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EVALUATION OF THE CEREBROPROTECTIVE ACTION OF 1-ADAMANTYLOXY-3-MORPHOLINO-2-PROPANOL HYDROCHLORIDE IN RATS WITH EXPERIMENTAL HEMORRHAGIC STROKE ACCORDING TO THE DYNAMICS OF NEUROCYTOLYSIS, NEUROAPOPTOSIS AND NEUROPROLIFERATION MARKERS

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Acute cerebrovascular accident (CVA) is an important medical and social problem due to its high frequency, severe consequences and treatment complexity. Cerebral stroke affects 33 million people worldwide every year [1]. In terms of the development severity, the course and consequences of cerebral circulation disorders the most dramatic is a hemorrhagic stroke (HS). At that about 30-35% of the affected people die in the first 30 days and half of them in the first 2 days [2-4]. A significant role is played the compression of the surrounding brain matter accompanied by a decrease in cerebral circulation and the development of secondary ischemic damage. Ischemia around the hemorrhage zone triggers the typical pathobiochemical cascades in the neurons: changes in the glutamate and calcium metabolism, free radical reactions, lipid peroxidation, excessive formation of nitric oxide, activation of astro- and microglial cell pools and associated immune shifts. Besides, the situation is aggravated by the accumulation of catalytically active iron which intensifies the lipid peroxidation processes and formation of the free radicals. One of the possible options for such patients management is the earliest possible surgical intervention, however there are the number of limitations for a surgical removal of hematomas. Unfortunately, now there is no specific drug treatments for intracerebral hemorrage (ICH). That is why the search, development and practical application of the drugs with proven neuroprotective activity is a correct approach. A promising drug is 1-adamantyloxy-3-morpholino-2-propanol hydrochloride (ademol). The neuroprotective action of ademol was established on model analogs of ischemic stroke clinical forms, transient ischemic attacks and traumatic brain injury. This action is associated with a modulating effect on the NMDA receptors activity, stimulation of cerebral blood supply, elimination of energy deficiency, metabolic acidosis, and oxidative damage of neurons by nitric oxide metabolism correction, preservation of cytoarchitectonics of the cerebral cortex, including apoptosis reduction [5]. The efficacy of ademol in hemorrhagic strokes has not been previously studied. Our preliminary works have proven the ability of ademol to reduce the lethality of animals with model HS, improve a cerebral blood supply, and to reduce neurologic deficit in the ICH [6]. Therefore there is every reason to hope for the cytoprotective properties of with ICH conditions.

It is known that ICH is accompanied by significant destructive changes in the brain cytoarchitectonics [7-9]. This is reflected in rapid growth of NSE activity. Equally important is an evaluation of the drugs cerebroprotective effect on the modulation of neuron elimination types and the possibility for transformation of neurodestruction types, programmed (apoptosis) and spontaneous (necrosis). Nervous tissue is a highly specialized system formed by neurons, the cells with the maximum level of morphological differentiation.

Neurons are unable to divide and have low reparative potential. The only cellular element that is periodically restored is the neurogliocyte which is assigned to the neurogliocyte pool; neurogliocytes replace neurons died as a result of necrosis. Therefore the activity of neuroglia indirectly indicates the level and scale of

neurodestruction. This process can be traced back to the level of protein S100 protein which is expressed by the neuroglia.

Aim. To elucidate the presence and magnitude of ademol cerebroprotective effect in rats with experimental HS by the dynamics of neurocytolysis, neuroapoptosis and neuroproliferation markers.

Materials and methods. For the experiment, 196 Wistar male rats, 6-7 weeks of age, weighing between 160 and 180 g, were purchased from vivarium of National Pirogov Memorial Medical University, Vinnytsya, Ukraine. Throughout the experiment, animals stayed under standard environmental conditions (temperature: 22.0 ± 2.0°C, relative humidity: 54–59%, and 12 h light/12 h dark cycle) with one-week adaptation before experiment. They were housed in cages made of polypropylene and had free access to feed and water ad libitum. The Ethical Committee of National Pirogov Memorial Medical University (protocol №3 from 25.05.2020) approved all protocols for animal experiment.

Evaluation of the protective properties of an industrial sample of 1.0% ampoule solution of 1-adamantiloxy-3-morpholino-2-propanol hydrochloride (Ademol-Darnitsa, Darnitsa, Ukraine) with an effective dose of 2 mg/kg was carried out under the conditions of experimental HS – intracerebral hemorrhage. ICH of moderate severity was modeled by autoblood injection (20 μl/100 g) into the brain internal capsule (stereotaxic coordinates of the projection H = 7.0 mm, L = 3.0 mm, a = 1.5 mm from bregma) [10].

Nimodipine (Nimotop-Bayer, Bayer AG, Germany), dose of 30 mg/kg, amantadine sulfate (PK-Merz, Merz Pharma, Germany), 10 mg/kg (i/v), and magnesium sulfate (Magnesium- sulfate Darnitsa, Darnitsa, Ukraine), 250 mg/kg were used as reference drugs. Treatment began 1 hour after the simulation of pathological condition, all drugs have been administered 2 times a day intravenously in a pre-catheterized (intravenous catheter UNOFLON 22 G, India) femoral vein with infusion system B. Braun McGaw (Germany) for 4 hours. The animal was in a free position in a special pen chamber (BIOPAK, USA) during infusion. If nimodipine is used the infusion system has been darkened according to the manufacturer's instruction. Animals of the control pathology group and falsely operated animals received a normal saline (2 ml/kg of body weight).

The NSE content (EC 4.2.1.11) and serum protein S100 level were determined by the immunoenzyme method using NSE ELISA KIT (DAI, USA), S100 ELISA KIT (Fujirebio Diagnostics Inc., Sweden). The serum was obtained from blood taken by direct venipuncture from the central femoral vein and centrifuged at 1600 g, 10 min, 19-21°C. The serum was transferred to Eppendorf tubes and stored at – 25°C until the study onset with the device of Hipson company (Czech Republic).

At the 4th day (96 hours) after ICH reproduction, the brain was removed from the cranium for flow cytometric analysis, a bilateral lobectomy was performed, and the cortex was removed for further study. A suspension was prepared from this biomaterial with the addition of original CyStain DNA reagents (Partec, Germany) on a Partec flow cytometer (Germany). S Phase has been evaluated, the percentage of the DNA synthesis phase (DNA content > 2 c and < 4 c) to all cells of the cell cycle, and the SUB-G0G1 period – an indicator of nuclear DNA fragmentation (cell nuclei with DNA content < 2 c).

All the results are expressed as mean ± standard error of mean (SEM). All data were analyzed statistically by one-way ANOVA followed by Dunnett's *t*-test. Pairwise comparison of means among the groups was analyzed by one-way ANOVA followed by post hoc Tukey's HSD test. *P* values <0.05 was considered as significant.

Results. Under ICH conditions, the study of neuromarker activity in rats showed that 96 h (4th day) after modeling the pathology, the level of NSE activity probably increased relative to that of in pseudo-operaged animals by 18.1 times while at the end of experiment (21st day) day) NSE activity continued to be increased by

an average of 7 times (Table 1). The significant increase of NSE activity in the acute period of ICH is mainly due to the neurons destruction because of direct effect of ischemic factor on the intracellular metabolism. In the later period of acute CVA when adaptive and reparative processes are activated, the enolase content gradually decreases but does not decrease to the normal values. Our results regarding the nature of enolase level fluctuations in different periods of HS coincide with the literature data [11,12].

Such negative dynamics of NSE activity in conditions of the acute CVA according to hemorrhagic type indicates not only a significant size of the ischemic focus, but allows to give an unfavorable prognosis with a certain probability (lethal outcome, significant deterioration of cognitive functions, loss of adaptive abilities, etc.)

Table 1. Effect of ademol and reference drugs on the dynamics of NSE level (ng/ml) in the blood serum of the ICH rats femoral vein (M±m, n=15)

Animal groups	Term, days	
	4	21
Sham-operated animals + 0.9% NaCl, 2 ml/kg	0.156±0.008	0.134±0.012*
ICH+ 0.9% NaCl, 2 ml/kg (control pathology)	2.824±0.046°	0.939±0.026°[4]
ICH **+** ademol 2 mg/kg,	1.071±0.016°*#&	0.267±0.014°*#&[4]
ICH+ nimodipine, 30 μg/kg	1.154±0.046°*#	0.296±0.016°*#[4]
ICH + amantadine sulfate, 10 mg/kg	1.256±0.059°*#	0.308±0.007°*#[4]
ICH+ magnesium sulfate, 250 mg/kg	1.898±0.087°*	0.581±0.013°*[4]

Notes: ICH – intracerebral hemorrhage;
° – p<0,05 in relation to sham-operated animals;
* – p<0,05 in relation to the control pathology;
[#] – p<0,05 in relation to magnesium sulfate (250 мг/кг);
[&] – p<0,05 in relation to amantadine sulfate (10 мг/кг);
[4] – p<0,05 in relation to 4th day in respective group.

Therapeutic intravenous administration of ademol (conditional effective dose of 2 mg/kg) to animals with acute CVA was accompanied, as with nimodipine and amantadine sulfate, by a less intense increase in NSE activity: in 96 hours the enzyme level decreased in relation to the control group in 2.63, 2.45 and 2.25 times, respectively, and after 21 days in 3.52, 3.17 and 3.05 times (p <0.05). This effect of the studied drugs may indicate that they have a cytoprotective effect. The efficacy of ademol reliably prevails the amantadine and magnesium sulfate, on average, 1.17 and 1.77 times, respectively (4 days) and 1.15 and 2.18 times (21st day) in conditions of acute cerebral ischemia in terms of the ability to reduce the level of neurodegradation marker both in the acute and in the recovery period of acute CVA, and is comparable to the effect of nimodipine in the indicated periods. It is very important to study the prevalence of some processes of cell death over other ones (necrobiosis, apoptosis) and to assess the contribution of each process to the formation of ischemic focus in HS. The answer to this question have a practical value for the development of new pathogenetic stroke therapy. Equally important is the evaluation of cerebroprotective drug effect on modulation of neuronal

elimination types and the possibility of transformation of programmed (apoptosis) and spontaneous (necrosis) types of neurodestruction. It is known that neuronecrosis (neurocytolysis) is an avalanche-like chaotic process of cell destruction which is accompanied by an inflammatory response during the recovery period. On the contrary, cell death by apoptosis is not accompanied by a violation of the neurons membrane unity, therefore neuron apoptosis has quantitative and qualitative advantages over oncosis and is the "lesser evil" for CNS. Along with this according to some researchers [13, 14], cells apoptosis with stroke prevail over necrotic process and apoptotic tendencies have a negative connotation due to the fact that the latter can induce factors of delayed excitotoxic damage of neurons. That's why a leading direction of neuroprotection can be considered the development and clinical use of anti-apoptotic agents [15,16]. However it is known that if irreversibly damaged neurons cannot be eliminated by apoptosis, this triggers necrosis mechanisms and is undesirable event since the ischemic zone expands due to the attachment of additional cells to the penumbra focus [15,17, 18]. Thus, we believe that the apoptotic type of programmed cell death pathways prevention in ICH is an evolutionarily outdated direction of cerebroprotection. The introduction of agents that simultaneously inhibit all possible pathways of cell death (that is, both apoptosis and necrobiosis) is a promising way in this direction.

In view with the above, the next stage was a study of the antiapoptotic effect of ademol in ICH conditions (Table 2).

Table 2. Effect of ademol and nimodipine on DNA fragmentation in the nuclei of neurons in brain frontal lobes of the cerebral cortex of rats with ICH after 96 hours of therapy (M±m, n=8)

Experimental conditions	DNA fragmentation, %	DNA synthesis (S phase, %)
Sham-operated animals + 0.9% NaCl, 2 ml/kg	7.96±0.35	0.131±0.009
ICH + 0,9% NaCl, 2 ml/kg (control pathology)	14.58±0.61°	1.610±0.048°
ICH **+** ademol, ml/kg,	10.02±0.40°*	0.538±0.025°*^{a}
ICH + nimodipine, 30 μg/kg	11.32±0.44°*	0.906±0.027°*

Notes: ICH – intracerebral hemorrhage;
° – p<0,05 in relation to sham-operated animals;
* – p<0,05 in relation to the control pathology;
a – p<0,05 in relation to nimodipine.

The intensity of DNA fragmentation in the neurons nuclei in the frontal lobes of cerebral cortex significantly increased in animals of the control pathology group, on the average by 1.83 times; this indicates the process of intensive formation of the focus of ischemic penumbra (penumbra) to be precisely occurred due to neurons in a state of apoptotic death.

Therapeutic administration of ademol, like nimodipine to rats with HS had a modulating effect on the neuroapoptosis processes similar in direction and strength; this was indicated by a significant decrease in DNA fragmentation in the neurons nuclei in the frontal lobes of cerebral cortex in the study period by an average of 30, 0, and 22.34%, respectively (Table 2). At the same time, the antiapoptotic effect of ademol exceeded the reference drug by an average of 13.0%, p <0.05. Obviously,

the apoptosis inhibition may indicate about reduction in the penumbra zone due to an increase in the number of functionally active neurons

Thus, the studied adamantane derivative suppresses not only the processes of neurocytolysis under ICH conditions, but also the neuroapoptosis phenomena which may be one of the mechanisms of cerebroprotective action.

The formation of a neurodestructive focus in the HS brain is a dynamic process that is formed from phenomena which at the first glance, are inherently opposite ones. So at the same time, destruction of neuronal membranes (neurocytolysis) occurs, and in response the parallel neuroproliferative changes are initiated due to the separation of glial elements. At the next stage we studied the activity of neuroproliferative processes in the brain of rats with ICH. The S100 protein level was used as a neuroproliferation marker.

The results showed the high levels of the S100 protein in blood serum along with high enolase activity that in the rats of the control pathology group with ICH of moderate severity is statistically exceeded S100 content in pseudo operated animals averaged 42.2 times, these facts confirm the neuroproliferation onset (Table 3).

Table 3. Effect of Ademol and reference drugs on the level of S100 protein in blood serum from the femoral vein of rats with ICH during the acute stroke period (96 hours of experiment - 4th day), M±m, n=7

Animal groups	Level of S100 protein, ng/ml
Sham-operated animals + 0.9% NaCl, 2 ml/kg	0.239±0.016
ICH + 0.9% NaCl, 2 ml/kg (control pathology group)	10.091±0.185 ^{o}
ICH **+** ademol 2 ml/kg,	3.516±0.081 $^{o*\#\&a}$
ICH +nimodipine, 30 mcg⁄kg	4.757±0.20 $^{o*\#\&}$
ICH + amantadine sulfate 10 mg⁄kg	5.763±0.158 $^{o*\#}$
ICH + magnesium sulfate, 250 mg⁄kg	8.08±0.154 ^{o*}

Notes: ICH – intracerebral hemorrhage;
^{o} – p<0,05 in relation to sham-operated animals;
* – p<0,05 in relation to control animals;
$^{\#}$ – p<0,05 in relation to magnesium sulfate (250 mg/kg);
$^{\&}$ – p<0,05 in relation to amantadine sulfate (10 mg/kg);
a – p<0,05 in relation to nimodipine.

The therapy with the mentioned neuroprotective agents inhibited the increase in the S100 protein level. Ademol had the greatest depressing effect. Against the background of its infusion, the serum S100 protein level was lower in relation to the control pathology by an average of 2.87 times, p <0.05. In terms of its ability to inhibit neuroproliferation it significantly exceeded nimodipine by 1.35, amantadine sulfate by 1.64, and magnesium sulfate by 2.3 times (Table 3).

Flow cytometric analysis makes it possible a quantification of the ischemic focus parameters which is no longer represented by destructively altered neurons, but by neurogliocytes with the onset of recovery period. When conducting flow cytometric analysis the increased activity of neurogliocytes is evidenced by the appearance of

nuclei in the synthesis phase (phase S of the cell cycle). In the animals of the control pathology group, the number of dividing cells with DNA in the suspension obtained from the frontal lobes of the cerebral cortex significantly increased, on average 12.3 times as compared to the same values in the group of pseudo operated animals. The obtained data may indicate the activity of the processes of ischemic penumbra focus formation already at the end of the acute period. (4th day of ICH) (see Table 2).

Ademol therapy had a depressing effect on the structure of the ischemic focus, primarily by reducing of irreversibly damaged neurons quantity. So, against the background of the ademol administration, the content of nuclei with DNA in the S phase turned out to be lower in relation to similar indicator in control pathology group, or in animals that received nimodipine therapy, on average, 3.0 and 1.68 times, respectively, $p < 0.05$. Low activity of neuroglia against the background of ICH therapy with ademol solution confirms its cerebroprotective activity as a result of which neuronecrosis is significantly reduced and the morpho-structural integrity of the brain is preserved. Ademol is significantly exceeded nimodipine in cytoprotective action. Thus, the studied adamantane derivative under ICH conditions promotes the suppression of neurocytolysis processes and associated neuroproliferation, and this function is one of the cellular mechanisms of its cerebroprotective action.

Conclusion and future prospects.

1. Under experimental ICH conditions, ademol at the dose of 2 mg/kg exhibited neurocytoprotective activity which is confirmed by the dynamics of neurodestruction and neuroproliferation biomarkers, namely the decrease in NSE activity, and the decrease in the level of S100 protein.

2. Therapeutic i/v administration of ademol at the dose of 2 mg/kg, and nimodipine (30 μg/kg, i/v) resulted in the positive modulating effect on the processes of neuroapoptosis in the cerebral cortex of rats with ICH. The ability of ademol to reduce the percentage of apoptotic neurons in the cerebral cortex of rats with ICH may be one of the mechanisms of its cerebroprotective action. Taking into account the pronounced anticytolytic and antiapoptotic ademol properties under ICH it can be classified as a primary neuroprotector which makes the drug promising for the pathogenetically directed interruption of early mechanisms of cell death, and provides a reasonable opportunity for its future application at the first stages of CVA.

Conflict of interest

All authors declare that no conflict of interest exists.

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