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NEUROPROTECTIVE EFFECT OF ORGANIC ACIDS DIAMIDES. FOCUS ON CHANGING MITOCHONDRIAL FUNCTION

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Abstract

Ischemic stroke is the most common angioneurological pathology. Recent studies show the significant therapeutic potential of neuroprotective agents in the treatment of stroke. This study focused on evaluation the effect of organic acids diamides on the change of the respirometric function of mitochondria under experimental cerebral ischemia. As a result, it was found that the use of the studied diamides contributed to the restoration of ATP-generating activity, maximum respiration level, respiratory capacity, ATP turnover, respiratory control and coefficient of oxidative phosphorylation efficiency, and a decrease in brain necrosis zone in group of rats treated by substances NA-1, NA-2 , NA-3, NA-5 and NA-6 by 27.79% ($p < 0.05$), 15.44% ($p < 0.05$), 17.34% ($p < 0.05$), 18.29% ($p < 0.05$) and 24.23% ($p < 0.05$), respectively. Moreover, the studied compounds had low toxicity (LD50 in the range from 2428 ± 125.951 to 6131 ± 256.911 mg / kg).

Keywords: *neuroprotection; mitochondria; mitochondrial dysfunction; amides*

Introduction

Stroke is a common cerebrovascular disease, which is one of the main causes of mortality and primary disability of the population [1]. As a rule, a stroke is defined as the development of focal neurological symptoms, due to occlusion of the cerebral vessels, a thrombus, or an embolus and the resulting cerebral infarction zone formation [2]. There are two fundamentally different pathogenetic variants of stroke - ischemic and hemorrhagic. Approximately 70-80% of cases of stroke occur in its ischemic variant, however, that the number of hemorrhagic stroke currently tends to increase [3]. Morphologically ischemic stroke is characterized by the presence of two main areas of brain tissue damage - the zone of cerebral infarction (occurs during the first hours after the cessation of cerebral blood flow) and the area of ischemic «penumbra» - an ischemic area with functioning neurons and reduced metabolic potential [4].

The main treatment for ischemic stroke is thrombolytic therapy by recombinant tissue plasminogen activator drugs (Retaplase, Alteplase, Tenecteplase), which are administered no later than 4.5 hours after an ischemic attack [5]. However, despite the high therapeutic efficacy, thrombolytic therapy can provoke the development of clinically more unfavorable neurological, cognitive and metabolic disorders, combined by the concept of the phenomenon of ischemia / reperfusion [6]. At the same time, the development of this phenomenon opens up certain prospects for the targeted administration of drugs that preserve the functional activity of brain tissue - neuroprotective drugs [7]. It is known that the zone of ischemic penumbra is the target for cerebroprotective therapy. At the same time, metabolic disturbances in the penumbra zone are observed when cerebral blood flow reaches 35% of the physiological level and is manifested in the form of anaerobic oxidation activation, a decrease of pH and an increase of a lactic acid level. Further progression of these pathological changes leads to a decrease in ATP concentration critical for cellular activity, which is observed at a cerebral blood flow level of 15-20% from normal [8]. These metabolic changes lead to the activation of the apoptotic signal, which contributes to the loss of neuronal integrity and an increase in the area of ischemic cerebral infarction. Moreover, since mitochondria play a key role in cellular bioenergetic processes, it can be assumed that the therapeutic influence on these cellular organelles aimed at restoring their functional activity may be a promising direction of neuroprotection in ischemic stroke [9].

Materials And Methods

Experimental animals.

The experiment was carried out on 79 Balb / c male mice (20-22 grams) and 100 Wistar male rats weighing 210-230 grams, 6 months of age. The animals were obtained from the «Rappolovo nursery» and were kept under standard vivarium conditions with a natural change in the daily cycle, an ambient temperature of 22 ± 2° C and a relative humidity of 60 ± 5%. Rats were kept by 5 individuals in macrolon boxes with free access to food and water. The keeping of animals and the manipulations were in accordance to the requirements of Directive 2010/63 / EU of the European Parliament and of the council on the protection of animals used for scientific purposes, September 22, 2010.

Studied objects.

In this work, the studied compounds were diamides of organic acids obtained at the Department of Organic Chemistry, PMPI. The characteristics of the test substances are given in table 1. The structures of the compounds were confirmed by NMR, IR, UV spectroscopy.

Study design.

This study was performed in 2 stages. At the first stage, the acute toxicity of the test compounds was evaluated with the determination of LD_{50}. Then, at the second stage, the influence of the test substances on the change of the respirometric function of mitochondria in rats with cerebral ischemia and cerebral necrosis zone were evaluated. In the second phase of the study, the studied diamides of organic acids were administered per os at a dose of 1/100 of the LD_{50} after 30 minutes the ischemia modelling and further for 3 days (one administration in day). On the 4th day of the experiment, rats were decapitated, the brain was removed, and changes in mitochondrial function and cerebral infarction area were assessed. Aminophenylbutyric acid at a dose of 25 mg / kg was used as a reference drug, which was administered

according to a similar scheme to the test substances [10]. During the study, the following experimental groups were formed: SO - sham-operated animals; NC- negative control (did not receive therapy); APBA is a group of rats treated by aminophenylbutyric acid; groups of rats that are received of the test substances NA-1 - NA-7. The number of animals in each group was 10 individuals.

Evaluation of acute toxicity

The study of the test-compounds was carried out using the available method for evaluation the oral toxicity of chemical substances in an acute experiment - the «Up and down» procedure. According to this protocol, two tests were conducted: limit testing and basic testing. Since diamides of organic acids a rather low toxicity is described, in the conditions for determining acute toxicity, a limit dose of 5000 mg / kg (*per os*) was chosen. The analysis and the LD_{50} calculation corresponded to the recommendations set out in OECD №425 [11].

Model of cerebral ischemia.

Rat cerebral ischemia was modeled using the modified Tamur method based on irreversible occlusion of the middle cerebral artery. Surgery was performed in animals anesthetized with chloral hydrate (350 mg / kg, ip). In the area below and to the right of the eye, wool was removed. an incision was made, soft tissues were dissected, exposing the process of the zygomatic bone, which was removed. Then, through the trepanation hole, thermocoagulation of the middle cerebral artery was performed under the place of its intersection with the olfactory tract. The surgical wound was sutured in layers and treated with an antiseptic solution (povidone-iodine, 10%). Before awakening, the animals were left under a warming lamp [12].

Biomaterial sampling and sample preparation

The animals brain was used in this work like test-material. Rats were decapitated, the cranium was opened and the brain was removed. The brain was homogenized in an isolation medium (1 mmol EDTA, 215 mmol mannitol, 75 mmol sucrose, 0.1% BSA solution, 20 mmol HEPES, with a pH of 7.2). Next, the resulting homogenate was centrifuged at 1000g for 3 minutes, then the post-nuclear supernatant was re-centrifuged at 13000g for 15 minutes. The mitochondrial fraction was resuspended in the isolation medium. All procedures were performed at 4°C [13].

Respirometric analysis

Analysis of the mitochondria respiratory function was carried on the system of laboratory respirometer (AKPM1-01L manufactured by «Alfa Bassens», Russia). The mitochondrial respiration was evaluated by the change of oxygen consumption (OC) in the test-secondary supernatant against the injection of uncouplers of mitochondrial respiration. The uncouplers in this study were: 4 - (trifluoromethoxy) phenyl) hydrazono) malononitrile (FCCP- concentration 1 μM); oligomycin - concentration 1 μg / ml; rotenone - concentration 1 μM; sodium azide - concentration 20 mmol. The following parameters were calculated during the respirometric analysis:

- ATP-generating ability - the difference of OC after FCCP and oligomycin addition;
- The maximal respiration level - the difference of OC after FCCP and rotenone addition;
- The respiratory capacity - the difference of OC after FCCP addition and the basal level of respiration.
- ATP turnover - the difference of basal OC and OC after oligomycin addition
- Coefficient of oxidative phosphorylation - ATP turnover divided basal OC
- Respiratory control- OC after FCCP addition divided proton leakage.

OC was determined in ppm with followed conversion to protein concentration in the sample. The protein content was determined by the Bradford method [14, 15].

Necrosis zone evaluation

The size of the necrosis zone was determined by the triphenyltetrazolium method. The brain was removed, cut off the cerebellum, divided the hemispheres. Both hemispheres were weighed, then separately homogenized and placed in cups with 10 ml of a 1% solution of triphenyltetrazolium chloride in phosphate buffer (pH 7.4). Sample were placed in a water bath for 20 minutes at 37 ° C. Tne samples were centrifuged at 5000 RPM / 10 min. Next, 3 ml of cooled chloroform was added to the obtained supernatant and incubated for 15 minutes at 40 C with periodic stirring. The resulting mixture was re-centrifuged and the optical density of the chloroform extract of formazan was measured at

492 nm against pure chloroform. The calculation of the necrosis zone was expressed as a percentage of the total mass of the hemispheres by formula .

$$x = 100 - \frac{\varepsilon_1 M_1 + \varepsilon_2 M_2}{\varepsilon_1 (M_1 + M_2)} 100$$

where x is the size of the zone of necrosis as a percentage of the total mass of the brain;

ε1 is the optical density of the sample with an intact hemisphere;

ε2 is the optical density of the sample with a damaged hemisphere;

M1 is the mass of the intact hemisphere;

M2 is the mass of the damaged hemisphere [16].

Statistical methods

The obtained data were expressed as M (mean) ± SEM (standard error of mean). Comparison of averages was carried out by the ANOVA method with post-hoc Newman-Keuls test for multiple comparisons. Differences were considered statistically significant at $p<0.05$. During the statistical analysis the STATISTICA 6.0 software (StatSoft, USA) for Windows was used.

Results

Evaluation of test-compounds acute toxicity

When assessing the acute toxicity of the studied diamides of organic acids, it was found that none of the tested compounds passed the limit test (LD_{50} <5000 mg / kg). In the conditions of the main test, it was found that the administation of the test substances in an increasing dose range: 175; 550; 1750; 5000 mg / kg, an insignificant death of animals was observed, which may indicate the low toxicity of the estimated acid diamides. Dead animals were subjected to necropsy, according to the results of which no significant deviations of the internal organs state in mice treated by the test objects. Visually, there was a slight increase in the liver and spleen, swelling of the gastric mucosa. The results of the acute toxicity assessment of the studied diamides of organic acids are represented in table 2.

Thus, based on the LD_{50} values, the studied compounds can be assigned to the 5th chemical hazard class according to the GSH classification nomenclature (Globally Harmonized System of Classification and Labeling of Chemicals (GHS) Part 3 Health Hazards, United Nations, 2017).

The results of evaluating the effect of the studied compounds on the change in the respirometric function of mitochondria and the brain necrosis zone in rats.

When assessing the effect of changes in the respirometric function of mitochondria under the conditions of cerebral ischemia correction by the studied diamides of organic acids, it was found that in rats of the NC group, in comparison with SO animals, the ATP-generating ability, the maximum level of respiration and respiratory capacity decrease by 7.58 times ($p<0.05$); 9.05 times ($p<0.05$) and 6.21 times ($p<0.05$), respectively (Table 3). Also, in the NC group, a decrease in ATP turnover by— 8.35 times ($p<0.05$); respiratory control - 4.71 times ($p<0.05$) and the coefficient of oxidative phosphorylation efficiency - 6.8 times ($p <0.05$) was observed.

When using aminophenylbutyric acid, an increase in ATP-generating ability, a maximum level of respiration and respiratory capacity by 2.07 times; 3.06 times and 1.93 times, respectively ($p <0.05$, all indicators relative to the NC group) was noted. At the same time, in animals treated by aminophenylbutyric acid, ATP turnover, respiratory control and oxidative phosphorylation efficiency coefficient were superior to those of the rat group that had not been treated by 2.13 times ($p <0.05$); 1.44 times ($p <0.05$) and 1.83 times ($p <0.05$) respectively (Table 3).

Against the background of the test compound NA-1 administration, restoration of the respiratory function of mitochondria in rats was observed, which was reflected in an increase in the indices characterizing the processes of cell respiration. ATP-generating ability increased by 3.2 times, the maximum level of respiration - 4.2 times, respiratory capacity - 2.6 times; ATP turnover - 3.4 times; respiratory control - 1.7 times and the coefficient of oxidative phosphorylation efficiency - 3.2 times (all indicators $p <0.05$, relative to the NC group of animals).

Similar changes of mitochondria the respirometric function were obtained in the conditions of NA-2 and NA-3 compounds administration to rats with cerebral ischemia. So against the background of the NA-2 administration, the ATP-generating ability increased by 2.11 times ($p<0.05$), and when using NA-3, by 2.08 times

(p<0.05). The maximum respiratory rate in rats treated by compounds NA-2 and NA-3 increased by 2.39 and 2.41 times (p<0.05, both parameters), respectively, compared with the NC group. At the same time, the respiratory reserve against the background of the use of substances NA-2 and NA-3 tended to not increase so much as the maximum level of respiration and ATP-generating ability. Against the background of administration of compounds under laboratory codes NA-2 and NA-3 to rats, an increase in respiratory capacity by 66.9% (p <0.05) and 46.8% (p <0.05), respectively was observed. It is worth be noting that ATP turnover, respiratory control, and the oxidative phosphorylation efficiency coefficient when using the NA-2 substance exceeded the similar parameters of NC rat group by 2.9 times; 1.4 times and 2.7 times, respectively (p <0.05, all indicators). Similarly, ATP turnover, respiratory control, and oxidative phosphorylation efficiency coefficient changed with the administration of NA-3 compound, these indicators increase by 3.0 (p <0.05); 1.5 times (p <0.05) and 2.8 times (p <0.05) respectively compared with the group of animals that did not undergo pharmacological correction (Table 3).

In animals treated by the studied object under the code NA-4, an increase in ATP-generating ability - by 1.7 times (p <0.05); the maximum level of respiration and respiratory capacity - 2 and 1.9 times, respectively (p <0.05) was observed. Moreover, with the use of compound NA-4, an increase in ATP turnover— by 3.1 times (p <0.05); respiratory control - 1.5 times (p <0.05) and the coefficient of oxidative phosphorylation efficiency - 2.9 times (p <0.05) was noted.

With the administration of the test substance NA-5 in animals, the respiratory function of mitochondria was restored, which was expressed in an increase in ATP-generating ability, the maximum level of respiration and respiratory reserve by 2.61 times; 3 times and 1.8 times, respectively (p <0.05, all indicators relative to the NC group of rats). At the same time, in animals treated by NA-5, ATP turnover increased by 3 times (p <0.05), respiratory control - 1.3 times (p <0.05) and oxidative phosphorylation efficiency coefficient - 2.7 times (p <0.05) in relation to the same indices of the NC group (Table 3).

Against the background of the administration of NA-6 into animals, in comparison with a group of animals lacking pharmacological support, an increase in ATP-generating ability - by 2.58 times; the maximum level of respiration - 2.1 times; respiratory reserve - 2.1 times (p <0.05, all indicators) was observed. At the same time, when using the studied object NA-6, an increase in ATP turnover - by 3 times (p <0.05); respiratory control - by 34.4% (p <0.05) and the coefficient of oxidative phosphorylation efficiency - 2.8 times (p <0.05) was noted.

In rats treated by the compound NA-7 normalization of parameters characterizing the respirometric function of mitochondria was noted (Table 3). So against the background of the use of NA-7, there was an increase (relative to the NC group) of ATP-generating ability - by 1.7 times (p <0.05); the maximum level of respiration - 2.3 times (p <0.05); respiratory reserve - 2 times (p <0.05); ATP turnover - 2.9 times (p <0.05); respiratory control - by 37.3% (p <0.05) and the coefficient of oxidative phosphorylation efficiency - 2.7 times (p <0.05).

As a result, the observed changes in mitochondrial function contributed to a decrease in the area of cerebral infarction (Fig. 1) in rats treated by the studied substances and aminophenylbutyric acid. Thus, when animals were treated by the substances NA-1, NA-2, NA-3, NA-5 and NA-6, the area of brain necrosis decreased relative to the NC group of rats by 27.79%, 15.44%, 17.34% , 18.29%. 24.23%, respectively (p <0.05. All indicators). At the same time, in animals that are received aminophenylbutyric acid, the zone of cerebral necrosis decreased by 23.04% (p <0.05). It should be noted that the use of NA-4 and NA-7compounds did not significantly affect the change of the value of the cerebral infarction zone in rats (Fig. 1).

Discussion

Mitochondria are two-membered organelles that play the key role of cellular regulators of energy production, apoptosis reactions, and redox cell potential [17]. In the present, a significant role of the violation of structural integrity, but primarily the functional activity of mitochondria in the pathogenesis of cerebrovascular diseases, for example, ischemic stroke has been established [18]. Insufficient tissue oxygenation contributes to a decrease in the mitochondrial membrane potential, which in turn leads to the dissociation of electron transport reactions along the mitochondrial

respiratory chain. Under the current conditions, inversion of the F_1F_o ATP-synthase activity is noted, which leads to a significant decrease in ATP synthesis and an increase in the production of oxygen free radicals and increased lipid peroxidation [19]. As a result of energy deficiency and ROS hyperproduction, activation of apoptosis is observed, which is accompanied by an increase in the zone of cerebral infarction.

In connection with the significant role of mitochondrial dysfunction in the ischemic cascade of brain damage, it can be assumed that targeted pharmacological correction of this condition will help maintain neuronal integrity, thereby ensuring a neuroprotective effect. Thus, the work of *Naoi M, et al., 2019* shows that polyphenolic compounds have a neuroprotective effect associated due to the restoration of the activity of neuronal mitochondria [20]. Apigenin, resveratrol, ferulic acid, and many other polyphenols modulate the mitochondrial apoptotic signal, reactions of the tricarboxylic acid cycle (primarily catalyzed by succinate dehydrogenase), restore the activity of respiratory complexes and the activity of endogenous antioxidant enzymes [21]. Also *Zhu Y et.al., 2018* demonstrated the high neuroprotective potential of mitochondria-oriented peptides of the SS series - SS-31 (D-Arg-Dmt-Lys-Phe-NH2; Dmt-2 ', 6'-dimethyltyrosine), the use of which experimental traumatic brain injury reduced the hydratation of brain tissue, the severity of neurological deficit, releasing cytochrome C, accompanied by the restoration of the antioxidant potential of mitochondria [22].

Based on the current trend of mitochondrial medicine, a study of the influence of seven diamides of organic acids on the change in the functional activity of mitochondria (respiratory function) under experimental cerebral ischemia were conducted. As a result, it was found that the use of the studied compounds in low doses (from 24 to 61 mg / kg) contributed to the normalization of the respirometric function of mitochondria, which was expressed in the restoration of ATP-generating activity, maximum respiration level, respiratory capacity, ATP turnover, respiratory control, and coefficient the effectiveness of oxidative phosphorylation. It is known that these indicators allow the screening version to most fully investigate the state of the respiratory function of the mitochondria of the cell. So ATP-generating ability - characterizes the ability of mitochondria to synthesize ATP during electron transport reactions along the mitochondrial respiratory chain; maximum level of respiration - shows the limit of the functional capabilities of mitochondria, i.e. demonstrates a measure of the stress effectiveness of mitochondria; respiratory capacity - is a «metabolic reserve» which can be involved with a significant shortage of oxidation substrates, in particular glucose; ATP turnover - characterizes the «lifetime» of ATP molecules in a cell; respiratory control - shows the ability of mitochondria to utilize ADP and the coefficient of oxidative phosphorylation efficiency - is a measure of the efficiency of oxidation and phosphorylation coupling and the maximum possible synthesis of ATP under given conditions [23].

Since the restoration of these indicators against the background of the use of all the studied diamides of organic acids, it can be assumed the presence of mitochondria-positive properties in test substances. It is worth noting that the acid diamides studied in this work are low toxic compounds, which opens up certain prospects in the development of neuroprotective drugs based on them and, in addition, a neuroprotective effect of some amides of organic acids, realized by reducing the cytotoxic effect of exciting neurotransmitter amino acids [24] has been established. At the same time, among the studied substances, the compound with the code NA-1 (N-acetyl-2-phenylacetamide) has the most pronounced pharmacological effect, against the background of which a significant restoration of the respiratory function of mitochondria was noted, which exceeded the indices of the referent, aminophenylbutyric acid.

Conclusion

The study showed the promise of further study of diamides of organic acids as neuroprotective compounds that restore mitochondrial function. At the same time, for the studied compounds, primarily N-acetyl-2-phenylacetamide, low systemic toxicity was established (5-class toxicity according to the GHS classification). However, despite the promising obtained data, further studies are needed to evaluation of other aspects of the mitochondriotropic and neuroprotective effects of

diamides of organic acids. Thus, it seems relevant to assess the effect of acid diamides on changes in anaerobic metabolism, the activity of mitochondrial respiratory chain complexes, and enzymes of the tricarboxylic acid cycle.

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Conflicts Of Interest

The authors statement no conflict of interest with the submitted manuscript.

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Table 1. Characterization of the test substances.

Chemical name	Physicochemical properties	Laboratory code
N-acetyl-2-phenylacetamide	Odourless white crystalline powder. Slightly dissolve in water. Soluble in ethanol, DMSO.	NA-1
N- (2-phenylacetyl) propanamide	Odourless white crystalline powder. Slightly dissolve in water. Soluble in ethanol, DMSO.	NA-2
N- (2-phenylacetyl) butanamide	Odourless white crystalline powder. Slightly dissolve in water. Soluble in ethanol, DMSO.	NA-3
2-methyl-N- (2-phenylacetyl) propanamide	Light yellow, odourless crystalline powder. Slightly dissolve in water. Soluble in ethanol, DMSO.	NA-4
N-acetyl-2- (2-oxo cyclopentyl) - acetamide	A light yellow, odourless crystalline powder. Slightly dissolve in water. Soluble in ethanol, DMSO, chloroform.	NA-5
N-propanoyl-2- (2-oxo-cyclopentyl) - acetamide	Odourless yellow crystalline powder. Slightly dissolve in water. Soluble in ethanol, DMSO.	NA-6

Table 2. Results of acute toxicity evaluation of the test compounds

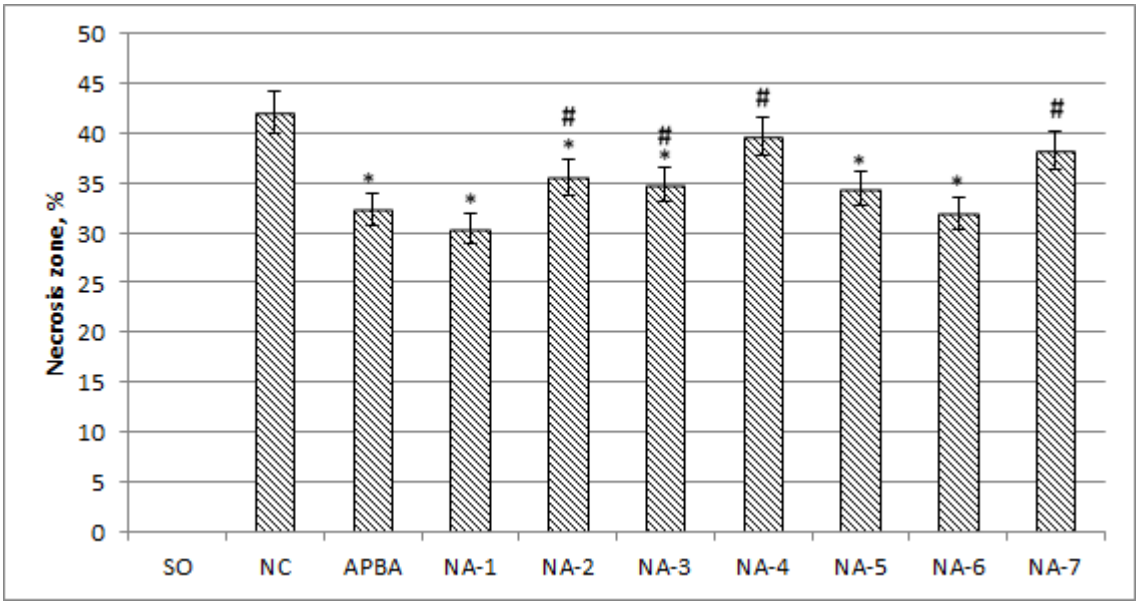
Test-compound	LD_{50} mg/kg (*per os*)	Dose for the second stage, mg/kg *per os* (1/100 from LD_{50})	Number of animals used for the test
NA-1	6131±256,911	61	15
NA-2	3574±198,928	36	12
NA-3	3021±203,917	30	10
NA-4	2428±301,188	24	8
NA-5	2562±127,292	26	10
NA-6	2831±133,921	28	12
NA-7	3361±266,122	34	12

Table 3. Changes in the respirometric function of mitochondria against the background of correction of cerebral ischemia with the studied diamides of organic acids

Group	ATP-generating activity, ppm/g of protein	Maximum respiration level, ppm/g of protein	Respiratory capacity, ppm/g of protein	ATP turnover, relative units	Respiratory control, relative units	Coefficient of oxidative phosphorylation efficiency, relative units
SO	93,2±2,768	89,6±4,55	92,5±4,562	1,92±0,008	24,5±2,898	0,204±0,004
NC	12,3±2,592#	9,9±1,038#	14,9±2,255#	0,23±0,005#	5,2±0,930#	0,03±0,006#
APBA	25,4±9,536*	30,3±5,855*	28,7±5,879*	0,49±0,009*	7,47±0,786*	0,055±0,005*
NA-1	39,36±4,486*	41,19±5,231*	38,82±6,311*	0,78±0,004*	8,894±1,003*	0,097±0,006*
NA-2	26,01±2,091*Δ	23,68±4,159*Δ	24,88±5,115*Δ	0,67±0,001*	7,234±1,398*	0,082±0,004*
NA-3	25,63±4,418*Δ	23,85±4,299*Δ	21,88±7,335*Δ	0,68±0,002*	7,838±1,683*	0,083±0,007*
NA-4	20,5±4,993*Δ	20,17±4,201*Δ	27,94±7,914*Δ	0,72±0,002*	7,996±1,724*	0,087±0,004*
NA-5	32,16±2,755*	29,55±4,013*Δ	26,67±6,992*Δ	0,7±0,001*	6,905±1,277*Δ	0,081±0,003*
NA-6	31,77±4,458*	20,73±6,509*Δ	30,63±6,137*	0,69±0,002*	6,998±1,562*Δ	0,083±0,006*
NA-7	20,4±3,689*Δ	22,88±5,436*Δ	29,37±6,449*	0,67±0,004*	7,139±1,393*	0,08±0,006*

Note: # - statistically significant relative to theSO animals ($p < 0.05$, Newman-Keuls test);
* - statistically significant relative to the NC group of animals ($p < 0.05$, Newman-Keuls test).
Δ - statistically significant relative to the group of animals treated by NA-1 compound ($p < 0.05$, Newman-Keuls test).

Figure 1. The effect of the studied compounds and the reference drug on the change of the size of the brain necrosis zone in rats.



Note: ***** - statistically significant relative to the NC group of animals ($p < 0.05$, Newman-Keuls test).
- statistically significant relative to the group of animals treated by NA-1 compound ($p < 0.05$, Newman-Keuls test).