen peroxide. After conditioning with Ultra V Block, incubation with antibodies was carried out in accordance with the instructions of the antibody manufacturer. Visualization was performed using the UltraVision Quanto HRP + DAB system with additional antibody manufacturer. Visualization was performed using the software Statistica for Windows 13 (StatSoft Inc., No. JPZ804382130ARCN10-J). Obtained data were expressed as median (Me), lower and upper quartiles (Q1; Q3). Comparison between 3 groups or more was performed using one-way Kruskal–Wallis analysis of variance. The differences between the compared values were considered statistically significant at the level of 95 % (p < 0.05).

Results

The subarachnoid hemorrhage led to pronounced structural changes in the neuronal population of the hippocampus. Focal or total hyperchromatosis was noted in a significant part of neurons (Fig. 1). The neurons were of different sizes, there were “shadow cells” and areas of neuronal loss, which disrupted the orderly cellular architecture of the hippocampus. In preserved individual neurons, condensation of chromat in and different sized nucleoli were noted, as well as nuclear and cytoplasmic vacuolation.

Modeling of subarachnoid hemorrhage led to statistically significant changes in the GFAP, NeuN and CASP3 expression. The dynamics of the protein’s expression was different depending on the experimental group and terms. Minimal cytoplasmic expression of CASP3 in neurons was noted in the intact group, which was comparable to the background staining (Fig. 2). SAH led to a significant increase in CASP3 levels in neurons of control groups 5 and 6 – respectively 8.8 and 7.2 times higher than in intact animals (Fig. 3).

After treatment by acelysin and nimotop on the 4th day of the experiment, the expression of CASP3 in neurons was increased compared to intact group by 2.3 and 5.7 times, respectively. On the 7th day, these indicators were 1.8 and 3.9 times, respectively, which reflected the beneficial effect of these drugs in terms of prevention against apoptotic neuronal death. The antiapoptotic effect of acelysin was expressed to a greater degree compared to nimotop. Thus, in the 1st and 2nd groups, CASP3 expression in neurons was smaller by 59.64 % and 53.84 %, respectively, compared to the 3rd and 4th observed groups. Uneven expression of CASP3 in the neurons of the affected hippocampal zones could be explained by the regionally different tolerance of the neurons to ischemia.

GFAP expression in astrocytes showed increase in all observation groups compared to the intact group, where expression was equal to 1.4 % (Fig. 4). In the 1st and 2nd groups, the expression was increased by 4.57 and 4.71 times, with a statistically unreliable difference between these two periods. In the 3rd and 4th groups, GFAP expression was increased by 6.21 and 4.71 times, respectively, with a statistically significant difference between the two observation periods. In control groups, on the 4th and 7th day the expression was increased by 8.14 and 5.85 times, respectively, in relation to intact group. Thus, the most pronounced expression of GFAP was noted in the control 5th group on the day 4. In these observations, the layer of pyramidal neurons was sur-
rounded by a dense network of astrocyte bodies and their processes (Fig. 5).

NeuN immunoreactivity in neurons was mainly nuclear and to a lesser extent was detected in the perinuclear cytoplasm. In control observations, the NeuN expression was minimal: 1.7 % and 0.8 % on the 4th and 7th day, respectively, which indicated the growth of delayed cerebral ischemia in the indicated periods. The dynamics of NeuN expression in neurons when using acelysin and nimotop indirectly corresponded to the dynamics of CASP3 expression in these cells. Thus, the increase in neuronal NeuN was associated with decrease in their CASP3 expression. This supports the mention of protective properties of acelysin and nimotop in terms of reducing the apoptotic neuronal death. Quantitative indicators of the expression of the studied markers are provided in Table 1 and Fig. 6.

Table 1. Levels of CASP3 and NeuN in neurons and GFAP in astrocytes in the hippocampus of rats with SAH on the 4th and 7th day and in intact group

<table>
<thead>
<tr>
<th>Groups</th>
<th>CASP3</th>
<th>GFAP</th>
<th>NeuN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1, SAH + acelysin,</td>
<td>2.3(2.0; 2.4)*</td>
<td>6.4 (5.7; 7.7)*</td>
<td>4.2 (3.9; 4.5)*</td>
</tr>
<tr>
<td>day 4, n = 5</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Group 2, SAH + acelysin,</td>
<td>1.8 (1.5; 2.2)</td>
<td>6.6 (6.1; 7.2)*</td>
<td>7.8 (7.7; 8.3)**</td>
</tr>
<tr>
<td>day 7, n = 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 3, SAH + nimotop</td>
<td>5.7 (5.0; 6.5)**</td>
<td>8.7 (8.0; 9.2)*</td>
<td>2.5 (1.2; 2.9)*</td>
</tr>
<tr>
<td>day 4, n = 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 4, SAH + nimotop</td>
<td>3.9 (3.6; 4.2)**</td>
<td>10.4 (10.0; 10.7)**</td>
<td>4.0 (3.5; 4.3)**</td>
</tr>
<tr>
<td>day 7, n = 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 5, control,</td>
<td>8.8 (8.1; 9.4)</td>
<td>11.4 (10.5; 12.1)</td>
<td>1.7 (1.3; 2.0)</td>
</tr>
<tr>
<td>day 4, n = 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 6, control;</td>
<td>7.2 (7.0; 7.7)</td>
<td>8.2 (7.9; 8.7)</td>
<td>0.8 (0.7; 1.1)</td>
</tr>
<tr>
<td>day 7, n = 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact, n = 5</td>
<td>1.0 (0.7; 1.2)</td>
<td>1.4 (0.9; 1.7)</td>
<td>8.5 (7.7; 8.9)</td>
</tr>
</tbody>
</table>

*: p < 0.05 compared to the control; **: p < 0.05 compared to 4 days SAH animals.

Fig. 1. Hyperchromatosis of hippocampal neurons on the 4th day of experimental SAH. Hematoxylin and eosin staining. Mag. ×400.

Fig. 2. Hippocampal expression of caspase 3 in the intact rat. Mo Monoclonal Antibody Anti-Caspase 3 p17 (D-12) sc-373730. Mag. ×100.

Fig. 3. Hippocampal expression of caspase 3 in control rat (group 5, day 4). Mo Monoclonal Antibody Anti-Caspase 3 p17 (D-12) sc-373730. Mag ×100.

Fig. 4. Hippocampal expression of GFAP in the intact rat. Mo Monoclonal Antibody Anti-GFAP (2E1) sc-33673. Mag. ×100.

Fig. 5. Hippocampal expression of GFAP in control rat (group 5, day 4). Mo Monoclonal Antibody Anti-GFAP (2E1) sc-33673. Mag. ×100.
Discussion

We obtained the data regarding the CASP3 and NeuN expression in neurons and GFAP in astrocytes demonstrate predictable changes in the hippocampus cell populations in response to subarachnoid hemorrhage and the associated ischemic damage. The multidirectional expression dynamics of individual markers recorded on the experimental days 4th and 7th, to some extent reflect the processes of intensification of both neurodegeneration and neuroplasticity, as well as the influence of the studied drugs. It is known that in the acute period of ischemia, the processes of cellular death are started in the form of necrosis/apoptosis with subsequent hyperexpression of proapoptotic proteins, including caspases. Increased expression of caspase, which also takes part in postischemic activation of neuroplasticity, gradually decreases during the subacute period. Increased caspase expression associated with ischemic damage has been described both in experimental studies [14] and in patients with stroke [15]. However, according to several authors, cell apoptosis can play an important role in brain damage after SAH [16]. The decline in neuronal CASP3 under action of acelysin and nimotop indicates a decrease in the processes of apoptosis when using pharmacological treatment. Herewith, the most pronounced decrease in CASP3 was observed 7 days after the use of acelysin, which supports the possibility of effective use of this drug to alleviate ischemic brain trauma after SAH. In the same period (7 days), the maximum expression of NeuN in neurons indicates the minimally expressed processes of cell death in this period under the conditions of acelysin use. It is believed that neuronal NeuN acts as a protein synthesis and protein expression modulator during cellular response to ischemia.

The effectiveness of the aspirin use in SAH is controversial, which follows from numerous scientific studies devoted to this issue [17,18,19]. Some data indicate that short-term (up to 3 months) aspirin use is associated with an increased risk of aneurysmal SAH [20]. Another study emphasizes that long-term aspirin use is not associated with mortality or complications after SAH [21]. Although the exact effect of antplatelet agents on the outcome after aneurysmal SAH is not sufficiently elucidated, there is still an assumption that platelet adhesion inhibitors reduce the risk of delayed cerebral ischemia and, thus, have a beneficial effect on clinical outcomes [22].

Upregulation of GFAP is widely considered as indicative sign for reactive state and morphological remodeling of astroglia in response to different neuropathologies with ischemic and complex mechanisms [23]. Significant increase in GFAP expression in the acute period of SAH also points to reactive astroglial remodeling. Literature data show that the level of GFAP significantly increases in the blood of patients with SAH during the development of hemorrhage and 24 hours after it. Elevated blood GFAP levels at hospital admission predicted mortality and poor outcome in patients with SAH [24,25]. Experimental studies evidenced, that GFAP levels in astrocytes increased after SAH, with subsequent recovery to the initial level [26].

The role of astrocytes in the recovery after SAH remains controversial, since reactive astrocytes are classified into at least 2 types: neurotoxic A1 and neuroprotective A2 [27]. A1 astrocytes induce cell death by releasing proinflammatory factors after SAH. Acelysin and nimotop increased GFAP expression on day 4 and day 7, respectively, but it is not known which type of reactive astrocytes prevailed during experimental SAH. This question requires the use of specific markers for A1 and A2 subtype astrocytes, respectively.

Apparently, the anti-apoptotic effect of L-lysine and its derivatives is realized through an increase in the expression of HSP70 in the cytosol and mitochondria of neurons of the sensorimotor cortex and hippocampus. HSP70 blocks the Fas/Apo-1 apoptosis trigger receptor and also inhibits apoptosis in mitochondria. HSP70 acts in the step between cytochrome c release and procaspase-9 cleavage [28].

Our results to some extent reflect the molecular mechanisms of neurodestruction in SAH at different time-points of the modelled pathology, as well as represent an experimental justification for the use of nimodipine and acelysin as effective neuroprotectors in SAH.
Conclusions

1. Experimental SAH is accompanied by the abnormal expression of CASP3 and NeuN in neurons and GFAP in astrocytes. The expression depends on the duration of the reaction to the therapy on the prevention of apopotic death of hippocampal neurons.

2. The use of acetyliln and nimodipine does not affect the decrease in CASP3 expression in neurons on the 4th day by 1.84 and 1.95 times on day 7 and 1.54 times compared to the control group, and on the 7th day by 4.00 and 1.84 times, respectively, which indicates a more pronounced neuroprotective effect of acelysin on hippocampal neurons.

3. Increased GFAP expression in astrocytes during SAH reflects the reactivity of astrocytes, which can have a negative and a positive effect on the recovery due to different functionality of reactive astroglia. The use of acelysin and nimodipine does not affect the decrease in GFAP expression in astrocytes on the 4th and 7th day of SAH and its return to baseline values.

4. Experimental SAH is accompanied by the abnormal expression of neutrophil nuclei antigen NeuN is inversely related to the dynamics of CASP3 expression in neurons. An increase in CASP3 expression in neurons is accompanied by the disappearance of the nuclear protein from the positive effect of the therapy on the prevention of apopotic death of hippocampal neurons.

5. The results of the experimental study represent a theoretical justification for the feasibility of including immunodipeptide and acelysin in the basic therapy of SAH.

Conflicts of interest: authors have no conflict of interest to declare.

Original research

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